Strategies for Monitoring of Endocrine Disrupting Chemicals in Aquatic Environment

Hallgren, Pär

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Pär Hallgren

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LUND UNIVERSITY

Department of Analytical Chemistry

Centre for Aquatic Biology and Chemistry

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List of Publications

This doctoral thesis is based on five papers which are referred to in the text by Roman numerals (I-V).

I. Improved spectrophotometric vitellogenin determination via alkali-labile phosphate in fish plasma -a cost effective approach for assessment of endocrine disruption

Hallgren, P., Mårtensson, L., Mathiasson, L.
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II. A new spectrophotometric method for improved indirect measurement of low levels of vitellogenin using malachite green

Hallgren, P., Mårtensson, L., Mathiasson, L.
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III. Crucian carp *(Carassius carassius)* as a test species for endocrine disruption

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2009, Manuscript.

IV. Steroid hormone determination in water using an environmentally friendly membrane based extraction technique.

Zorita, S., Hallgren, P., Mathiasson, L.
Journal of Chromatography A,
2008, 1192, 1-8.

V. Measuring bioavailability of polychlorinated biphenyls in soil to earthworms using selective supercritical fluid extraction

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Contribution by the author to the different papers

Paper I. The author was responsible for the experimental design, performed all the experiments, and wrote the major part of the manuscript.

Paper II. The author was responsible for the experimental design, performed all the experiments, and wrote the major part of the manuscript.

Paper III. The author was responsible for the experimental design, performed all the experiments, and wrote the major part of the manuscript.

Paper IV. The author was partly involved in method development, partly involved in sampling and participated in the writing of the manuscript.

Paper V. The author was responsible for the experimental design, performed all the experiments, performed most of the analysis, and wrote the major part of the manuscript.

List of abbreviations

AhR	Aryl hydrocarbon receptor
ALP	Alkali-labile phosphate
DDT	dichloro-diphenyl-trichloroethane
DNA	Deoxyribonucleic acid
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE2	17α-ethinylestradiol
EC_{50}	Concentration that gives an observable effect in 50% of the individuals
EDC	Endocrine disrupting chemical
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
ELISA	Enzyme-linked immunosorbent assay
ELRA	Enzyme-linked receptor assay
EPA	Environmental Protection Agency
ER	Estrogen receptor
ER-CALUX	Estrogen receptor-mediated chemically activated luciferas gene expression
ERE	Estrogen responsive element
EROD	Ethoxyresorufin-O-deethylase
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GC-MS/MS	Gas chromatography – tandem mass spectrometry
GSI	Gonado Somatic Index
HF-MMLLE	Hollow fibre microporous membrane liquid liquid extraction
LOEC	Lowest observed effect concentration
LSI	Liver Somatic Index
MFO	Mixed function oxygenases
MS	Mass Spectrometry
NP	Nonylphenol
PAH	Polycyclic aromatic hydrocarbon
pKa	Acid dissociation constant
PCB	Polychlorinated biphenyl
PBDE	Polybrominated diphenyl ether
PEC	Predicted environmental concentration
POP	Persistent organic pollutants
PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
SBSE	Stir bar sorptive extraction
SFE	Supercritical fluid extraction
SHBG	Steroid hormone-binding globulin
SPE	Solid-phase extraction
SPME	Solid-phase micro extraction
STP	Sewage treatment plant
TBT	Tributyltin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalence factor
TSH	Thyroid-stimulating hormone
VTG	Vitellogenin protein biomarker
WHO	World Health Organisation
YES	Yeast estrogen screen

Populärvetenskaplig sammanfattning

År 1962 utkom Rachel Carson med boken ”Silent spring” som genom att ge en samlad bild av hotet från miljögifter blev startskottet för den moderna miljörörelsen. Storsäljande ”Our stolen future” av Theo Colburn från 1996 kan betraktas som en aktuell uppföljare. Här beskrivs hur kemikalier genom sin hormonstörande verkan kan leda till försämrad spermiekvalitet, minskad fertilitet, bröst- och prostatacancer och utvecklingsskador hos barn. Även om boken kan uppfattas som överdriven så kvarstår fakta att välkända miljögifter såsom bekämpningsmedel, flamskyddsmedel, båtbottenfärger och polyklorerade bifenyler (PCB) har hormonstörande effekter redan vid extremt låga koncentrationer. Vi vet även att industrikemikalier, som t.ex. ftalater och nonylfenol, som produceras i stora mängder och finns överallt runt om kring oss, också har hormonstörande egenskaper.

Vad vi inte vet är om det är dessa typer av ämnen som bidrar till att svenska mäns spermier håller sämre kvalitet än finska mäns spermier. Denna typ av orsakssamband är väldigt svåra att påvisa för människor men det har framkommit klara bevis för reproduktionsstörningar i djurlivet. Hormonpåverkan på fisk nedströms reningsverk har påvisats i Sverige och då har läkemedelsrester (främst p-piller) utpekats som trolig orsak. För att skydda oss själva och de akvatiska ekosystemen måste vi därför kunna mäta, spåra och förhindra spridningen av hormonstörande ämnen. Ökad medvetenhet om dessa faror skapar en stor efterfrågan på analysmetoder som inte bara är korrekta utan även enkla och mindre kostsamma. Kunskapen om spridning av dessa ämnen är i nuläget kraftigt begränsad av hur många prover man har råd att analysera. Analytisk kemi handlar traditionellt mestadels om att kvantifiera enskilda kemiska ämnen men ofta är detta inte tillräckligt. I utsläppet från t.ex. ett reningsverk förekommer en hel ”cocktail” av en mängd ämnen varav somliga

kan ha hormonstörande effekter. För att få en helhetsbild om hälso- och miljörisker så kan man med fördel använda sig av metodik där man mäter den sammantagna effekten av denna blandning. Man kan då använda sig av en biomarkör som är ett biologiskt ”svar” orsakad av en viss typ av miljögift och därmed ger ett mått på exponeringen och ibland även på den toxiska effekten. Eftersom en biomarkör kan ge utslag för flera olika miljögifter och över en längre period kan man minska antalet mätningar.

För att mäta den biologiska effekten av hormonstörande ämnen i vatten är vitellogenin en vedertagen biomarkör. Vitellogenin är ett ägguleprotein som normalt bara produceras av en könsmogen fisk av honkön. När hannar eller icke könsmogna individer utsätts för östrogenhärmande miljögifter såsom PCB, dioxin, ftalater, nonylfenol påträffas vitellogenin i blodet. Kvantifieringen av vitellogenin kan vara kostsam men i den här avhandlingen presenteras två nya billiga mätmetoder för att indirekt mäta vitellogenin i blodprover från fisk. Med den nya metodiken kunde hormonstörande effekter på regnbågslax (*Oncorhynchus mykiss*) upptäckas efter att dessa placerats ut i Vallkärrabäcken, som mottar ett visst flöde av lakvatten från den övertäckta soptippen på Sankt Hans Backar i Lund. Biomarkören vitellogenin har även studerats i fisken ruda (*Carassius carassius*). Rudans extrema tålighet mot låg syrehalt gör den mer användbar än de relativt störningskänsliga laxfiskarna. Efter att ha injicerat östrogen i rudor kunde det utläsas att fisken är lämplig att använda vid miljöövervakning då den reagerar kraftigt på hormoner. Med rudan kan vi genomföra mätningar i vatten som andra fiskarter inte skulle klara av.

Biomarkörer är användbara men kan inte avslöja vilka ämnen i vatten-, mark- eller sedimentprover som orsakar den hormonstörande effekten. För att få svar på det måste man använda kemiska analyser. I den här avhandlingen presenteras en metod för att mäta halten av naturligt östrogen samt den aktiva substansen i p-piller i avloppsvatten. Om den hormonella effekten i miljön inte

orsakas av denna typ av ämnen utan istället av ”gamla” organiska miljögifter är det lämpligt att göra en riskbedömning av hur benägna dessa ämnen är att frigöras från jord- eller sedimentpartiklar, bli biotillgängliga och spridas i miljön. I den här avhandlingen presenteras även en kemisk mätteknik som utnyttjar superkritisk koldioxid för att mäta biotillgängligheten av miljögifter i jord.

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1 Introduction – Endocrine Disrupting Chemicals

Are we humans slowly poisoning ourselves to a degree where we cannot longer reproduce? At least it would be interesting to find out why the sperm quality is better, and the incidence of testicular cancer much lower, in Finland compared to the rest of Europe [1]. Considering other vertebrates there is certainly proof that environmental pollution can seriously effect reproduction, like in the well known example of eggshell thinning in birds [2, 3]. However, there is a great challenge in trying to understand in which cases ecological effects can be attributed to environmental pollution and the challenge may be even greater concerning chemicals with endocrine effects. Compared to historical knowledge of environmental pollution this research field means new pollution sources, new toxicological mechanisms, lower effect concentration and new strategies for analysis. Heaps of dead fish are floating right next to the place where the local evil factory spews its toxic waste from a rusty pipe and into the river. Fortunately, this is not a fair description of the effects of pollution in the rich part of Europe today. The most toxic compounds has been phased out although we still have to deal with the relics of an ancient time present as chlorinated pesticides, polychlorinated biphenyls (PCBs), and heavy metals that still bioaccumulate in fish. The dirtiest form of mass production for mass consumption has been exported to low-wage-countries so they do no longer pollute our nose or eye but perhaps our conscience. In the developed countries the environmental concern has shifted from the specialised use of very toxic chemicals to the “passive” leaching of high volume industrial chemicals from products produced locally or imported. As an example chlorinated pesticides have been substituted with new and “safe” alternatives. However these formulas may contain high volumes of estrogenic alkylphenols as detergent [4]

so the hazard has not been *removed*, merely *moved*. In leachate water from a waste dump or in a sewage treatment plant, the flow of chemicals used in modern society converges to form a mysterious cocktail. When trying to evaluate the environmental impact of these chemicals in natural waters, scientists will face many challenges. In comparison with the platitude of dead fish and toxic waste pipe, the cause and effect is now separated in time and spatially. Further, the effects studied are not acute effects caused by high concentration of single substances but rather chronic effects from a mixture of compounds present at very low concentrations. The study of low concentration exposure is necessary especially since chemicals with endocrine effects have been found to be active already at extremely low concentration. The endocrine system is delicate and the inhibition of a single enzyme might turn a whole population sterile as shown for marine gastropods exposed to tributyltin (TBT) [5]. In the long run, failure of a fish to reproduce is also a form of dying, although less dramatic. It is not known how serious effects of endocrine disruption are on populations relative to other environmental factors such as habitat degradation and food availability [6].

Whether investigations are launched to answer this large question or with the aim to evaluate the problem more locally the research budget will set the frames for methods that can be used. During the research that is presented in this thesis the lack of a generous budget for buying equipment or buying services of analysis has not been a problem but rather a driving force to find new ways to cost efficient environmental analysis. The aim for cost efficient and simple methods also means that the methods could be of interest for less equipped laboratories or developing countries.

Chemical analysis, as well as other sciences, becomes more and more dependent on advanced technology and biotechnology and as a consequence the cost for analysis of samples increases. Hormones are active at very low

concentration (ng/L) and quantification demands for advanced equipment such as gas chromatography – tandem mass spectrometry (GC-MS/MS) which is indeed expensive equipment (100 000 €). The affordable number of samples to be analysed in a study will restrict the knowledge that can be acquired from an experiment. The independent quantification of several estrogenic compounds in a water sample can be substituted with a single measurement of e.g. total estrogenic load using a bioassay and in this way money can be saved. Still, bioassays have not fully taken the step into large scale monitoring from its experimental use. Also the use of biomarkers sometimes still depends on quantification with expensive immunoassays. The development of cost effective methods would help us to get more information out from what is invested into research and monitoring. This is more or less true independently of weather e.g. a biomarker as vitellogenin (VTG) is used for laboratory evaluation of endocrine effects of chemical substances, screening for endocrine effects in recipients, or large scale surveys to evaluate the ecological impact of endocrine disruptors. A cheap alternative to a more advanced immunological quantification of VTG as a measure of endocrine disruption may be a very attractive alternative for less equipped laboratories, municipalities and industry. This has been the starting point for the work presented within this thesis.

1.1 Chemicals with endocrine effects

Endocrine disruptive chemicals (EDCs) are a diverse group of compounds that have little in common except their ability to affect the endocrine system. A definition given by World Health Organisation (WHO) reads: “exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)-populations” [7]. EDCs share the common feature of ability to disrupt the endocrine system but the mechanism of action may be very different. As will be

discussed below in section 4.4 the actions of EDCs can be through: "mimicking" a natural hormone by activation of a hormone receptor, blocking the natural signal by inactivation of the hormone receptor, interfering with hormone production, interfering with hormone metabolism, interfering with hormone transport, or by other means. These exogenous substances can be anything from natural plant compounds to synthetic pesticides. Some are easy degradable and some are very persistent in the environment. Some are widespread and have weak potential effect while others are rare but with high potential. The gist of this is that while e.g. PCBs and polyaromatic hydrocarbons (PAHs) are groups of chemicals that share a specific chemical structural resemblance, it is not possible to present an overview of EDCs categorized on basis of chemical structure. This will also mean that for an analytical chemist it is fairly easy to present a sample preparation technique for quantification of all PCBs in a sample since structural resemblance will also mean resemblance in water solubility, stability, boiling point etc. but impossible to present a sample preparation technique for all EDCs in a sample. In analytical chemistry the physiochemical properties of chemicals is often of great importance, but in this thesis chemicals will be discussed mainly with focus on their common sources and common biological effects, rather than on structural resemblance. To give a comprehensive introduction, different types of EDCs are presented below in groups on basis of their origin.

1.1.1 Natural hormones

Natural hormones are not persistent and it may sound farfetched to talk about risks concerning biomolecules that naturally circulate in animals. However, like in all cases, it is the dose that makes the poison. Natural steroid hormones have been discussed as potential EDCs in two contexts. Firstly, the sewage treatment plant in an urban area collects and sums up the collective dose of hormones from the whole population in a city, creating a much higher hormone load than

from a sparsely-populated area. A pregnant woman can excrete up to 5 mg 17β-estradiol (E2) per day [8] and the sewage treatment plant is the bottleneck through which all natural hormones from the local citizens must pass and the outlet point will inevitably create a local hot spot even in cases where the recipient as a whole is likely to remain unaffected.

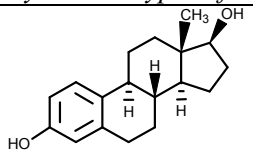
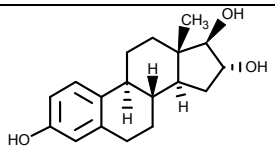
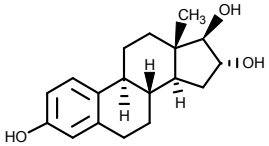
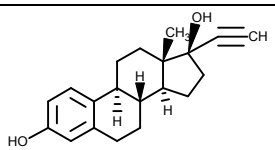
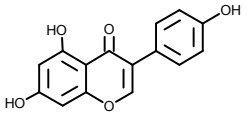
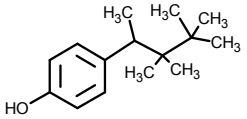
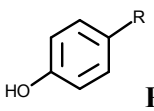
Secondly, there is also some evidence that in areas with intense agriculture, and a dense livestock, natural hormones from manure may reach biologically active concentrations in surface waters [9]. Lange *et al.* [10] calculated that the total estrogenic activity excreted by all livestock in the USA corresponds to 49 ton per year, the largest contributor being pregnant cattle.

Naturally, the risk will increase the denser the livestock/citizen population is and the smaller the water recipient is. In addition to the expressive statement that it is the dose that makes the poison, it can also be hold for true that it is the wrong compound at the wrong place and at the wrong time that makes the poison. Natural hormones from one species, like cows, can cause effects in remotely related species, like fishes. All vertebrates excrete steroid hormones but for those fish species that spawns only for a limited period during the year it is crucial that the hormonal signal do not circulate in the blood stream all year round. The reason for steroid hormones of human origin or from livestock being a threat towards other species is simply evolutionary. The endocrine system in different vertebrates is surprisingly similar and the regulation of reproductive function and maturation through the estrogen steroid hormone and the estrogen receptor, is thought to be the most ancient of the steroidal functions [11]. Evolution has created many differences in the estrogen receptor but still estrogen has a vital role in the reproduction for both humans and primitive animals like echinoderms [11].

Estrogen is the collective name used for estrone (E1), 17β-estradiol (E2), and estriol (E3) which are all primarily female sex hormones. Estrogen and other

steroid hormones like testosterone, cortisol and progesterone are all derived from cholesterol and share the common structure of three fused rings with 6 carbons and one ring with 5 carbons as depicted in Table 1.

Table 1. Natural and synthetic compounds that can interact with the estrogen receptor and their relative estrogenic potency in relation to E2. Relative potencies were estimated with a yeast assay ([12], [13], [14],) or are presented as the range given by several types of bioassays [14].*

17β-estradiol (E2) natural female sex hormone		**Relative potency** 1
Estrone (E1) natural female sex hormone		$1x10^{-2}$-$1x10^{-1}$ [14]*
Estriol (E3) natural female sex hormone		$3.7x10^{-1}$ [14]
17α-ethinylestradiol (EE2) synthetic female sex hormone		$1.9x10^{-1}$-1.9 [14]*
Genistein phytoestrogen present in e.g. soy bean		$8.6x10^{-6}$ [12]
Nonylphenol *4-(3,3,4,4-tetramethylpentan-2-yl)phenol* industrial chemical		0.28 [13]
Nonylphenol *commercial mixture* Industrial chemical	 **R=C_9H_x**	$1.9x10^{-3}$ [13]

The natural estrogenic steroids have different potential regarding their biological activity and among them, E2 has the largest ability to interact with the estrogen receptor (ER). The results from comparison with E2 in various bioassay, as presented in Table 1, show that E1 and E3 have less estrogenic potency. Estradiol is not only produced naturally in humans but is also administered in hormone replacement therapy. However, orally administration of natural estradiol is not very effective since the first pass through liver will eliminate a large fraction of the delivered dose. For therapeutic use synthetic estrogens have been synthesised. The synthetic hormones can be administered orally and still withstand the first pass metabolism in the liver so that they are effective enough to prevent conception.

1.1.2 Synthetic hormones

Synthetic hormones are used mainly as the active compound in contraceptive pills but also in hormone replacement therapy. 17α-ethinylestradiol (EE2) is the active compound in most contraceptive pills. This compound shares the basic structure of E2 but has been substituted with an ethinyl group to make it less susceptible for degradation and hence biologically active for a longer period in the body. The persistence of EE2 in plasma is thought to be one of the reasons why an oral dose of 40 μg of EE2 has the same estrogenic potency as an oral dose of 4mg of E2 [15]. Sometimes the synthetic hormone used in drug formulas is the inactive prodrug mestranol which is the 3-methyl ether of ethinylestradiol. The prodrug is even more effectively taken up from the gastro-intestinal tract and is not active until the liver has demethylized the inactive precursor. The desired increase in stability inside the human body also has the less favourable effect of increased stability in the environment. The stability results in longer life time of EE2 in a sewage treatment plant (STP) as compared to natural hormones. In a large Danish survey estrogens were measured and the contribution of different estrogens to the total estrogenic load

was estimated by multiplying the measured concentration with the estrogenic potential observed in a yeast bioassay [16]. The results showed that in the samples of untreated sewage where EE2 was detected (in 7 out of 12 samples), only about 10% of the total estrogenic load (estrone + 17α-estradiol + 17β-estradiol + EE2) was accounted for by EE2, but in treated water EE2 occasionally accounted for 50% of the estrogenic load. If estrogenic load had been evaluated with a whole organism bioassay like e.g. a fish, it is reasonable to believe that the significance of EE2 would have been even larger. This would be expected since the synthetic estrogen is more biologically stable. The estrogenic response in fish exposed to diluted effluent from a STP in western Sweden was mainly attributed to the presence of EE2 as apposed to the detected natural hormones and xenoestrogens [17].

Due to its potency and persistence the Swedish Medical Products Agency has identified EE2 as a compound constituting an environmental risk after comparison of predicted environmental concentration (PEC) and lowest observed effect concentration (LOEC) and this despite that the annual sale of EE2 is merely 6.4 kg (year 2002) in Sweden [18]. This figure has been estimated by considering the annual sales of pharmaceuticals containing synthetic hormones and the amount of active compound in the most popular products. It is reasonable to assume that close to 100% of the contraceptive pills are actually consumed. This would e.g. not be the case for the fraction of antibiotics and painkillers that are returned to the drugstore or worse disposed with solid waste or through the sewage.

A risk assessment of the potential ecological hazard of EE2 from a typical Swedish STP is illustrated below by calculation of the predicted environmental concentration (PEC) in the effluent. The contraceptive pill typically contains 35 μg of EE2 but, since the pill is taken in cycles where 3 weeks of active substance is followed by one week without, the estimated average daily dose is

around 26 µg/day [19]. It can be assumed that 40% is not metabolised within the consumer [19]. In Sweden, in year 2004, the number of women "on the pill" represented 10.9% of the total population [20]. The sewage treatment plant in Kristianstad, Sweden, handles 23000 m^3 of waste water from 53000 inhabitants every day. The average removal efficiency of EE2 in different sewage works with activated sludge treatment in developed countries has been estimated to 82% [19]. Applying these figures on removal on the conditions in Kristianstad would mean that the PEC in effluent from the sewage works is 26 µg x 0.4 x 0.109 x 53000 x 0.18 / $23x10^6$ L = 0.5 ng/L. EE2 is a very potent chemical and it has been shown that as low concentration as 0.125ng/L is enough to effect the growth in the gastropod *Bithynia tentaculata* [21]. This means that the concentration of EE2 in the effluent is potentially harmful to the environment but in most cases the effluent is diluted in the recipient.

1.1.3 Xenoestrogens

A large number of man-made chemicals have been identified as potent EDCs with the ability to work as agonists or antagonists to endogenous hormones. Among these are herbicides, insecticides and industrial chemicals such as alkylphenols, phthalates, bisphenol-A, PCBs, TBT and polybromintated diphenyl ethers (PBDEs) [22]. The European Commission has prioritized 146 EDCs that are of concern for human and wildlife exposure due to their persistence or high volume use [23]. Among these substances are both "newer" high volume industrial chemicals such as alkylphenols and phthalates as well as "old" contaminants such as PCBs and pesticides. It has since long been known that PCBs and chlorinated pesticides are persistent organic pollutants with serious ecotoxicological impact and the endocrine effects caused by these compounds can be regarded as just another other proof of their since long known toxicity. The endocrine effect of phthalates and bisphenol-A, on the other hand, is perhaps much more alarming since these chemicals have been

considered as “safe” and are used in vast amounts. Estimations show that the annual release of nonylphenol from imported textiles to Swedish waters is between 3 and 9 tons [24].

Overall, there is a huge knowledge gap concerning endocrine effects of chemicals. In the United States the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) has advised that 87000 chemicals should be prioritized for endocrine disruptor screening [25]. Even after sorting out the candidates for laboratory screening, this task is enormous and. The final results may still be inconclusive as tests for prenatal development is not included in the recommended screening battery of at least eight *in vivo* and *in vitro* assays. Furthermore, as will be discussed below, estrogenic effects are not always mediated through the estrogen receptor and could therefore be missed out with in-vivo tests.

1.1.4 Phytoestrogens

There are natural compounds in plants that can interact with the estrogen receptor. These compounds are called phytoestrogens and are not as potent as the intrinsic steroid hormones but on the other hand they are very frequent in provisions. These compounds are mostly discussed in context of hormone replacement therapy and even cancer prevention. Epidemiological studies have shown that phytoestrogens in soy-products may have a preventive action against both prostate cancer and breast cancer [26]. Perhaps the most studied group is the isoflavones found especially in beans, peas, alfa-alfa and peanuts. Genistein is the major isoflavone in soy bean and has a similar structure to the natural estrogen by its several aromatic rings and phenol substituents at both ends (Table 1). When it comes down to fish health and phytoestrogens it is not very likely that the naturally occurring phytoestrogens would have a significant impact on the endocrine system of wild fish. However, soy bean and seed meal from cotton, lupin, and alfa-alfa is often used to produce fish feed and the

presence of phytoestrogens and other non-beneficial substances in plant-derived fish feed has been discussed in relation to aqua-culture [27]. This will have to be investigated further, since the use of plant derived feed must increase in order to radically decrease the use of fish meal in aquaculture which has led to overfishing. The suspicion of influence from fish feed on in vivo tests has been confirmed in a study using a yeast estrogen-screening test, where estrogenic effects were detected in all (n=17) commercial fish feed tested [12]. Phytoestrogens in fish feed must also be avoided in laboratory tests and field experiments where fish is used to evaluate estrogenic effects.

1.2 Ecological effects of EDCs

Is the reproduction of humans or other species threatened by EDCs? This is a fundamental question that has been circulating especially after the release of the famous, or by some referred to as in-famous, book Our Stolen future in 1996 by Theo Colburn [28]. While the number of publications on endocrine disruption is ever-increasing, the research-community is far from consensus when it comes down to estimating the magnitude of the problem. Unfortunately, the answer to this question can not be given in this thesis. If the question is rephrased to: Are there proofs of population-level effects of EDCs?, then some answers can be given. Yes, there are examples where a specific chemical has been linked to serious effects on population level and the most well documented case is perhaps that of TBT from antifouling, boat bottom paint. TBT is bioaccumulated in gastropods, and besides the acute toxic effect, low levels cause endocrine disruption through elevation of the testosterone level, leading to imposex [29]. The first reports on masculinisation of molluscs was reported in 1970 as penis-like structures were observed in the sea snail dogwhelk (*Nucella lapillus*) [30]. In a more resent study of marine gastropods in Israel all female whelk (*Hexaplex trunculus*) were found to be sterile within a distance of

ca 1 km from an old marina [5]. The link between TBT, imposex and a decline in local gastropod population is strong enough to claim that this causal effect has been identified with certainty [31]. Concerning fish, it is difficult to evaluate the ecological impact of the estrogenic compounds mentioned above like natural hormones, synthetic hormones, xenoestrogens and phytoestrogens.

Research on estrogenic endocrine disruption is mainly restricted to evaluation of estrogenic or anti-estrogenic compounds by studying their ability to interact with estrogen receptor at basic molecular level. This is a major problem since conclusions on ecological relevance are uncertain from data gathered at the molecular level. Therefore, there is a need for more laboratory tests that study the impact of EDCs on reproduction in terms of physiological effects but, just as important, also on behavioural effects. It has been shown that pollutants can affect the reproductive behaviour in fish such as courtship and parental care [32]. In a nicely designed study with waterborne estrogen exposure, Kramer et al. [33] were able to link the measurement of the vitellogenin biomarker, by quantification of alkali-labile phosphate (ALP), with egg production as an indicator for reproductive performance. More of these types of studies are needed since the biomarker itself might be a physiological response that lies within the natural homeostatic response of the organism and does not automatically mean that the condition is critical for the fish.

Another major problem to address is that laboratory studies almost exclusively evaluate exposure to *single EDCs*. In real life, organisms would not realistically be exposed to a single compound but to a mixture of chemicals and the effects of a combined exposure to several chemicals are seldom studied. Data from investigations on whole organisms, where synergetic effects are studied are scarce. The few laboratory exposure studies available that study the combined antagonistic, additive or synergistic effects have been conducted with cell-based bioassays (see section 3.2 below) e.g. using liver cells [34] and are not entirely

ecologically relevant. It is of course more complex and costly to design experiments aiming at an investigation of the combined effect of mixed exposure of EDCs to a whole organism but these types of experiments are really needed.

Perhaps, knowledge of the ecological impact of EDCs is preferably gained by vast field measurements in order to draw conclusions from field data. Studies of point sources by typically comparing the up-stream and down-stream data will not answer questions on reproductive changes in a whole river system. To do this it would be necessary to compare two separate river systems with different pollution status or large scale sampling of whole river systems. This type of studies has proven that there is a widespread sexual disruption in fish as a result of water pollution, by linking the high frequency of intersexuality in wild populations of fish, to chemicals in rivers of the United Kingdom [35]. This is an example of the type of large scale monitoring investigations that are very informative but scarce. Biological tools and chemical measurement of EDCs are both needed for better understanding of the ecological impact of EDCs on a local and global perspective and for pollution preventive actions.

1.3 Strategies for monitoring of EDCs

Basically, there are two main strategies for monitoring of EDCs in water. In one, a chemical analysis is performed to provide a measure of concentration of analytes with endocrine effects. In the other, biological methods are used to give a measure of total estrogenic load.

Analytical chemistry has traditionally dealt with chemical analysis and has aimed for accurate detection and quantification of very low levels of single chemical compounds. The process of sampling, sample preparation, instrumental analysis and data interpretation can be very tedious and has to be adjusted according to the properties of the compounds of interest. As mentioned

in section 1.1. above, EDCs are a heterogeneous group of compounds and the leachate water from a landfill or from raw sewage can be considered as a cocktail of numerous different organic and also metallic pollutants. As a consequence it is not realistic to identify and quantify all compounds with suspected endocrine effects in one water sample. For example, natural estrogens are not as lipophilic as PCBs and could not be retrieved from a water sample using the same sample handling. Methods like the one presented in Paper IV are very useful e.g. for the comparison of the treatment efficiency in different STPs regarding a single analyte but are less likely to give a good answer on the total estrogenic load in the effluent. In short, variability in pKa, polarity and stability among estrogenic compounds precludes test methods based on molecular structure for estimation of total estrogenic load and turns the focus towards test methods based on functional similarity. For estimation of the total estrogenic load it would therefore be more efficient to utilise biological methods. In cases where the estrogenic load is high it might be necessary to expand the measurement to include the whole battery of analytical methods available to track the most important pollutants in the sample. This is necessary for preventive actions.

Among the ecotoxicological methods to evaluate water quality and the hazards of chemicals, it is still common to use unrefined methods of acute toxicity testing. This would typically include the exposure of the common test species zebra fish (*Danio rerio*), to different concentrations of a chemical just to find out what is the concentration that will kill 50% of the tested animals. The outcome is the value of lethal concentration (LC_{50}). Since failure for a population to reproduce, in the long run, is just another way of slowly dying, chronic tests are just as important for evaluation of chemicals as tests for acute toxicity. Aquatic organisms with short reproduction cycle, like the water flea (*Daphnia magna*), may well be used to test chronic exposure effects over

several generations [36]. Ecotoxicological testing has also incorporated techniques from molecular biology and microbiology and different miniaturised in-vitro methods have been developed. These bioassays have also been embraced by analytical chemists and, as will be obvious in this thesis, the fields of ecotoxicology, biochemistry and analytical chemistry have converged when it comes to environmental monitoring. To exemplify the cross-breeding of analytical chemistry with biochemistry and to illustrate the purpose of a bioassay it is convenient to refer to the well known measurement of dioxins where bioassays have been used for quite some time.

The total load of dioxin-like toxicity in an environmental sample can be measured with an enzyme-linked immunoassay that has been constructed in accordance to the receptor-ligand interaction between the Aryl hydrocarbon receptor (AhR) and dioxin like compounds [37]. This biological quantification step is cheaper and faster than individual quantification of all dioxin-like compounds in the same sample using high resolution chromatography - MS.

Suppose that one wants to evaluate the total dioxin-like toxicity in a sample. There are generally speaking two options at hand. One option is to use chromatographic methods to quantify all individual dioxins, dioxin-like PCBs, and furans and multiplying each and all of them with their specific toxic equivalence factor (TEF) value that describes their potency to interact with the AhR. The other option is to use an immunoassay that will measure the total potency of all compounds in the sample towards the AhR. The estrogen receptor is a nuclear receptor just like the AhR and receptor-based bioassays for measurement of estrogenic EDCs have been developed and are further described in section 3.1 below. It should be mentioned that these types of bioassays with ER or AhR are often still depending of the traditional sampling and sample preparation procedures of analytical chemistry.

One disadvantage with chemical quantification of EDCs in a water samples is that you will only find those compounds that your method has been developed for. However, with biological methods the endocrine effect of unknown chemicals in the sample might also be accounted for. Some of the biological monitoring tools discussed below will also distinguish between antagonistic and synergistic effects.

To find out more about the ecological impact of EDC there is a need for large scale monitoring strategies. The extent of monitoring is obviously restricted to the number of samples that can be afforded and therefore there is a need for cost effective methods.

Cellular and non-cellular bioassays, very suitable for laboratory evaluation of endocrine effects in industrial chemicals, are presented in chapter 3 below. These methods are very helpful in finding the potential hazardous compounds among the thousands of chemicals not yet evaluated. Whole organism bioassays are more relevant from a physiological point of view and by using them it will be safer do draw conclusions on population-level effects.

2 Objectives

In our research group working with applied environmental analytical chemistry recent research has resulted in new strategies for monitoring of water pollutants such as a protocol for evaluation of leachate water from landfills [38]. Further, the inclusion of a biological test for acute toxicity [39] in the protocol has widened the field of application. Still there has been a lack of suitable tools in the protocol for evaluation of chronic toxicity in this type of water, especially to meet the growing concern for chemicals with endocrine effect. What is needed is a bioassay that is cost efficient, has ecological relevance and is sensitive for endocrine disruption but still robust enough to withstand the less favourable conditions of dirty water. It was early discovered that the indirect measurement of the biomarker vitellogenin by spectrophotometric quantification of alkali-labile phosphate (ALP) had great potential for cost efficient evaluation of endocrine effects in water.

The first main objective was to develop the ALP method for indirect quantification of vitellogenin and then to use the method for measurement on exposed fish. This resulted in Paper I that presents an improved, standardized and validated method for indirect measurement of VTG using molybdate in the spectrophotometric quantification with subsequent application on crucian carp (*Carassius carassius*), as presented in Paper III. Further, the quest for lower detection limit in the ALP methodology led to the development of a new quantification method using malachite green. This work is presented in Paper II together with application on rainbow trout (*Oncorhynchus mykiss*) from a field study in Vallkärra brook, Lund, Sweden.

The second main objective was to broaden the use of vitellogenin as a biomarker to include the use of a fish species that has a high tolerance for dirty waters. This would enable field measurement and characterization of water

which is too dirty to host traditional test organisms like rainbow trout. This led to an investigation of whether the very tolerant cyprinoid fish crucian carp (*Carassius carassius*) could be suitable for detection of endocrine disrupting chemicals with estrogenic effects. In Paper III the results from a laboratory test with injection of steroid hormones in crucian carp is presented and the suitability of using this species for monitoring of estrogenic effects is discussed.

A third main objective was to develop a chemical sampling and analysis technique for steroid hormones with sufficiently low detection limit for use in future tests with crucian carp exposed to water-borne steroid hormones in laboratory tests or in field exposure tests. Paper IV presents a method for measurement of steroid hormones by using hollow-fibre microporous membrane liquid-liquid extraction (HF-MMLLE) in combination with GC-MS.

The last objective was to develop an instrumental technique with the ability to differentiate the bioavailable fraction of persistent organic pollutants with endocrine effects from the non-bioavailable fraction. This would serve as a very useful tool in risk assessment of contaminated soil and sediment. Paper V presents a method for measuring bioavailability with supercritical fluid extraction (SFE).

3 Overview of biological techniques for monitoring of EDCs

A biological assay has several advantages over chemical analytical methods.

1/ The assay might in the best case provide the simultaneous measurement of the total estrogenic load in the water tested.

2/ The assay may include compounds not yet identified as estrogenic that would have been neglected in a chemical analytical method.

3/ With the exception of non-cellular bioassays, a biological assay can be designed to include synergistic or antagonistic effects in a complex mixture of substances.

An acute- or chronic toxic response in a multicellular organism starts at the molecular level. The starting point may involve the ligand-receptor interaction as in the case of a xenoestrogen binding to the estrogen receptor. (As will be discussed in section 4.4.3 below this is not the only mechanistic explanation of endocrine disruption.) The effect is then mediated to higher organisational level and will subsequently influence the function of an organelle or a whole cell. At the next level the effect is apparent at organ level and this may be discovered by e.g. using Liver Somatic Index (LSI) as a biomarker for toxic response. In severe cases the effect is also visible at the next organisational level which means that the whole organism may stop swimming/flying/drinking beer and so on. The use of bioassays for monitoring of EDCs can be divided according to the organisational level at which the endocrine effect is studied, as further discussed throughout chapter 3. A bioassay at the lowest organisational level would only contain biomolecules in a system for recognition of EDCs with immunological recognition or interaction with the estrogen receptor. A bioassay at the next organisational level would contain complete cells where the receptor interaction is an integrated part of a cellular response. A bioassay at the highest

organisational level would be one that involves a complete multicellular organism. Roughly it can be stated that the higher up in the organisational level the effect is studied, the higher the ecological relevancy. It is easier to predict the negative impact of endocrine disruption on fitness and reproduction from observations made at a higher level. On the other hand, it is not possible to understand the mechanism of action for an endocrine disruptive chemical at this organisational level due to the complexity of a whole organism bioassay. An overview of the types of bioassays that are available for studying estrogenic and anti-estrogenic effects is presented here below.

3.1 Non-cellular assay

In some quantitative assays it is desirable to eliminate the effect of different membrane permeability for the chemicals. In a non-cellular assay the chemical or mixture of chemicals do not have to penetrate a cell membrane to give a response. In an ELISA (Enzyme-linked immunosorbent assay) antibodies has been raised against estrogenic compounds and incorporated into a test kit for quantification of concentrations down to 20-40 ng/L [40]. This is 200 times higher detection limit than what can be achieved with chromatographic instruments and not sufficient for evaluation of most recipient waters without prior sample enrichment. This type of ELISA will only quantify immunologically recognised substances and will not discriminate between substances of high and low estrogenic activity. The estrogen receptor can also be used as the biomolecule for recognition in non-cellular assays. For easy interpretation the receptor system may be coupled with luminescent quantification in an ELRA (Enzyme-linked receptor assay) which enables quantification down to 20ng/L of E2 [41]. A similar system is an assay called ERBA (Estrogen-receptor binding assay) which contains sheep uterus estrogen receptors [42]. Organs, like the uterus, that are highly influenced by sex

hormones, contain much of the estrogen receptor and therefore cells from these tissues can be used in cellular assays.

3.2 Cellular assay

Breast cancer tumours are influenced by estrogen and therefore cellular bioassay for testing of estrogenic effects most often contain human breast cancer cells and sometimes kidney cells. The E-screen bioassay [43] contains breast cancer cells and in this system the cancer cells will respond to estrogenic compounds by increased proliferation. The degree of proliferation is compared to a standard typically exposed to known amounts of 17-β-estradiol and a negative control. A variation of this is the commercially available ER-CALUX® (Estrogen responsive chemically activated luciferase expression) assay [44] that contains breast cancer cells which have been bioengineered to contain a reporter gene. When cells are exposed to an estrogenic chemical the reporter gene will express the enzyme luciferase and after addition of the substrate luciferin the cells will luminesce [45]. Yeast cells are easy to culture and are therefore an attractive alternative. The YES (Yeast estrogen screen) assay [46] contains cell types which originally do no have the estrogen receptor so the cells have first been genetically modified to contain the receptor gene. Furthermore, a plasmid has been inserted that carries the estrogen response element (ERE) which in turn regulates a reporter gene expressing the enzyme β-galactosidase. Estrogenic compounds will bind to the estrogen receptor (ER) and two of these combined molecules will in turn form a dimer. The dimer will attach to the ERE in the plasmid leading to the expression of β-galactosidase. After addition of substrate the enzyme will catalyse the formation of a coloured product that can be quantified with spectrophotometry. The yeast assay is suitable for cheap evaluation of a large number of compounds and was used for evaluation of how the interaction with the estrogen receptor is dependent on the

structure of the alkyl chain in 37 different alkylphenols [13]. This is how the nonylphenol 4-(3,3,4,4-tetramethylpentan-2-yl)phenol in Table 1 was identified as the most potent alkylphenol.

Cellular bioassays can be used in combination with the quantification of the vitellogenin biomarker. This is possible since vitellogenin also can be expressed in natural or manipulated cell lines *in vitro*. Liver is the primary tissue for production of vitellogenin and hepatocytes from carp (*Cyprinus carpio*) [47] and rainbow trout [48] have been used in bioassays with expression of VTG as endpoint. This type of cellular bioassays might give good predictions on what to expect from *in vivo* tests using the same species and chemicals in a whole organism assay. The advantage with this approach over using the whole organism is the reduction of inter-individual variation, lower cost and less laboratory requirements. Additionally there is an ethical responsibility to try to reduce the use of animals in laboratory tests.

3.3 Whole organism assay

Fish is the most obvious choice of test organism for monitoring of chronic exposure to water pollutants. In toxicological studies fish is a well known test organism and standardized tests for acute and chronic toxicity e.g. in zebra fish (*Danio rerio*) has been around for quite some time. The use of fish in assays for endocrine disruption has been reviewed by Schultz et. al [49] and Wheeler et. al [50]. However, much of the knowledge about vitellogenesis and other physiological responses to steroid hormones in vertebrates has been gained from studies on the South African clawed toad (*Venous laevis*). The use of Amphibians in studies of endocrine disruption has been reviewed by Kloas and Lutz [51]. There are many similarities between the endocrine system of fish and that of invertebrates and vitellogenin is therefore also a suitable biomarker for estrogenic effects in invertebrates and this field has been reviewed by

Matozzo et al. [52]. An in vivo assay with a whole organism is the only way to study endocrine effects over several generations. These studies are very important since endocrine disrupting chemicals effects may have multi-generational effects [36].

3.4 Discussion and comparison of biologically based assays

Non-cellular assays will provide good quantification of EDCs and are not influenced by differences in metabolism, persistence and toxic responses. On the other hand these are factors that are beneficial to a whole organism biological assay in order to produce ecologically relevant data on estrogenic load. A non-cellular assay cannot distinguish between agonistic and antagonistic binding, whereas a cellular assays with vitellogenin production as endpoint has this advantage. Of course the choice of suitable bioassay is depending on what questions that need to be answered. A whole organism assay will not only reflect the external exposure but will also incorporate the effect from EDCs that have bioaccumulated in the organism. This type of assay has high ecological relevance but suffer from many insecurities and possible interfering factors. Moving along towards using “simplified” biological assays such as cell systems, or even further to just using biological molecules such as antibodies, the assays become less complex, easier to interpret but at the same time less relevant from an ecological- or ecosystem perspective. As an example a test system where the number of eggs and fitness of offspring from fish exposed to sewage water is evaluated, will give a legitimate answer to the question whether the mixture of chemicals in the sewage has a detrimental effect on the fish community in the recipient. However, no information will be provided on what chemicals are responsible for the effect and on the mechanism behind the observed effect on the eggs. A simple biological assay

with e.g. breast cancer cells containing the estrogen receptor can give a straight answer to whether a certain chemical has direct estrogenic potency but it cannot be concluded if the same chemical is persistent and bioavailable to a living organism in a recipient. The choice of test method may give very different results when comparing the estrogenic potency of different substances. A bioassay with cancer cells, Estrogen receptor-mediated chemically activated luciferas gene expression (ER-CALUX) assay, was compared with a bioassay with transgenic zebra fish in which the same luciferase reporter gene had been introduced as in the cancer cells [44]. The difference in potency of the natural hormone E2 and the synthetic hormone EE2 was compared in the two bioassays respectively. The synthetic hormone was 100 times more potent than the natural hormone in the test with whole fish but in the cellular assay they were equally potent. It is not surprising that the synthetic compound is more potent *in-vivo* since this is a medical compound designed to be somewhat stable in the human body to achieve the desired effect. (see the discussion on synthetic hormones in section 1.1.2. above). The importance of this comparison is that it highlights the drawbacks of using a simplified system. A comparison of the reaction in different assays to the same compound will give an indication of what bioassay that is capable of detecting very low concentrations. In Table 2 the EC_{50R} values for different bioassays are presented. The EC_{50R} is here defined as the concentration of agonist that provokes a response halfway between baseline and maximum response in the dose-response curve of the bioassay. The comparison of EC_{50R} values for different assays has been used by Gutenfdorf [53] as an indication of sensitivity.

Table 2. "Sensitivity" of different bioassays for 17β-estradiol. The EC_{50} value represents the concentration of 17β-estradiol needed in a bioassay to produce a signal equal to 50% of the maximum response in a dose-response curve.

Method	Endpoint	EC_{50R} 17β-estradiol	**Ref**
Non-cellular assay			
ERα assay	Binding to human receptor	3.5 nM (950 ng/L)	**[53]**
ERβ assay	Binding to human receptor	65 nM (18 ng/L)	**[53]**
Cellular assay			
Carp hepatocyte assay	Vitellogenin	100 nM (27 µg/L)	**[47]**
Yeast assay	Galactosidase activity	150 pM (41 ng/L)	**[54]**
Yeast assay	Galactosidase activity	100 pM (27 ng/L)	**[45]**
Breast cancer cell line	Luciferas activity	15 pM (4.1 ng/L)	**[54]**
Human transgenic cell assay	Luciferas activity	5-40 pM (1.4-11 ng/L)	**[53]**
ER-CALUX	Luciferase activity	6 pM (1.6 ng/L)	**[54]**
E-Screen	Growth induction in human breast cancer cells	5 pM (1.4 ng/L)	**[53]**
Whole organism assay			
fathead minnow	ALP	251 ng/L	**[33]**
fathead minnow	Inhibited egg production	120 ng/L	**[33]**
zebra fish	VTG	640 pM (174 ng/L)	[54]

From Table 2 it can be argued that the cellular assays are the most sensitive. There was also a difference when ER-CALUX was compared to a yeast assay. The former was more sensitive and gave an EC_{50R} value of 6 pM (≈1.6 ng/L) for 17-β-estradiol, which was 20 times lower than for the yeast assay [45]. The carp hepatocyte bioassay can detect compounds with an estrogenic potency of ≥ 2×10^{-5} [47] relative to E2, but as shown in Table 2 it is the least sensitive assay in this comparison. The benefit of this assay is that the relative potency of estrogens tested seems to correlate well with experiences from in vivo testing and therefore it might serve as a relevant tool for screening of EDCs. Cellular assays which are not sensitive enough to screen for EDCs in water samples may be complemented with a sample preparative step, e.g. with solid phase extraction (SPE), to enrich the analytes before analysis [55]. However, this will have the negative effect of also enhancing the concentrations of other chemicals

that might be toxic to the cells, a problem that is illustrated in the example below.

A yeast assay used for evaluation of the total load of EDCs in untreated sewage can suffer from serious interference from other toxic compounds. The yeast cells are living cells and some compounds can disturb the cell metabolism. In Figure 1 the measurement of estrogenic and androgenic effect in water from a municipal sewage treatment plant and untreated leachate water is presented.

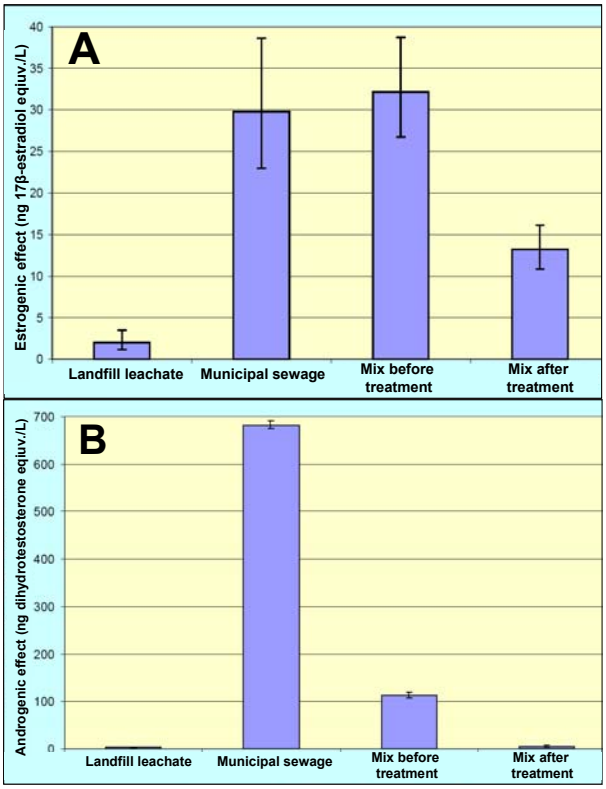


Figure 1. Modified yeast bioassay were used to evaluate estrogenic (A) and androgenic (B) effects in different flows in the sewage treatment plant of Fornby, Siljanäs, Sweden. The first column represents leachate water from Lindbodarna landfill before treatment in the plant and the second column represents municipal sewage before treatment. The third column represent the mixture of the two streams (where leachate water constitute approx. 10%) before treatment and the fourth column represent the mixed flow after treatment. (Figure from [56], used with permission from the author)

In Figure 1A the estrogenic effect is measured with a yeast bioassay in untreated municipal sewage and in the same sewage stream after it has been mixed with leachate water from a landfill. Since the estrogenic effect of the leachate water alone is much smaller than in the municipal sewage it might be expected that mixing the two streams would lead to a decrease in the overall estrogenic effect but in contrast to this the yeast assay shows an increased estrogenic load. (Since the variation is quite large in this data set the trend is a bit speculative but nevertheless the figure is very illustrative.) This is unexpected and it might be possible that the estrogenic effect in the leachate water is in fact much larger than shown by the yeast assay but the presence of substances that are toxic to the yeast cells will hide this effect. When the two streams are mixed, the toxic chemicals in the leachate water are diluted so that they no longer have a detrimental effect on the bioassay.

Figure 2B definitely shows that there are substances in the leachate water that are toxic to the yeast assay designed to measure androgenic effect. The municipal sewage water is only diluted 10% by the mixing with leachate water but the measured androgenic effect drops by more than 80%. It is very likely that this is caused by chemicals in the leachate water having growth inhibiting effects on the cell culture in the bioassay or that the chemicals in leachate have specific anti-androgenic action. This is an example of one of the drawbacks with using a cellular bioassay.

4 Biological monitoring of EDCs with the vitellogenin biomarker

A cellular bioassay with fish hepatocytes was described in section 3.2 above. The amount of the protein vitellogenin produced by the liver cells was used as an endpoint to evaluate the estrogenic effect. It is far more common that this female specific protein is quantified in whole organism assays and the use of increasing levels of the female specific protein VTG in male or immature fish has been widely accepted as a biomarker for estrogenic endocrine disruption, as reviewed in several publications [50, 52, 57-59]. The main part of my research has been directed towards the use of vitellogenin as a biomarker and the quantification of this biomarker. Therefore the entire chapter 4 in this thesis is dedicated to this subject.

4.1 Definition of a biomarker

For monitoring purposes a toxicological response in the environment can be used as an alarm signal. In chapter 3 above, the hierarchy of the toxicological response was mentioned for the sake of classifying different bioassays. The same sort of classification can be used to define and differentiate a biomarker from a bioindicator. The disappearance of certain invertebrate fauna in a river may be an alarm signal for deterioration of the water quality and this is a study of a bioindicator. By definition, a bioindicator is an endpoint studied at the hierarchic level above an individual organism and consider responses at population-, community-, or ecosystem level [60]. A biomarker is a biological response to an environmental chemical, studied at the hierarchical level of a whole organism or below, used for monitoring purposes. Elsewhere, the definition of a biomarker is stricter and draws the line below whole-organism

level, hence excluding endpoints of survival, growth and reproduction. In this case vitellogenin and other biomarkers are defined as: “biochemical, physiological, or histological indicators of either exposure to or effects of xenobiotic chemicals [61].

4.2 Vitellogenin as a biomarker

In oviparous animals the developing embryos are totally dependent on the nutrients and “building blocks” stored in the yolk. It is also well established that the yolk is not produced within the actual egg cell [62]. Instead the “building blocks” for the yolk are produced elsewhere, predominantly in the liver, and then transported through the blood as precursor molecules before uptake into the growing oocyte. In all teleost fish the most important of these precursors is vitellogenin. Female fish invest in their offspring by allocating a lot of energy into producing vitellogenin and this process is under hormonal control. This hormonal control is challenged by endocrine disrupting chemicals which are capable of disturbing the vitellogenin production as well as other vital functions under hormonal control. As will be discussed below in section 4.3, describing natural vitellogenesis, estrogen is the hormone that controls the production of vitellogenin. The natural level of estrogens circulating in the blood of male or immature fish is very low and so is the vitellogenin concentration. A most obvious type of endocrine disturbance is when xenoestrogens induce the unnatural production of vast amounts of vitellogenin in male or immature fish. Hormones are effective already at low concentrations in the blood system and so are xenoestrogens. Therefore the increase of vitellogenin in male fish is a sensitive biomarker for endocrine disruption.

A sensitive biomarker will respond already at low exposure levels and therefore the physiological response within the test organism may very well be within the homeostatic response. This means that the biomarker not necessarily indicates

detrimental effects for the organism. On the positive side this means that the biomarker serves as an early warning signal before acute toxic effects appear. A good biomarker will also be a specific one. Vitellogenin is a very specific biomarker for estrogenic effects. On the negative side is that the high specificity and sensitivity of the vitellogenin biomarker makes it difficult to predict what the measurement of the biomarker can tell us about effects on population level. Measurement of Liver Somatic Index (LSI) as a biomarker for toxic response in fish means measuring at a higher level of hierarchy. The measurement is conducted at organ level in contrast to VTG that is a measurement at molecular level. The LSI biomarker represents a measurement of total toxic stress in the organism and does not have the same specificity as the VTG biomarker but on the positive side is that more reliable predictions on population effects can be made. It is a general argument within ecotoxicology that there is a trade-off between specificity and ecological relevance when choosing a biomarker [60].

Even if elevated levels of VTG in male or immature fish is a clear signal of endocrine disruption this does not have to mean that the fitness and reproductive capacity of the fish has been affected. Biomarkers can be classified into biomarkers of exposure and biomarkers of effect (meaning: toxic effect) [60], even though it might be difficult to distinguish between the two at times. If the biomarker is intended to be used as an indicator/predictor of effects the biomarker response must be tightly linked to responses at higher organisational levels [63]. It can be discussed whether vitellogenin can be considered as a biomarker of exposure or as a biomarker of toxic effect. It is a great challenge to evaluate to what extent VTG can be used to predict the negative impact of EDCs on fish reproduction. In laboratory experiments the ability of male Cunner (*Tautogolabrus adspersus*) to fertilize eggs was not correlated with VTG levels but there was a negative correlation between VTG levels and Gonado Somatic Index (GSI) [64]. These results are inconclusive

because the first result can be used as an argument that VTG levels in male fish is not a biomarker of toxic effect while the second result speak in favour of this. Since the biomarker obviously is linked to a response at a higher organisational level, GSI, the criteria for a biomarker of effect is fulfilled. The effect at a higher organisational level may not have to be a physiological one. Vitellogenin is a biomarker of effect, with ecological relevance, if increasing levels in blood can be linked to a change in animal behaviour. Kramer [33] found a negative correlation between plasma levels of ALP in male fish and the number of eggs laid by females in a laboratory study. An explanation was suggested in that male behaviours such as courtship and defensive behaviour are responsible for inducing female egg laying. The use of vitellogenin as a tool for monitoring of EDCs, as discussed in this thesis, is not depending on whether this biomarker is defined as a biomarker of exposure or effect. It is however important to understand the processes of both natural and induced vitellogenesis in fish.

4.3 Natural- and induced vitellogenesis in fish

Vitellogenesis in fish is the hormonally controlled process whereby the vitellogenin macromolecule containing protein and lipids essential for the growing eggs are formed in liver cells, transported in the blood, taken up as vesicles in the ovary and finally split into the yolk components, which are stored in yolk platelets inside the evolving oocytes. At later stages of oocyte development this is obviously a massive event in female fish, since the ovaries may grow in size from 1% to 20% of the total body mass [62]. This growth can mostly be attributed to all the vitellogenin that has been produced in liver and shuttled through the blood system. The magnitude and seasonal trend in vitellogenesis is of course variable. The reproduction strategies are diverse among the 20 000 species [65] of teleost fish and some differences between a

single- and multiple spawner are discussed in connection with Paper III and section 4.6.3 below.

It could be argued that it would be "evolutionary suicide" to allocate all this energy to vitellogenin production throughout the whole year and perhaps this can help understanding the complicated system of hormonal control. In Figure 2 a simplified description of the hormonal control of vitellogenesis is depicted. As in many other cases of hormonal control there are many feedback mechanisms that helps balancing the system but in this description they have been left out for simplicity.

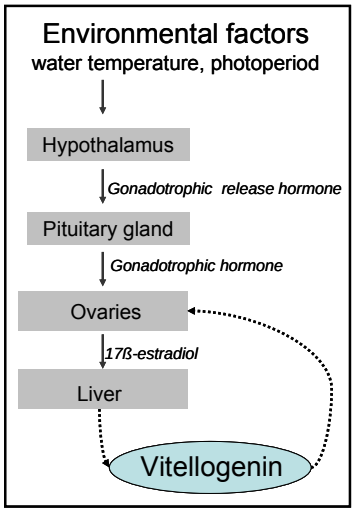


Figure 2. Natural vitellogenesis in fish.

As especially obvious in the climate of Northern Europe, the start of the reproductive season may be indicated by environmental factors such as a change in water temperature or photoperiod. Figure 2 shows how the sensory stimuli will cause hypothalamus to secrete gonadotrophin-releasing hormones. This signal will reach the pituitary glands which in turn releases gonadotrophins

into the blood system. When the gonadotrophins reach the gonads, or more precise the follicle cells, the ovaries start to produce and release 17β-estradiol and to a lesser extent other estrogens. The production of vitellogenin in liver cells is under hormonal control by estrogen and therefore the complete liver cells as well as the organelles mitochondria and Golgi apparatus will enlarge in response to 17-β estradiol. In liver cells the onset of vitellogenesis can also be observed in proliferation of secretory vesicles and rough endoplasmatic reticulum together with an increase of ribonucleic acid (RNA), lipids and glycogen [62]. Hormones may have several interaction sites and gonadotropins simultaneously regulate the ovarian uptake of vitellogenin from the bloodstream and will have a negative effect on blood concentration of vitellogenin [66]. Hence, the amount of vitellogenin in the bloodstream is not controlled by the estrogens alone. For the discussion in Paper III and section 4.6.3 below it is important to remember that the amount of vitellogenin present in the blood at a certain time is the net balance of vitellogenin production and vitellogenin accumulation. Laboratory estrogen-injected females will therefore stimulate vitellogenin production but not accumulation in oocytes [67].

When vitellogenesis is studied in laboratory test or in field-caged animals it is important to be aware that the link between environmental factors and hormones also means that stress can also influence vitellogenesis. A sudden change in water parameters, handling or confinement of fish can trigger the release of stress hormones such as the glucocorticoid cortisol. Stress factors such as confinement [68] or acidic stress [69] can suppress vitellogenin levels in female rainbow trout. This negative effect can be due to decreased levels of estrogen or decreased susceptibility in the estrogen receptor mediated response in the liver. In chub, that has high basal cortisol levels, a stress induced increase of cortisol coincided with a decrease in estrogen level in confined females [70]. In contradiction, for confined female rainbow trout cortisol levels increased,

vitellogenin decreased while estrogen levels were unaffected [68]. Decreased susceptibility of the estrogen receptor response by the action of glucocorticoid stress hormone has been indicated in a detailed study on rainbow trout [71]. In the normal estrogen response in liver cells, estrogen binds to the estrogen receptor but estrogen also has a positive effect on the transcription of more estrogen receptors. This is because estrogen binds to the promoter that promotes the transcription or the estrogen-receptor gene. When cortisol binds to the glucocorticoid receptor this interferes with, and will have a negative effect on, the promoter gene responsible for the transcription of the estrogen receptor gene. Also in captive female lizards, stress caused a reduction in the vitellogenin synthesis [72].

Another factor that might influence natural vitellogenesis as well as the interpretation of the biomarker response is the nutritional state of the organism [73]. For rainbow trout it has been shown, that a diet deficient in essential poly unsaturated fatty acids may lead to reduced concentration of serum vitellogenin [74].

The general process of vitellogenesis is very similar in fish and amphibians. Many studies on the South African clawed toad (*Xenopus laevis*) has helped in understanding the process in fish. However there are differences between amphibians, birds and fish in the chemical composition of vitellogenin as well as differences among different fish species. The properties of the vitellogenin biomarker is described here below for better understanding of the basis for indirect quantification described in section 4.6.

Vitellogenin is often referred to as “a protein” while the correct biochemical description is a glycolipophosphoprotein. The protein backbone synthesised in the liver is a polypeptide with two major subunits namely lipovitellin and phosvitin. From studies with South African clawed toad, it has been estimated that the lipovitellin protein monomer has a molecular weight of about 200´000

and can be subsequently split into one non-phosphorylated peptide of 120´000 and one phosphorylated peptide of 31´000 [66]. The dominating protein-phosphorus content in the vitellogenin macromolecule can be attributed to the other monomer phosvitin which has a molecular weight of about 35´000-40´000. This monomer is rather unique since over half of the amino acids are serine. The phosphorous content is 9.5% and this means that nearly all serine are phosphorylated [75]. In trout, the whole macromolecule has a phosphoprotein phosphorus content of about 0.6% [76]. The process of protein modification after translation has been studied in rooster and it was found that phosphorylation took place after glycosylation and thereafter the vitellogenin was quickly excreted from the hepatocyte [77]. The phosphorylated serine amino acids are especially interesting for the indirect quantification of vitellogenin as will be discussed in section 4.6 below. In addition to this there is also a large fraction of phosphate present as components of phospholipids. The whole macromolecule contains 18-21% lipids of which two-thirds are phospholipids [78]. The lipid content of vitellogenin is higher in teleost fish in comparison with other oviparous vertebrates such as the domestic hen (15%) [79].

While still circulating in the plasma it has been suggested that the lipid material is non-covalently bound to the lipovitellin sequence of the polypeptide chain [62]. The complex also binds calcium and to some extent it functions as a shuttle for lipid-soluble substances such as carotenoids in salmonids [80].

Even though the general process of vitellogenesis is similar in *Xenopus laevis* and teleost fish it has been shown that the yolk proteins in teleosts are atypical. Teleost lipovitellins seem to be less phosphorylated while the phosvitins vary in protein-phosphorus compared to amphibians [66]. This leads to a larger overall variability of phosphorylation in fish vitellogenin as compared to more consistent features in the vitellogenins of amphibian and avian species [81].

Even if to a lesser degree than for *Xenopus laevis*, teleost yolk proteins are very rich in phosphoserins and the phosvitin domain in rainbow trout VTG consist of about 50% serins [82]. The high variability of phosphoserins may just be coincidental mutations or may in fact have evolutionary relevance. Teleost species reproducing in freshwater are suggested to have vitellogenin poorer in phosphorus as compared to non-teleosts vitellogenins [76]. Hypothetically, differences in the vitellogenin molecule could be the result of evolutionary selection for e.g. high phosphorylation because of the need of the high binding energy of phosphate-protein during embryonic development for some species [83]. The characteristics of the yolk proteins in the platelets will also determine their water solubility and evolution could favour water soluble proteins in free-floating eggs because oocytes are adjusted for the right buoyancy by great accession of water fluid [84].

Atypical yolk proteins in teleosts are also probably reflected in high variability in antibody cross reactivity. An immunoassay developed for vitellogenin measurement in sera from rainbow trout (*Salmo gairdneri*) showed little cross reactivity with sera from the closely related atlantic salmon (*Salmo salar*) [85].

4.4 Modes of action for endocrine disruption

Natural vitellogenesis as initiated by the action of natural estrogens, and the induction of the biomarker in male or immature fish by xenoestrogens, are both processes that are mediated via the interaction with the estrogen receptor. For better understanding of the concept of endocrine disruption this mechanism needs to be discussed further as well as other types of endocrine effects that are not mediated through the estrogen receptor.

4.4.1 Nuclear receptor interactions

The estrogen receptor (ER) is a member of the nuclear receptor family, a group of receptors with a common mode of action. A good review on the interaction between chemicals and nuclear receptors was presented by Janosek [86]. After binding to a ligand this type of receptor is transferred to the nucleus, where they will bind to a specific responsive element on the DNA and in this way trigger gene expression. (See also the explanation of a cellular bioassay with estrogen receptor in section 3.2 above) There are about 47 other nuclear receptors that have been identified to this day. The nuclear receptors are subdivided into three classes where class I represents the steroid hormone receptors. Class II includes among others the aryl hydrocarbon receptor (AhR), thyroid receptor and vitamin D receptor. The receptors for which the ligands have not yet been identified are called Class III nuclear receptors, or orphan receptors.

Compounds can bind to a steroid receptor and elicit the same effects as the endogenous hormone. This is referred to as an agonistic action. The binding of a chemical to the estrogenic receptor does not automatically classify the compound as being estrogenic. Some chemicals are antagonistic and bind to ER without triggering gene transcription. In this way the receptor is blocked from interaction with endogenous estrogens. The use of the term “estrogenic” effect is not very strict and sometimes refers to compounds that can mimic the endogenous hormone and interact with ER. In other cases “estrogenic” might just mean that the compound has the same overall effect as an estrogen, e.g. an increase of VTG production. It is possible to test whether a chemical has direct estrogenic effects mediated via coupling to the ER or if the estrogenic effects observed are caused by other mechanisms. A suspected xenoestrogen can be tested in a cellular- or organism- bioassay alone, or in combination with tamoxifen which is an estrogen antagonist that will block all ER interaction. If the estrogenic effect of the EDC is blocked by tamoxifen, the effect was

mediated through the estrogen receptor. Even if the binding of a chemical to a receptor leads to a limited gene expression, the overall effect can be said to be antagonistic if many receptors are blocked and hindered from binding to endogenous estrogens with a potential stronger effect.

Much research has been conducted in relation to the biochemical lock-and-key interaction of a receptor and ligand, not the least in medical sciences. Do we have knowledge enough to make predictions on the binding capability to ER of different EDCs just on basis of the structure of the xenobiotic compound? Based on the high diversity among the known ligands it has been argued that this is not likely [43].

Predictions are further complicated by the fact that the estrogen receptor is not identical in vertebrates. For instance the resemblance in amino acid sequence between the receptor in rainbow trout and the receptor in lizard is 88 % and furthermore, closely related lizards express different amounts of the receptor [87]. These facts speak for very diverse susceptibility to estrogenic compounds in different species.

The estrogenic effect is not only controlled by the amount of estrogenic ligand but also by the number of available receptors. The number of receptors are in no way static and in section 4.3 above it is described how estrogens may up regulate the number of receptors. It is also well known that in humans different tissues respond in different ways to estrogens. This is because there are two subtypes of estrogen receptors and the allocation of the two different ERα and ERβ subtypes will influence the response to estrogens. An endocrine disruptor that by some mechanistic action will lead to an up regulation of the number of estrogen receptors can therefore be said to be an estrogenic chemical. A reversed effect would label the compound as being anti-estrogenic. For example it has been suggested that the anti-estrogenic effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) stems from its ability to decrease the

number of available receptors and this without interacting directly with the ER [88]. Dioxins also seem to manifest indirect estrogenic effects according to Ohtake [89] but otherwise dioxins are most well known for their strong interaction with the Ah-Receptor which historically is referred to as the dioxin receptor. The AhR is also a nuclear receptor and some PCBs are known to bind to both the AhR and to ER. This "cross talk" between these two receptors is a very interesting research field and the similarities between these receptors suggest that one could expect to find many more compounds with ability to interact with both of them. It has been shown that AhR after activation by dioxins can directly interact with both ERα and ERβ [89].

4.4.2 Interactions with hormone production or hormone metabolism

Endocrine disruption can be the result of disturbance of the production or metabolism of endogenous hormones. Cholesterol is the product from which all androgens and estrogens are synthesised by a chain of different enzymatic reactions. The amount of cholesterol available for initial steroidogenesis can be altered by EDCs. Further steroid transformations are executed by enzymes in the cytochrome P450 family which also can be affected by EDCs. The most well known example of this is the ability of tributyltin to inhibit the P450 aromatase enzyme and thereby the transformation of testosterone to E2. The indirect effect of chemicals that are considered toxic but not normally referred to as potent EDCs deserves an extended discussion. The liver cells are not only responsible for detoxification of xenobiotics but are also the site for deactivation of steroid hormones. The ability of xenobiotics to trigger the P450 system through the AhR is well known [90]. By this mechanism the detoxifying enzymes are up regulated as e.g. manifested by the increase in EROD activity after exposure to PAHs. What is often forgotten in this context is that this activation might also lead to an endocrine effect since many of the P450

enzymes are responsible for both synthesis and deactivation of steroid hormones. The mechanism of the P450 system in vertebrates is nearly always mentioned in the context of xenobiotic substances that has to be neutralized or excreted by the detoxification system of an organism. Most knowledge of how this system work has been gained from experiments with xenobiotic substances and it is well known how liver cells in vertebrates react to the exposure of dioxins through the AhR and how this leads to an upregulation of mixed-function oxygenases (MFOs) [90, 91]. As mentioned earlier, the receptor itself has from time to time been referred to as the dioxin receptor. This nomenclature is not ideal since it is not very probable that the receptor has evolved through natural selection for a defence mechanism dealing with dioxin! The MFOs did not evolve to protect the organism from man-made chlorinated toxins like dichloro-diphenyl-trichlororethane (DDT), PCB, or PBDE, invented during the last 50 years nor did they evolve to facilitate the bioactivation of medical substances invented during the last 20 years. It is instead more reasonable to consider the AhR, and the enzymes that it regulates, as a system for the metabolism of biochemicals normally present within the organism. Maybe it just happens to be that PAHs have a similar ring structure as steroid hormones and therefore they are both substrates for the same enzymes. Kime [4] argues that toxicologist tend to focus too much on the MFOs as indicators of toxic exposure and therefore forgetting about the tight connection between the metabolism of xenobiotics and intrinsic hormones. He states that a stimulation of MFOs by pollutants almost inevitably leads to some kind of disruption of the endocrine system! It is therefore a bit unlucky that most discussion on EDCs has been almost solely related to compounds that are able to interact with the estrogen receptor.

4.4.3 Other examples of mechanism in endocrine disruption

The discussion on endocrine disruption has mainly been focused on the estrogen receptor and compounds capable of interacting with the same. The interaction between the receptor and an estrogenic "impostor" is indeed an important mechanistic explanation for the disruption in vertebrates and this mechanistic explanation is also relatively easy to explain to the general public. How important is the ligand-receptor interaction really if compared to other ways that xenobiotics can interact with the hormone system?

EDCs may interfere with the endocrine system anywhere along the hormonal axis by disturbing hypothalamic release factors, gonadotropins, feedback mechanisms or hormone binding proteins. A double effect of PCBs can be used to illustrate the importance of both hormone binding transporter proteins and feedback mechanism. PCBs have a negative effect on total plasma concentration of thyroid hormone. The mechanism behind this is the competitive binding to the thyroid transporter protein which leads to increased glucorination and excretion of thyroid hormone. This will also lead to an elimination of the negative feedback loop that thyroid hormone has on the pituitary thyroid-stimulating hormone (TSH) secretion. The overall effect will hence be an increase in the size of the thyroid gland as the gland struggles to compensate for the loss of thyroid hormone [92].

In many cases the endocrine effects of are not mechanistically fully understood as in the case of nonylphenol (NP). Nonylphenol is known to bind to the ER but this interaction is not very strong. Still, strong estrogenic effects have been shown in exposure studies and an extended discussion on NP is motivated for the sake of illustrating complex interactions leading to estrogenic effects.

Spontaneous intersex or sex reversal has never been observed in the Japanese Medaka (*Oryzias latipes*) but after laboratory exposure to 0.05 mg/L of 4-nonylphenol, 50% of the individuals developed both testicular and ovarian

tissue in the gonad [93]. Considering NP, the pollution source is from sewage, sewage sludge and sea sediments close to urban areas. As large amounts as 13´000 µg/kg has been found in sewage-impacted coastal areas in Jamaica [94]. Nonylphenol can indeed bind to the ER in an agonistic way but the ligand-receptor affinity is 1000 times less potent than for the natural endogenous ligand E2 [95]. Instead, one important feature of NP is the capability to indirect capability to induce changes in the endogenous hormone concentration rather than having a direct agonistic effect on the ER. In a study with fathead minnows the exposure to 0.05-3.4 µg/L of NP resulted in an average increase in estrogenic potency in the plasma of 900% [96]. Only 4 % of this increase in E2-equivalents could be attributed to the actual increase of plasma 4-NP and the rest was attributed to an indirect effect resulting in increased levels of E2. The matter is further complicated by the fact that only bioavailable compounds can interact with the receptor. In the blood E2 is bound to the steroid hormone-binding globulin (SHBG) with high affinity, while alkylphenols bound less tightly to the same protein [97]. Another factor that is important for the comparison of estrogenic potency between E2 and NP is the persistence. NP, being an industrial chemical is not as easily degraded in the vertebrate body. Bioaccumulating chemicals can affect an organism during long time and if they are passed on to the eggs they pose an immediate threat to the evolving offspring. The biological half-life of a chemical is a factor that will explain why different test methods give different answers when evaluating how estrogenic a compound is. Kepton was shown to be around 100´000 times less potent as E2 when evaluated by an in vitro test [43]. The same compound was only 1000 times less potent when evaluated by in vivo testing with rats [98].

4.5 Direct vitellogenin measurement with immunological methods

There are several methods for quantification of the biomarker vitellogenin and measurement often comprises antibody-based immunoassays or radioimmunoassay. Antibodies can be raised against VTG and will have minimal interference from other proteins. Reproducibility can be achieved with available antibody-base commercial kits for vitellogenin even though they are expensive to buy and expensive to keep fresh during transport. These kits are species-specific but even when there is a high cross-reactivity for other species, they will not provide a 100% quantitative measurement of the unique vitellogenin, if not the right standard is used. If a 100% quantitative measurement is asked for, vitellogenin from the species of interest need to be extracted and purified for use as quantitative standard and this without affecting the antibody-binding capabilities of the macromolecule. There is of course the alternative to develop species-specific antibodies but not all laboratories have this possibility. As long as the protein standard utilised to generate a calibration curve for the immunoassay is from the same species as the measured fish, it is not necessary to use anti-bodies raised for that species. A somewhat lower degree of recognition for the anti-bodies will affect the sample and standard to the same extent.

The cross-reactivity of anti-bodies in test kits for measuring VTG is somewhat unpredictable. High cross-reactivity has been shown for different species within the cyprinid family [99] but in another immunoassay developed for vitellogenin measurement in sera from rainbow trout (*Salmo gairdneri*) there was little cross reactivity with sera from the closely related Atlantic salmon (*Salmo salar*) [85]. There has been some success in the process of developing a universal antibody capable of detecting VTG in any oviparous vertebrates [100] but the need for a

species specific standard in form of a purified protein is probably difficult to circumvent. As a consequence it might be necessary to accept that a universal method for VTG is not 100% quantitative without the proper standard solution. If cost-effectiveness is necessary it might be wise to look for alternative methods in stead of pursuing the ultimate immunoassay.

There are different methods to quantify vitellogenin and who wouldn't agree with the statement made by Marin and Matozzo that "the choice of the best method to measure vitellogenin levels need to take into account feasibility, sensitivity, reproducibility and cost-effectiveness [101]? An indirect measurement relying on small volumes of bulk chemicals, simple separation techniques and spectrophotometric detection is an attractive cost-effective alternative. In fact, such a method was used long before there were any antibody-based methods around.

4.6 Indirect vitellogenin measurement through Alkali-Labile Phosphate

VTG is very dominant in the plasma after the onset of vitellogenesis and the protein is heavily phosphorylated. These two prerequisites enables indirect quantification through measurement of alkali-labile protein bound phosphate (ALP) as an alternative to the more expensive enzyme linked immunosorbent assay. The amount of protein-bound phosphate is a good estimation of how much VTG that is present in plasma and this measurement is conducted either on the complete isolated protein fraction from a volume of plasma or alternatively on the fraction of lipoproteins that has been extracted from plasma. This quantification may appear as a non-specific and blunt tool to quantify a certain protein but is feasible due to the unusual features of this molecule. VTG is in fact the most phosphorylated amino acid sequence in nature and this is

because the amino acid backbone of the phosvitin subunit is so unusually rich in serine amino acids of which nearly all are phosphorylated [102].

It has even been stated that VTG is the *only* phosphorous containing protein in the blood of oviparous vertebrates [103]. Even if this statement turns out to be not entirely true, the usefulness of ALP measurement is still unchallenged. Up till now the ALP method has been used in at least 27 publications for indirect quantification of VTG in fish e.g. [33, 104-106] but also for measurement in mussel [107-109], in clam [110-113], in crustacean [114] and in lizard [115]. It has often been stated that the good correlation between ALP and direct quantification, when both methods have been used simultaneously, justifies the use of the indirect method. Good correlation coefficients, with r-values between 0.73 and 0.99, have been obtained for measurements in species like *Centropomus undecimalis* [116], *Salmo gairdneri* [117], *Scyliorphinus canicula* L.[118], *Oncorhynchus mykiss* [103] and *Sceloporus occidentalis* [119].

In studies where the information about the absolute quantity of VTG is crucial, traditional measurements with immunological techniques are of course preferable. However, in exposure studies where exposed and unexposed groups are compared the ALP method may be just as good. In 2002 Verslycke et al. [103], showed that direct and indirect methods have similar sensitivity for detecting induction of VTG in rainbow trout. The ALP method has mostly been used in applied publications and less focus has been direct to the analytical method itself why there is certainly room for improvements.

4.6.1 Method development of a molybdate based ALP method

As mentioned, the ALP methodology has been widely used. The cited spectrophotometric method for quantification of phosphate has most often been the one developed by Stanton from 1968 [120], but there has been a large variation in how the sample preparation steps has been executed. As stated by Porcher et al. [121] all VTG quantification methods need to be standardized before they can be used in regulatory tests. In principal there are two ways to initially separate the protein fraction of interest from the plasma sample. One alternative is to extract the lipoproteins with a non-polar solvent like t-butyl methyl ether and the other is to precipitate the complete protein fraction in plasma. (Figure 3 describes the latter alternative.) Thereafter the protein pellet needs to be washed in order to remove all traces of plasma. This is an important step, since free phosphate in the plasma present in concentrations from 40 to 256 µg/ml [122, 123], might disturb the measurement of protein bound phosphate. Paper I presents a standardized sample preparation procedure that was developed and validated. The complete scheme for this methodology is presented in Figure 3.

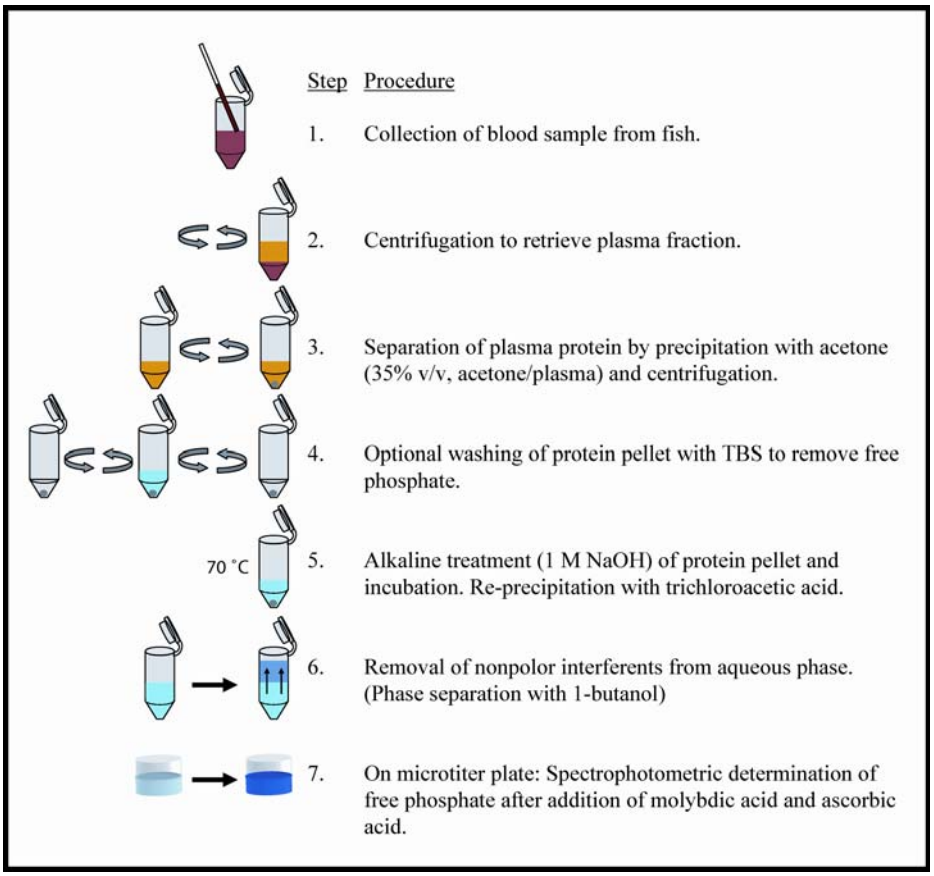


Figure 3. The complete scheme for determination of alkali-labile phosphate (ALP) in fish blood as developed and standardized. (Paper I).

After isolation of plasma proteins by precipitation and centrifugation, the addition of alkaline solution will break the bound phosphate groups. Phosphate is finally measured colorimetrically as a phosphomolybdate complex which produces a blue colour in its reduced form. The colorimetric determination of inorganic phosphate has a long history and as early as 1920 Bell and Doisy [124] presented a method for the colorimetric detection of inorganic phosphate in biological material based on the fact that phosphomolybdate is more easily reduced then the molybdate substrate. In 1925 Fiske and Subbarow [125]

presented an adjusted method that has been widely used. Later adjustments and variations include the suggestion of Lowry and Lopez in 1946 [126] to use ascorbic acid as reducer. The reducing agent along with pH, temperature and molybdate concentration controls the reduction and colour development of both phosphomolybdate and the molybdate substrate. Much of the work in preparation of Paper I involved the optimisation of these parameters. In Figure 4 the optimisation of ascorbic acid concentration is illustrated.

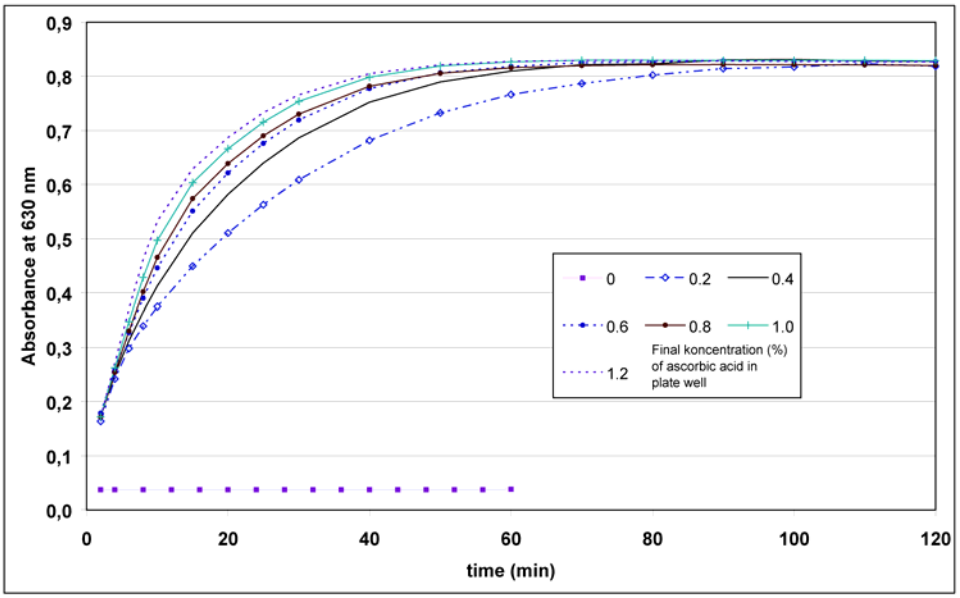


Figure 4. Influence of concentration of ascorbic acid (%) on the reduction of phosphomolybdate complex. Plate wells were filled with 25 µl phosphate standard in 1 M NaOH and decreasing volume of water. 50 µl of molybdate reagent in 2 M H_2SO_4 and increasing volume of a 4.9% ascorbic acid was added. Final well volume was always 245 µl and pH=0.61 (estimated with CurTiPot software[127]). The dotted line represents no addition of ascorbic acid.

The selectivity of ascorbic acid as reducing agent was obvious as no blue colour was formed by molybdate when the reagent was omitted (Figure 4) and this can be of great use. By analysing phosphate in duplicate plate wells the background absorbance can be calculated by simply omitting the addition of ascorbic acid to one of the well duplicates. This procedure is motivated since there was no difference between a phosphate standard without ascorbic acid and a reagent blank. If the absorbance in a sample without ascorbic acid added is slightly higher than for the reagent blank, this difference represents the matrix effect from plasma residuals. However, this way of estimating the non-phosphate related background absorbance can only be used when the interference is quite small. Previous ALP methods based on the spectrophotometric method of Stanton [120] includes two separate readings at different wavelengths to adjust for background interference. The suggestion made in Paper 1 to utilise the selectivity of ascorbic acid for estimation of background absorbance is a simpler and safer alternative to duplicate readings at two wavelengths but the best alternative would be a sample preparation that totally removes all interfering compounds. In cases where an emulsion is formed spectrophotometric reading is impossible and in Paper I it was investigated if this could be taken care of by introducing a phase separation step.

The blue phosphomolybdenium complex has an absorbance maximum around 800nm [120]. Figure 5 and 6 depicts the experiments preceding Paper 1 where absorbance spectra for samples with and without the introduced phase separation step were compared. These results demonstrated that without a phase separation step, a straightforward phosphate quantification using a single absorbance reading of the blue colour is unfeasible due to emulsion causing a high background absorbance. There is a high absorbance all through the spectrum even in a sample of human plasma that is expected to contain low levels of ALP (Figure 5, curve b). The same plasma spiked with phosphate generated a spectrum with a slight increase of absorbance in the 600-800 nm region and a quantification based on this increase solely would not be very accurate (Figure 5, curve a). In the method by Stanton [120] scattering correction can be calculated by simultaneously measuring absorbance at lower wavelengths but this is only feasible in cases where the disturbance is less pronounced.

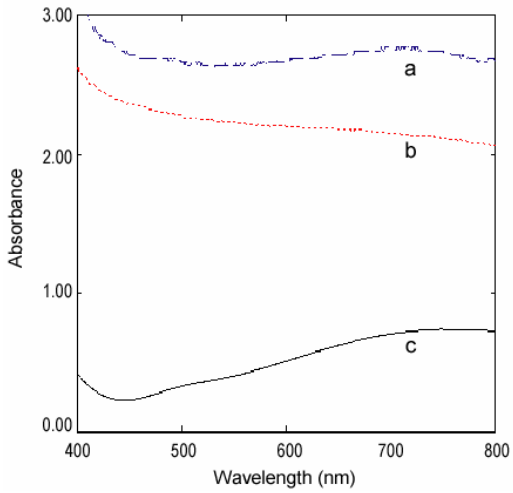


Figure 5. Absorbance spectrum of the blue phosphomolybdenium complex in: alkali-labile phosphate analysis without butanol extraction of human plasma spiked with phosphate (a), alkaline-labile phosphate analysis without butanol extraction of human plasma (b), and reagent blank spiked with phosphate (c).

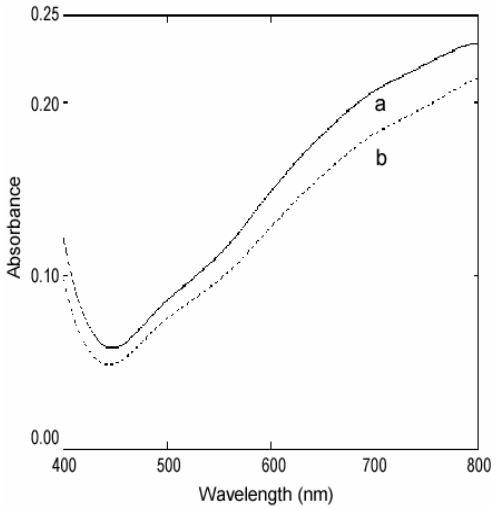


Figure 6. Absorbance spectrum of the blue phosphomolybdenium complex in: alkaline-labile phosphate analysis with 1-buthanol extraction of fish plasma (a) and reagent blank spiked with phosphate (b).

Absorbance spectrum for calibration solutions are shown in Figure 5, curve c and Figure 6, curve b. When a phase separation step with 1-butanol was introduced just before colour development the emulsion was removed successfully and the absorbance spectrum for fish plasma (Figure 6, curve a) was very similar to those of the calibration solutions. Centrifugation just before colorimetric reading was also tested as an alternative to phase separation for removing the emulsion, but in accordance with Stanton 1968 [120] this resulted in a loss of phosphomolybdate.

Figure 7 represents a typical measurement of ALP in wild captured Crucian carp according to the standardized procedure presented in Figure 3.

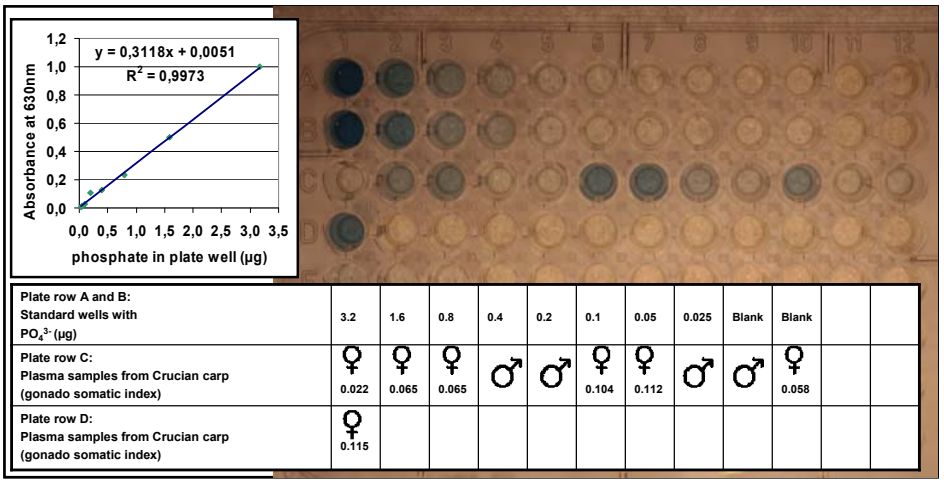


Figure 7. Indirect measurement of vitellogenin by alkali-labile phosphate in plasma samples from Crucian carp. Sample preparation according to Figure 3 and spectrophotometric quantification of phosphate with molybdate and ascorbic acid.

Figure 7 illustrates how samples from female fish are easily distinguished by higher plasma ALP levels as shown by the distinct blue colour. Further, females with higher GSI had higher levels of ALP. Phosphate standards for the calibration curve for are easily mixed directly on the plate by a series of dilution steps with a pipette (Figure 7, rows A and B). With a fast phase separation step the spectrophotometric phosphate determination using molybdic acid and ascorbic acid has been improved and practically all matrix interference has been eliminated. The common spectrophotometric measurement developed by Stanton [120] involves measurements at two wavelengths and calculation of a corrected absorbance value, and the simplified absorbance reading was one of the novelties presented in Paper I. The final ALP method presented here has a detection limit of 3.2 µg PO_4^{3-} / ml plasma which is six times lower limit of detection [33] and limit of quantification [117] than in previous methods. The variability during triplicate analysis of pooled plasma was 6% in this study and also in a former by Tinsley [128] with the difference that ALP levels in our

samples were 3 times lower. It can therefore be concluded that the developed method ensures a low variability.

Absorbance values from unknown samples might not fall within the range of the calibration curve and therefore it might be beneficial to create a dilution series of the unknown sample. This is most easily done on the plate but in this case it is important to assure that the dilution process does not affect the colour reaction.

4.6.2 Method development of a malachite based ALP method

The colorimetric estimation of protein-bound phosphate with molybdate as colour reagent typically has a limit of detection or limit of quantification of around 20 µg PO_4^{3-}/ml plasma [33] although the improvements presented in Paper I has decreased the detection limit to 3.2 µg PO_4^{3-}/ml [129]. However, for safe quantification of even lower background levels expected in some fish, e.g. in male Salmon [130], there is a need for a method with even lower detection limit than the developed molybdate method presented in Paper 1. One step in this direction is to add malachite green to the molybdate colour reagent. Phosphomolybdate forms a complex with malachite green and it was shown as early as in 1966 that this method is 30 times more sensitive for phosphate measurement [131]. The major drawback of using malachite green is the limited stability and solubility of the colour complex. However, this can be dealt with by addition of a surfactant (Tween 20) [131, 132] or polyvinyl alcohol [133, 134]. The latter was used in our case.

Malachite green has been used for bioanalytical purposes such as: quantification of inorganic phosphate in serum [131], quantification of lipid phosphorous [135] or estimation of enzymatic activity of alkaline phosphatase [132]. However, malachite reagent has never been used before for indirect quantification of vitellogenin. Protein bound phosphate has been analysed after their release from phosphoproteins by ashing [134] or preferably by alkaline

treatment [136]. The latter method has been used in medical biochemistry to determine the degree of phosphorylation in human plasma proteins [137], which is an application very close to the ALP method for indirect measurement of vitellogenin.

A method with low detection limit for ALP in fish plasma, using malachite green, was developed for environmental monitoring of endocrine disruption and presented in Paper II. To our knowledge this is the first time, where this quantification method from medical biochemistry has been used for ecotoxicological purposes. The same sample preparation as described in Paper I was used. The final method was validated by repeated analysis of sub-samples from pooled fish plasma and these results are presented in Figure 8.

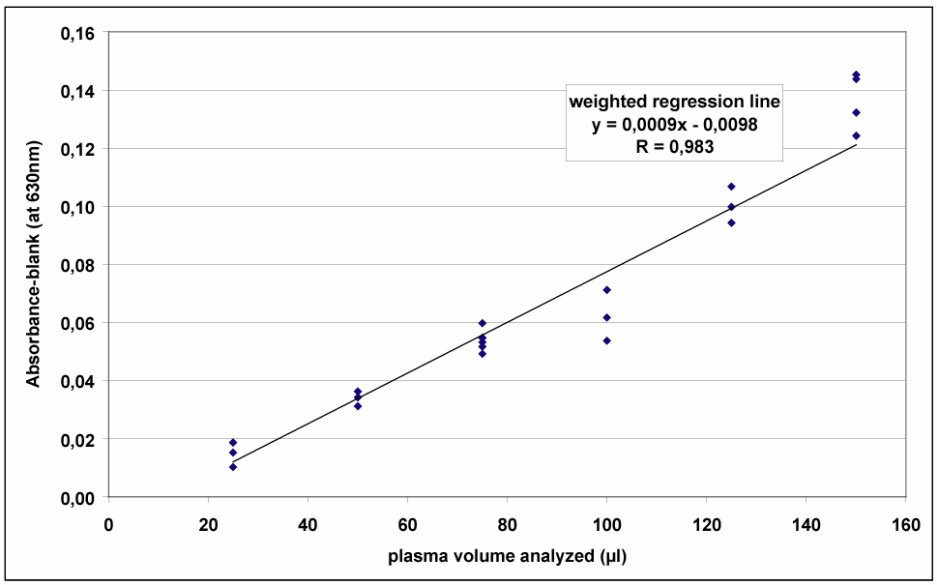


Figure 8. Intra-assay repeatability and linearity of alkali-labile phosphate determination with malachite green. Spectrophotometric determined after sample preparation of 25 µl (n=3), 50 µl (n=3), 75µl (n=6), 100 µl (n=3), 125 µl (n=3) and 150 µl (n=4) of pooled fish plasma. Absorbance values were subtracted by the non-specific absorbance in reaction blanks and the linear regression was calculated from weighted average values [138]. The weight was calculated as 1/RSD.

The detection limit is mainly influenced by background absorbance and matrix interference. In this case the method of standard addition is very useful [138]. Instead of standard addition with analyte, which is common practice, the concentration of analyte in the final solution was increased by increasing the plasma volume. Increasing volumes of plasma from the same pooled fish plasma was analyzed. From the regression line in Figure 8 the detection limit for the complete method was estimated to 0.3 µg of alkali-labile phosphate / ml plasma. (See Paper II for calculation).

Matrix interference would have resulted in that the origin was not included in the confidence interval for the intercept of the regression line, something that our method did not indicate (Figure 8). The same evaluation method as used in Paper II has previously been used by Tinsley (1985) for an ALP method using molybdate as colour reagent [128]. In that study the linearity was also good but the correlation coefficient was wrongly referred to as a proof of accuracy for the method. It is rather the closeness of the intercept to origo, which gives this information and such values were not given by Tinsley. No value for the detection limit was given in that study either, but in the referred method used by Tinsley the detection limit was around 20 times higher than in our study.

4.6.3 Application of the ALP methods

With the two indirect VTG determination methods presented in Paper I and II we had the tools necessary for applied studies on fish with different expected background levels of vitellogenin. The more sensitive spectrophotometric measurement with malachite was applied on rainbow trout and the molybdate method was applied on crucian carp.

4.6.3.1 Endocrine disruption in Vallkärra brook -malachite method on rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) has been widely used in field studies for measurement of different biomarkers. However, ALP levels in juvenile rainbow trout has been reported to be very low [130] and on the verge of what is possible to quantify with existing methods. Therefore the malachite method presented in section 4.6.2 above, with very low detection limit was very useful for measurement of ALP in this study. The brook of Vallkärra (Lund, Sweden) holds a population of wild brook trout (*Salvelinus fontinalis*) and the discovery of deformed fish in previous investigations [139] of the brook motivated further investigation of the water quality and of the nearby situated covered landfill. An extensive field study was conducted with the purpose of evaluating the suspected negative impact of landfill leachate on the small recipient by studying several biomarkers in rainbow trout. Cultivated rainbow trout were supplied by a fish farm and were held in flow-through tanks during 5 weeks (Feb-March 2008) at three locations along the brook (Figure 9). At the termination of the experiment a group of fishes from the fish farm was also sampled to serve as control. ALP was measured as one among several other biomarkers in a larger survey of fish health. Results from measurements of EROD (ethoxyresorufin-O-deethylase activity), LSI, haemoglobin and hematocrit are all presented by elsewhere [140]. An overview of the sample location is given in Figure 9. The results from ALP measurement and a subsequent discussion of endocrine disruption in Vallkärra brook is presented in Paper II.

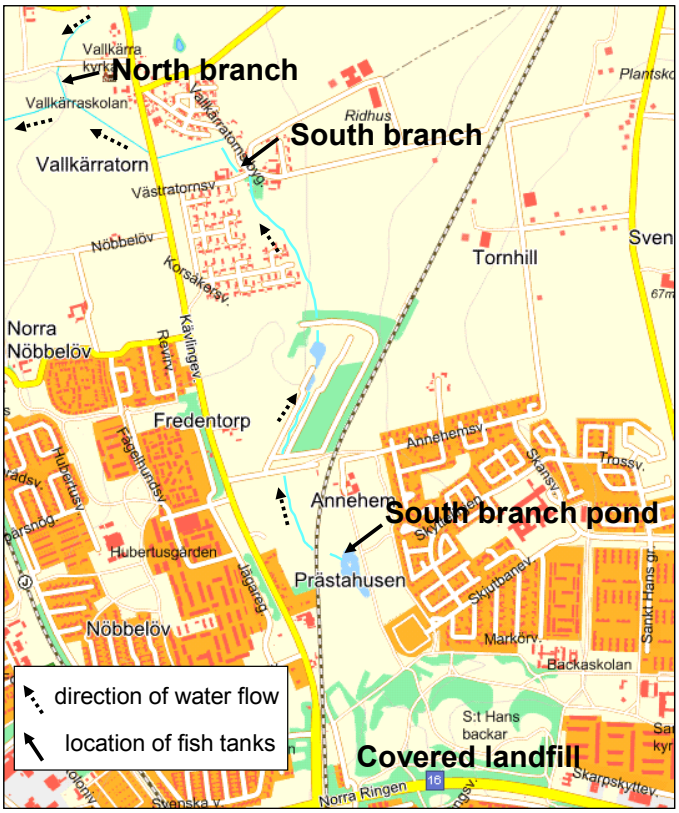


Figure 9. Sampling locations for rainbow trout held in flow-through tanks along Valkärra brook, Lund, Sweden.

Vallkärra brook (Figure 9) consists of two branches; a north branch that dewaters an agricultural area not influenced by urban runoff or the landfill, and a south branch which dewaters the covered landfill. The sample location for the first flow-through tank was at a distance of approximately 2 km from the landfill and along the presumed unaffected north branch of the brook. The second location was in the south branch 1.5 km downstream the landfill. The third location was in connection to a pond situated 400m downstream the landfill. The plasma levels of ALP in rainbow trout from the different sampling locations are presented in Figure 10.

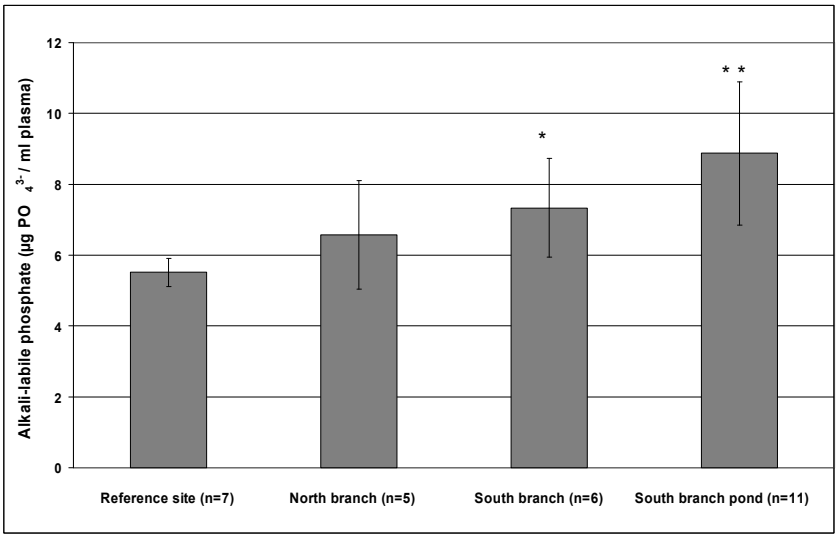


Figure 10. Mean values and standard deviation for ALP measurement with malachite green. Rainbow trout from fish farm (reference cite) and from fish placed in flow-through tanks at three locations along a brook exposed to landfill leachate (Vallkärra brook, Lund, Sweden).
** Denotes significant differences from reference site ($p < 0.05$).*
*** Denotes significant differences from reference site ($p < 0.01$).*

ALP in fish placed closest to the landfill area at the South branch pond was 8.9 PO_4^{3-} µg/ml (SD 2.0) which was higher than in fish from the reference site ($t = 4.94$, $p < 0.01$, d.f = 11). Also the fish placed further downstream in the South brook branch showed slightly higher levels of ALP, 7.3 (SD 1.4), compared to the reference ($t = 3.54$, $p < 0.05$, d.f = 6). This indicates an endocrine effect in the water. The variation within the groups of fish was very small in comparison to crucian carp (Paper III). The trout had a very homogenous age class and since they were from the same fish farm they are likely to be very similar genetically. The good repeatability in the malachite method (Figure 8) indicates that the variations within the means in Figure 10 represent a true variation of plasma ALP levels in individual fish. The malachite method enabled reliable quantification of low levels of ALP and without this method it would not have been possible to detect the difference between exposed fish and control. It is

also worth mentioning that a biomarker for endocrine disruption had not been included in the survey of fish health in this study, had it not been for the possibility to use the indirect VTG quantification. This demonstrates that the method is reliable and cost efficient. The spectrophotometric measurement with the molybdate method is still slightly easier to use although the detection limit is not quite as low. For application on a fish with higher background levels the molybdate method is therefore a better choice.

4.6.3.2 A new test species for endocrine disruption -molybdate method on crucian carp

The widely distributed cyprinid crucian carp *(Carassius carassius)* is found in ponds and lakes throughout most of Europe and central Asia [141]. This is a very tolerant species and this could be beneficial for monitoring of EDCs in aquatic environment that is less suitable for more delicate species. Insufficient cleaning of wastewater often result in low oxygen level and high ammonia concentration. Cyprinid species tend to be more tolerant to ammonia than salmonoid species and crucian carp is one of the most anoxia-tolerant vertebrates known [142]. In comparison to species like fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*), vitellogenin induction in crucian carp has been far less studied. The ideal species to be used should be tolerant in terms of survival but at the same time sensitive for endocrine effects. Crucian carp is a species which well fulfills the first criteria. The latter was studied in Paper III. The background levels of ALP were found to be low and stable and injection of 17β-estradiol showed strong inducability of ALP by estrogenic compounds. The background level of ALP in crucian carp was 14 µg/ml/ (SD 10) and 34 (SD 19) in male and female individuals respectively (Paper III). In Figure 11 these values are compared with the background levels in male, female and juvenile individuals of various other species.

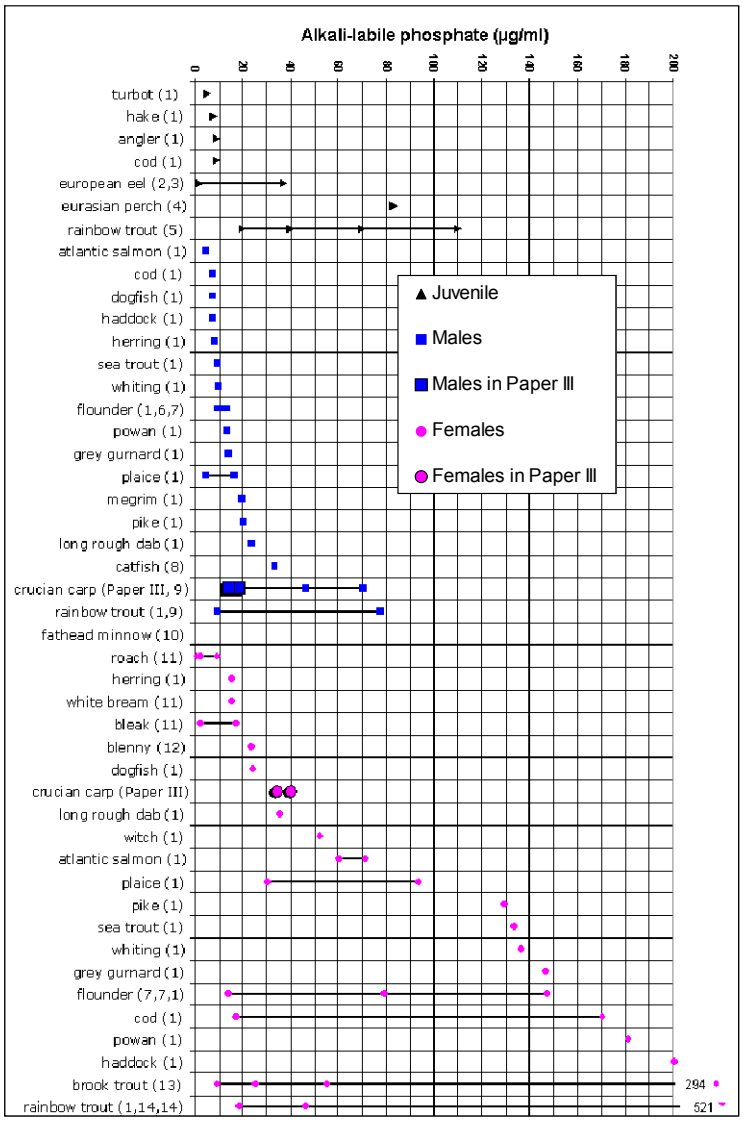


Figure 11. Compilation of literature values of background level of ALP in various species of fish. Highlighted values are from the study in Paper III. Some values were originally presented as µg of phosphorous and these have been recalculated to give µg of phosphate. References: 1=[130], 2=[143], 3=[106], 4=[105], 5=[103], 6=[144], 7=[145], 8=[104], 9=[128], 10=[33], 11=[146], 12=[147], 13=[148], 14=[117]. When data of different origin are given for the same species the first reference relates to the lowest value.

The background level of ALP in male crucian carp, presented in Paper III, is low which makes it easier to discover an increase caused by low level exposure of EDCs. The other values presented for male crucian carp in Figure 11, may not represent true basal levels due to possible phosphate contamination, as stated in the publication from which these values were obtained [128]. The background levels in female crucian carp were also relatively low and it would be interesting to investigate the seasonal trend of ALP. Since crucian carp is a sequential spawner [149] it might be that the plasma level of ALP in females does not change dramatically during the year. Several batches of eggs at different stages of maturation are present at the same time in the ovaries of crucian carp [150] and the recruitment of VTG from blood might therefore be more prolonged and less dramatic. The measurements in this study are insufficient for presenting a complete season-wide trend for ALP levels in female crucian carp.

As mentioned above, the test organism should be sensitive so that the increase in ALP levels after exposure of endocrine disrupting chemicals is large even at moderate exposure levels. The latter question is best answered with exposure to water-born ECDs at environmentally relevant concentrations in field experiments. The first step must be to prove a significant vitellogenin induction after injection of endogenous female steroid hormone. To exclude the possibility of stress related effects caused by the handling of fish it is customary to perform “blind” injections containing only the carrier in a group of fishes. The background level of ALP and the level in control injected fish were similar in our study as presented in Paper III. Here, male and female crucian carp were injected with 5 μg/g body mass of 17β-estradiol. In Figure 12 the induced vitellogenin production presented in Paper III is compared to similar studies.

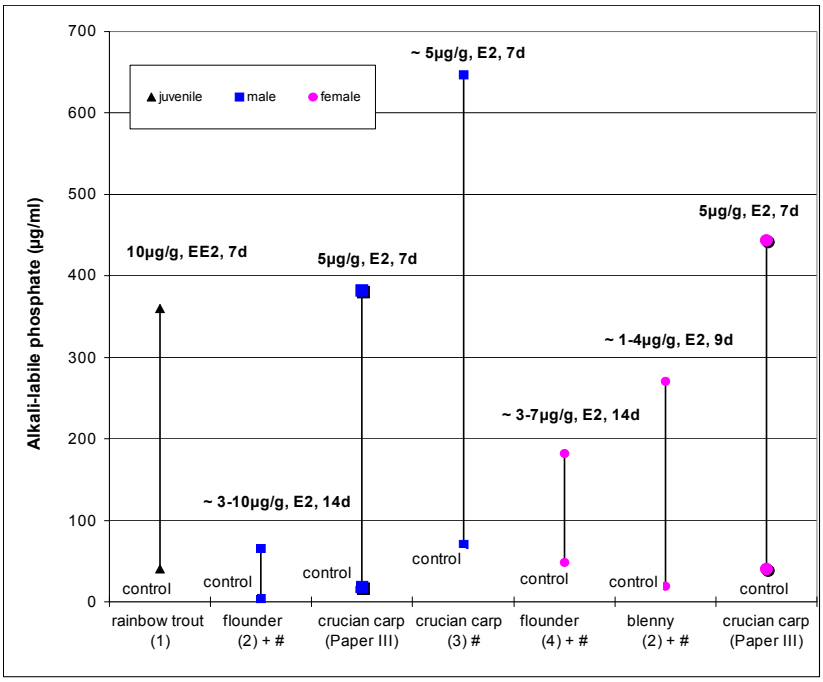


Figure 12. Induction of vitellogenin by injection of hormones. The study presented in Paper III in comparison to earlier studies. Vitellogenin measured indirectly by alkali-labile phosphate. Information given above each data point is: injected amount/fish weight, injected steroid hormone, duration of study. References: 1=[103], 2=[151], 3=[128], 4=[147]. (+) denotes that literature values are presented in µg PO_4^{3-} /ml serum instead of µl PO_4^{3-} /ml plasma. (#) denotes that the amount injected/g fish weight is an estimated average.

Both male and female crucian carp responded in a strong way to injections of female steroid hormone and ALP increased by a factor of 21 and 9 times respectively (Paper III). None of the studies illustrated in Figure 12 were performed in the exact same way but these are the most similar studies found that could be used for comparison of sensitivity in different species. From the experiments presented in Paper III and the comparison in Figure 12 it can be concluded that Crucian carp seems to be more sensitive to estrogenic hormones than e.g. juvenile rainbow trout that is often used for monitoring of EDCs. If an improved comparison of sensitivity is wanted, future studies would have to include crucian carp and another species where both are treated under exactly

the same experimental conditions. To summarise, in future exposure tests for evaluation of estrogenic effects in water it would be advantageous to use crucian carp because:

- crucian carp is a common fish widely distributed in fresh water.
- crucian carp can survive low oxygen levels and high ammonium levels and thereby enable an expansion of biological monitoring to more "rough" waters.
- the background level of ALP in male crucian carp is low.
- the background level of ALP in female crucian carp is fairly low and might be less fluctuating during the reproductive stages in comparison to single spawners. This might enable the use also of female individuals in exposure test something that is beneficial, since it is not possible to select only males before testing.
- estrogenic compounds induce a strong response in both males and females.

There is no absolute need for additional laboratory testing of how crucian carp respond to single compound exposure, before using this species as a tool for biological field monitoring of endocrine disruption. However, in one study where the salmonoid rainbow trout and the cyprinid roach were exposed to the same effluent from a waste water treatment plant, the salmonoid seemed to be the most sensitive to estrogens [152] and therefore if would be valuable to conduct laboratory experiments to find the effect concentrations (EC_{50}) for water-borne exposure of crucian carp to hormones. In such studies the nominal values after addition of chemicals need to be complemented with chemical quantification since deviations between the two are probable as shown previously [33]. A practical chemical method with low detection limit is needed and the method presented in Paper IV and chapter 5 below would be suitable for this purpose.

5 Quantification of steroid hormones in water

The low effect concentration of hormones, typically in the low ng/L range [153, 154] demands for quantification methods with sufficiently low detection limits. If the purpose is to trace the fate of estrogenic compounds in very dirty waters as sewage under treatment in a sewage treatment plant the analytical chemist is facing quite a challenge! The demand for low detection limit is in this case accompanied with a very complex sample matrix. The methods at hand has been reviewed by Lopez de Alda [155] and Petrovic [14]. Gas chromatography-mass spectrometry (GC-MS), with a detection limit of typically 0.5-74 ng/L [155] is the most widely used technique for final quantification of hormones in water samples. The benefit of adding a second step of mass fragmentation in the more powerful technique of tandem mass spectrometry (MS/MS) is about 5 times lower detection limit for analysis of these substances. Liquid chromatography-mass spectrometry (LC-MS) has the disadvantage of a lower analytical signal due to matrix effects and therefore this technique is less used for this application although this technique does not require the derivatisation step usually necessary in GC. The choice of quantification method is probably often made on basis of equipment available and affordable cost rather than always aiming for determinations using the state-of-the-art machinery. On the other hand there are many alternatives, when it comes to choosing an appropriate method for sample preparation. This is also the most time consuming step in trace analysis of micro-pollutants. Of course, first comes the actual sampling, which takes place far away from fancy expensive lab-equipment, with the less glamorous collection of sewage. The first decision is the choice of a suitable sampling strategy with respect to the problem considered. Samples can be collected as single grab samples, with discrete

sampling or as time-integrated or flow-proportional composite samples. Sample preparation often begins with filtration followed by extraction. If the total load of steroids in the sample is to be measured both the aqueous phase and the solids need to be extracted. The phases can also be extracted and analysed separately to give information on bound and free fraction. Solid-phase extraction (SPE) is the most common procedure for extracting steroid hormones. The typical extraction material is a disk or a cartridge but in order to minimize labour and the need for additional clean-up there are attractive alternatives such as solid-phase microextraction (SPME), stir bar sorbtive extraction (SBSE) and as presented in Paper IV a two-phase hollow-fibre membrane system. The method presented in Paper IV uses small pieces (~3.5 cm) of a hollow fibre with sealed ends, where the cavity and the fibre have been filled with di-n-hexylether. The hollow fibre is placed in 100ml of the aqueous sample and stirred for 2 hours. After the extraction process approximately the organic solvent in the lumen of the hollow fibre (ca. 10µl) is removed with a syringe. Then the analytes are derivatised before final analysis with GC-MS. The method detection limit for the whole protocol was 1.6, 3, and 10ng/L for 17β-estradiol, estrone and 17α-ethinylestradiol respectively which is good for such a complicated sample matrix as sewage water. The benefits of this approach are the minimum use of organic solvent and the avoidance of an extra clean-up step. Furthermore the method has good reproducibility and the enrichment factor for the analytes during the extraction process is similar for tap and sewage water which indicates a high selectivity.

Trace analysis with high resolution chromatography cannot be replaced by bioassays when it comes down to identification of unknown EDCs. A bioassay with yeast cells in the wells of a microtiter plate or with crucian carp in a cage might reveal endocrine effects in a water stream. This will then hopefully be followed up with preventive actions. These decisions need chemical analysis of

the water, river sediment or sewage sludge to point out the main suspects among the present EDCs. However, actions to prevent EDCs from reaching the aquatic environment cannot be planned solely according to what EDCs that has been identified and their concentrations. The issue of bioavailability of the identified pollutants must also be considered as discussed below.

6 Bioavailability of EDCs

Endocrine disrupting effects in an ecosystem is not caused by all of the present compounds with hormone effects. Rather, the observed effect is caused only by the bioavailable fraction of all present EDCs. The ecotoxicological definition of bioavailability is the fraction of a pollutant that is available for a biological uptake and hence the fraction that constitutes an environmental risk. When studies are conducted with the aim to identify what compounds that are causing an endocrine effect it is therefore important to distinguish the bioavailable fraction from the total present concentration of a chemical. This is relevant in cases where the suspected EDC is a persistent organic pollutant, but also when steroid hormones are suspected to cause endocrine effects in the environment.

Concerning the persistent organic pollutants in a contaminated sediment or soil, it is well known that these become tightly associated with particles in a process referred to as aging [156] and therefore becomes less bioavailable. Aging and reduced bioavailability will also reduce extractability and make the pollutants more difficult to extract in an analytical procedure [157]. To battle this problem, method development within the field of analytical chemistry, has mostly been striving towards exhaustive extraction techniques for accurate measurement of total concentration of pollutant in a sample. However, in Paper V a method for selective extraction and estimation of the bioavailable fraction of persistent organic pollutants (POPs) is presented. With selective supercritical fluid extraction (SFE) it was possible to estimate how large the fraction of the

total PCB content in a contaminated soil, that was available for uptake by earthworms (*Eisenia foetida*). This method could for example be utilised in a study of bioavailability of EDCs in river sediment. In the study of Vallkärra brook in Paper II the endocrine effect observed in fish was probably not caused by steroid hormones, since there is no contamination from sewage. It is more likely that the effect observed was caused by EDCs in leachate water from the landfill, since this type of water is known to contain endocrine disruptors like phthalates [158], PBDE [159], PCBs and phenols [160]. PAHs is another group of EDCs that might have impact on fish and the effect on vitellogenin production could be both negative and positive [161]. Metabolites of PAHs, measured with fixed wavelength fluorescence [162], were found in fish bile from exposed trout in a larger survey of fish health in the Vallkärra brook [140]. The source of these compounds may not only be the landfill itself but also sediments downstream that historically have acted as a sink for POPs and now are adding to the total load of pollutants in the brook. A chemical measurement of the total concentration of these compounds in the landfill or in the sediments might not identify the main responsible EDC since both PAHs and PCBs are known to become less bioavailable with the passage of time through the mechanism of aging [163-165]. Instead, the supercritical fluid extraction (SFE) methodology in Paper V would be suitable for estimation of how prone PAHs and PCBs are to migrate in the aquatic ecosystem. Selective SFE has with success been used for estimation of bioavailability of PAHs during bioremediation [164] and for estimation of bioavailability of PCBs to chironomids [166] and eels [167]. Sediment dwelling invertebrates will bioaccumulate the POPs in the river sediments and the pollutants are further biomagnified in fish feeding upon them. This exposure route will add to the load of pollutants taken up directly through the gills in the process referred to as bioconcentration. For a fish, the uptake through food may be an important route

of exposure. It has been shown that chironomid larvae will bioaccumulate PCBs [166, 168, 169] and PAHs [170]. Also oligochaete worms bioaccumulate EDCs as shown for PCBs [168] and PAHs [171]. However, in exposure studies like the one used in Paper II the use of flow-through containers will minimise the uptake through feeding and therefore the main exposure should be through gills. A thorough study of POPs in Vallkärra brook, conducted to explain the endocrine effects observed in fish, should thus preferably include both water measurements and estimations of bioavailability of POPs in sediment.

A discussion on the bioavailability of steroid hormones is relevant, since endocrine effects have been observed in fish as a result of exposure to sewage water. In this case it is important to distinguish between free and conjugated form of steroid hormones in water bodies. The conjugated hormones are not bioavailable in the sense that they can not interact with the estrogen receptor in e.g. a fish but they are easily deconjugated and can therefore be considered as a pool of potentially active hormones. If the total load of hormones in e.g. sludge is to be quantified the sample preparation need to include a deconjugation step. This is necessary independently of whether the final analysis is made by a chromatographic method as described in chapter 5 above or by any of the bioassays described in section 3.2 and 3.3. In a large survey of Danish waters, samples were analysed using both GC-MS and a yeast bioassay and each method was subsequently run with and without a preceding enzymatic deconjugation step rendering data of both bioavailable and total load of estrogens [16].

The conjugation/deconjugation process of steroid hormones is also important for understanding the fate of these compounds in the sewage system. The natural estrogens E1 and E2 are excreted through the urine mostly as conjugates with sulphate, glucuronide or sulphoglucuronide [172]. Free, unconjugated estrogens are poorly soluble in water and the formation of conjugated

molecules is the common biological mechanism to "detoxify" and facilitate excretion by enhancing water solubility. The formation of conjugates by esterification is performed, as in the case of detoxification of classical hydrophobic pollutants like PAHs, by enzymes in the cytochrome P450 system. Following conjugation and excretion the counteracting mechanism of deconjugation will begin driven by faecal bacteria like *Escherichia coli*. This process will actually start already in the body, for the fraction of hormones that are excreted via the bowel, since sulpfonated and glucuronated estrogens largely are deconjugated by the natural intestinal flora. The process of bacterial deconjugation will then continue all through the sewer system and the sewage treatment plant. Ingested EE2 is metabolized to 60% and the remaining fraction is excreted in both unchanged format and as conjugated hormones, in urine as well as in faeces. Logically, a larger fraction of the more water soluble conjugated EE2 is found in urine while free EE2 dominates in faeces [19]. In the sewage plant the steroid estrogens can: 1/ be degraded, 2/ after sorption to sludge leave the plant through the removal of excess sludge, 3/ leave the plant adsorbed to particles, 4/ leave the plant as freely dissolved in the water phase. The synthetic EE2 differs from the natural hormones in terms of its high persistence and bioaccumulation in aquatic oligochaete (*Lumbriculus variegates*) has been reported [173].

7 Conclusions and future perspectives

A cost efficient strategy for evaluation of endocrine disruption in aquatic environment has been developed as an alternative to the more costly chemical quantification of specific EDCs. This chronic toxicity test, utilising the VTG biomarker, can complement the test for acute toxicity previously developed within our research group. The cost for buying all chemicals and plastic consumer goods for setting up the indirect protocol for the analysis VTG in

1000 samples is only circa 350 euro. This is only 1% of the material cost for buying commercially available immunoassays for direct quantification of VTG in the same number of samples. The two alternatives for spectrophotometric determination of ALP, with molybdate or malachite, can be chosen on basis of the expected background level of ALP in fish plasma. The molybdate method is more robust but has ca 10 times higher detection limit than the malachite method. The latter method, which also has good repeatability, is more appropriate for measurements when low background levels of ALP are expected. The developed methodology enables the discovery of endocrine disruption with the aid of a biomarker, even in cases of a limited increase of the biomarker concentration and very low background levels, despite an indirect quantification technique. With the cheap methods presented in this thesis, it will now be more realistic to afford large scale monitoring with the aim to unveil the effects of EDCs on ecosystem level, within a normal research budget. Perhaps immunological methods for quantification of VTG are not suitable for use in developing countries and tropic climate, whereas the ALP methodology could enable monitoring of EDCs almost anywhere. Biomarkers are cheap tools and a simple spectrophotometric plate reader and a centrifuge are basic instruments that are far more likely to be found in a less equipped laboratory than a GC-MS/MS.

Crucian carp is a suitable whole organism bioassay for evaluation of dirty waters. Previous knowledge of the resistance to oxygen depletion has been complemented with evidence of high estrogenic inducability. This species is found in North America, Africa and Eurasia and may be the “survivor” needed for evaluation of endocrine effects in less treated waters abroad as well as in Sweden.

With hollow fibre microporous membrane liquid-liquid extraction it is possible to quantify hormones in the lower ng/L range. This method can be utilised in

future studies to evaluate the efficiency of STPs to remove hormones something that is important, since there is a discrepancy among different STPs. Furthermore, this method would be very useful for control of the exposure concentration during both laboratory and field experiments with fish and the vitellogenin biomarker.

Persistent organic pollutants that were released into the environment long time ago may still cause endocrine disruption. It is also a fact that with the passage of time pollutants may become tightly associated with soil and sediment and hence become less bioavailable. The result of this may be that the most contaminated sites are not necessarily the ones most toxic. For better risk assessment it is necessary to have tools for measurement of bioavailability. Selective supercritical fluid extraction is a straightforward tool for risk assessment of contaminated soils and sediments that can predict bioavailability with high precision. By considering both bioavailability and concentration of pollutants the limited funding for remediation purposes can be directed to the contaminated sites, which constitute the largest environmental risk.

Economy, risk and environment are three words that are tightly interlinked.

Firstly, the economic system of today is certainly a risk for the environment since companies will not benefit economically from pollution prevention, except in cases when this is done to avoid a fine.

Secondly, environmental deterioration leads to loss of income e.g. by decreasing the fish yield from the seas.

Thirdly, a strategy for monitoring of endocrine disrupting chemicals that involves biomarkers and bioassays is economical and will certainly be very helpful for evaluation of the environmental risk of these compounds in the future.

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