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Ecotoxicological test methodology for environmental screening of the European Water Framework Directive's priority substances adjusted to Swedish regional conditions

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Foreword

This report is a graduate project in Environmental Toxicology. It leads to a Master of Science degree in Biology from Uppsala University. This work was carried out in collaboration with the County Administration Board of Blekinge County, and it was part of a greater developmental project where 15 different County Administration Boards from different parts of Sweden were involved, in collaboration with the Swedish Environmental Protection Agency (SEPA) and the Swedish Environmental Research Institute (IVL). The goal of the developmental project was to develop a guidance document with practical advice for the implementation of the European Water Framework directive (WFD) in all the different regions of Sweden.

Supervisors for this project have been Jan Örberg, associate professor at the Department of Environmental Toxicology, Uppsala university, and Fredrik Andreasson, (PhD in Aquatic geochemistry), who is administrator of the WFD at the County Administration Board of Blekinge, Karlskrona, and Ann-Sofie Wernersson, (PhD in Ecotoxicology) who worked as ecotoxicologist at the County Administration Board of Västra Götaland, Gothenburg.

Firstly, I would like to express my gratitude to my three supervisors who made this exam work possible: Fredrik Andreasson, who arranged for me a space at the County Administration Board of Blekinge in Karlskrona during 8 months, and for his support, expertise knowledge, concern, time, instruction and encouragement during the work. I would like to thank Ann-Sofie Wernersson in Gothenburg for supplying me with abundant research material, for setting up the approach and giving her expertise knowledge in the field of effect-based biomonitoring. I would like to thank Jan Örberg at Uppsala University for his time, patience, kindness and comments, which improved my knowledge and the scientific quality of the thesis.

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Summary

The aim with this report was to make an inventory of and collect information about various bioassays that could be useful in river basin management by water authorities and County Administration Boards in Sweden. The purpose was also to make an evaluation of which measuring parameters and methods are most appropriate for various applications. Suggestions for ecotoxicological test methodology are made that will be used in future pilot testing within the development project. The inventory can be found in Appendix 2, 3 and 4.

The results and conclusions from the inventory and information search are as follows:

- Tier 1 screening of water systems should consist of a battery of a minimum of three tests from three trophic levels in order to be representative of the entire ecosystem under investigation. Suggestions for test batteries are made for whole sediment and surface water samples, adjusted to Swedish regional conditions, and these are in accordance with recommendations from the OSPAR Commission. Evaluation criteria are based on so-called environmental risk limits (ERLs), which determine whether the biological effects observed are neglible, maximum permissible or serious effects. The test battery should consist of a combination of short term acute and prolonged (sub) lethal tests in order to cover the most sensitive endpoints/species. The test batteries suggested are based on *in vivo* assays, but one or more *in vitro* assays can be added to the test battery in order to identify specific pollutants.

- Water extraction is the recommended method since the surface water samples can then be pre-concentrated up to a 1000-fold before being applied in the *in vivo* or in *vitro* assays. Without pre-concentration there may not be any effects in the assays, and the other advantage is that confounding factors such as salinity, pH fluctuations, high ammonium content, ion imbalance and hardness in the samples are avoided with water extraction. However some pollutants, especially metals may get lost in this process.

- Acute tests on microbial organisms such as bacteria which represent a third trophic level (decomposers) are less expensive, less labour-intensive and can be completed in a few hours up to 24 hours. Test kits which involve miniaturisation and microscale procedures, are available for prokaryotic genotoxicity assays, and also for assays on other trophic level organisms such as invertebrates, plants and algae. They can be performed in non-specialized laboratories and are a cheaper alternative compared to tests performed by accredited laboratories.

Abbreviations and terms

AA-EQS	Annual Average EQS
ASTM	American Society for Testing Materials
BFR	Brominated flame retardant
CALUX	Chemically activated luciferase expression assay
CAS nr	Chemical Abstract Service registry number
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE2	17α-ethinylestradiol
EDA	Effect-directed analysis
EDC	Endocrine disrupting chemical
EEQ	Estrogen equivalent
ELRA	Enzyme-linked receptor assay
EN	European Organization for Standardization
Endobenthic organisms	Organisms that live in the sediment on the floor of a water body such as lake or sea
Epibenthic organisms	Organisms that live on the surface of the sea-bed or bed of a lake
EQS	Environmental Quality Standard
Instar	An insect larva that is between one moult of its exoskeleton and another or between the final moult and its emergence in the adult form
ISO	International Organization for Standardisation
K_{oc}	Partition coefficient organic carbon-water
K_{ow}	Partition coefficient octanol-water
LOEC	Lowest observable effect concentration
MAC-EQS	Maximum Allowable Concentration EQS
NOEC	No observable effect concentration
OECD	The organization for Economic Cooperation and Development
PAH	Polycyclic aromatic hydrocarbon
PBDE	Polybrominated diphenyl ethers
PCDD	Polychlorinated dibenzo-*p*-dioxins

PCDF	Polychlorinated dibenzo-furans
PHAH	Polyhalogenated aromatic hydrocarbons
Pore water	The interstitial water present in the space between the sediment or soil particles
Sediment elutriate	The sediment is “flushed” with seawater in order to get out water-extractable pollutants from the sediment, and this water is then collected and tested
Spiked sediment	A material/chemical has been added to the sediment for the specific conditions of the experiment
SPM	Sediment particulate matter
SS	Swedish Standard
T_3	3,3´,5-triiodothyronine
T_4	Thyroxine
TBT	Tributyl tin compounds
TCDD	2,3,7,8,-tetrachlorodibenzo-*p*-dioxine
TEQ	Dioxin equivalent
TIE	Toxicity identification and evaluation
Whole sediment	Sediment sample has been manipulated as little as possible and therefore includes both sediment and pore water
WWTP	Wastewater Treatment Plant
YES	Yeast estrogen screen
YAS	Yeast androgen screen

1. Introduction

The Water Framework Directive (Directive 2000/60/EC) is a plan for water protection and management for all the countries within the European Union and was enacted on the 23rd of October in 2000. It covers inland surface waters, groundwater, transitional waters and coastal waters, all which are included in a river basin management plan. Each of the member states were obliged to identify all its river basins lying within the national borders by 2003, and form individual river basin districts lead by a competent authority. Sweden decided upon five such districts each covering several river basins. The purpose of the directive is in part to "prevent deterioration, enhance and restore bodies of surface water, achieve good chemical and ecological status of such water" by 2015 and to "reduce pollution from discharges and emissions of hazardous substances" (Homepage of the European Union, 2009). A list of priority substances was created by a combined monitoring- and modelling-based priority setting (COMMPS) technique which was based on the significant risk that the substances posed to the aquatic environment. The approach used to select priority substances is described in Article 16.2 of the Water Frame-work Directive (WFD) and should be based on a simplified environment risk assessment or a targeted risk-based assessment that considers:

1. Aquatic ecotoxicity or human toxicity of substance via water exposure paths
2. Environmental screening evidence of spreading of the contamination in time and space.
3. Production volume, consumption volume of substance and consumption patterns which may cause a spread in the environment (SEPA Report 5801).

A new daughter Priority Substance Directive (Directive 2008/105/EC) was developed under Article 16 of the WFD and enacted in 2008. In this directive Environmental Quality Standards (EQS) for the 33 priority substances are regulated within the field of water management. 8 additional pollutants which are also a part of the classification of chemical status of surface water are included in this document. Of the 33 priority substances 13 are regarded as hazardous priority substances and their emission and use must end before 2020.

Chemical analysis, presented in a screening report from 2009, has shown that the most problematic priority substances (that were most frequently found above the limit of quantification, (LOQ)) in surface water in Sweden are: Nonylphenol and tributyl tin compounds (TBT), followed by the heavy metals cadmium, lead and nickel, and also infrequently by DEPH (Di(2-ethylhexyl) phtalate). Substances in this study were measured in water and sediments at 15 different locations in Sweden during one year. Most water bodies had a yearly arithmetic mean that was above the EQS value for at least one priority substance. The substances found to be concentrated in surface water were also so in sediment. Substances that were frequently found in sediments but not in surface waters were: DEPH, octylphenol and the following polycyclic aromatic hydrocarbons (PAHs): anthracene, flouranthene, benzo(a)pyrene, benzo(b)flouranthene, benzo(g,h,i)perylene, and indeno(1,2,3-cd)pyrene. In still other matrices such as biota, mercury and methyl mercury (in fresh water fish) are found to be a problem. Fresh water systems in Sweden are generally more affected by priority substances than marine water systems. This is thought to be caused by the dilution that occurs in marine water systems, and that many point sources such as waste water treatment plants (WWTPs), urban run-off, landfill sites and industries are located near limnic water systems. One exception however is TBT which is concentrated in both marine and limnic water systems (SWECO 2009).

In this report ecotoxicological assays that may be employed in the monitoring of priority substances and specifically polluting substances in freshwater, transitional and coastal water recipients, in order to reach the goal of good surface water status by 2015 in Sweden are investigated. Propositions of test batteries for the purpose of tier 1 screening of water systems are made, and both water and sediments matrices are considered.

2. Method

Particular focus of this report is on *in vitro* tests on cultured cells and their applications in investigations of sediment and surface waters. The approach used in this study is divided in three steps. The first step involves an inventory of various types of bioassays and biomarkers, and in particular mechanistic tests on cultured cells used for different applications such as the assessment of dredging material, sediments of rivers, estuaries and coastal areas, waste water and water samples from various water bodies of interest. The inventory includes both standardized test issued by OECD (The organization for Economic Cooperation and Development), ISO (International Organization for Standardisation), SS (Swedish Standard), ASTM (American Society for Testing Materials) and others, and non-standardized tests performed within research projects. The second step involves identifying evaluation criteria for the tests and/or from the test protocols in order to determine whether the observed effects indicate a high, medium or low risk to the environment. As a third step, propositions for test batteries, based on findings in step 1 and 2 test methodologies, that could be applied within monitoring are made. The sources of contaminants considered in this report are (1) industrial plants and other point sources, like waste water treatment plants (WWTPs), (2) urban runoff water and other diffuse sources, and (3) landfill site leachate water.

3. Background

EQS values are defined as threshold levels that the substances cannot exceed in the water at any moment in time or average levels which cannot be exceeded during a period of time. There are thus two types of standards: (1) AA-EQS, the average concentration of substance calculated over a period of one year, and (2) MAC-EQS, the maximum allowable concentration of the substance measured at any point in time. The first one, AA-EQS is intended to protect against long-term exposure to toxicants whereas the second one, MAC-EQS is intended to protect against short-term exposure (Directive 2008/105/EC; SWECO 2009). EQS values are also differentiated into inland surface water EQS and other surface water EQS (which includes transitional and coastal waters).

Good surface water status according to the WFD means that the water course has both good chemical and ecological status. Good chemical status means that EQS values for priority substances and other pollutants determined at the EU level have been satisfied. Good ecological status means that the classification in accordance with Annex 5 of the WFD has been applied, which looks at many different biological parameters, and also at specifically polluting substances and priority substances that are a local and national problem in the water courses. EQS values for the specifically polluting substances (which means a significant amount of the polluting substance has leaked into the environment), also called river basin specific pollutants, can only be determined nationally. Table 1 (see page 13) lists priority substances found relevant for Sweden according to national environmental screening performed by County administration boards in collaboration with the Swedish EPA and the

Swedish Chemicals Agency, as well as the eight additional pollutants mentioned in the introduction, and their corresponding EQS values for fresh water systems.

In Sweden EQS values for 32 potential river basin specific pollutants (including 3 metals, 2 biocides and 18 plant protection products) have been proposed in 2007 by the Swedish Chemicals Agency, based on ecotoxicological studies from different trophic levels (SEPA Report 5799). These are listed in table 2 (see page 15).

In the daughter Priority Substance Directive EQS are only proposed for the water matrix except for hexachloro-benzene, hexachlorobutadiene and methyl mercury where EQS are also proposed for biota. There is however a possibility for individual member states to propose and apply EQS values nationally for the other matrices (sediment and biota) on the condition that it will provide the same kind of protection as the corresponding water EQS issued by the EU (SEPA Report 5973). According to the daughter Priority Substance Directive it would be important to monitor those substances which are highly lipophilic and have accumulation potential in sediments or biota in those particular matrices, since there is a risk for long-term effects on the ecosystem.

The policy now developed by the Swedish EPA is that national EQS value should be applied in those matrices where *the most sensitive organisms are exposed*, i.e. in water if daphnia, algae or fish are the most sensitive organisms, in biota if fish-eating birds, mammals or humans are the most sensitive organisms through secondary bioaccumulation/ biomagnification exposure, or in sediment if sediment-living organisms are the most sensitive organisms. A great deal of research, data collection and analysis must first be performed in order to determine these EQS values (SEPA Report 5973). Complementary EQS values have thus been proposed for those substances where the EQS values for water were not low enough to protect sediment-living organisms and/or humans or predators from secondary poisoning.

In the European Commission work is going on to increase the list of priority substances with an additional 18 substances from a current proposition list of 50 substances (Ann-Sofie Wernersson, personal communication).

3.1 Ecotoxicological Assays

Ecotoxicological assays are employed in combination with other biological, physiochemical and hydromorphological assessment tools, as well as chemical analysis of water samples within environmental monitoring and control of environmental pollutants in the water recipients in Sweden (NFS 2008:1; Ann-Sofie Wernersson, personal communicaton). Ecotoxicological assays can have several different applications, which are listed below:

- In order to help prioritize when choosing test stations and thereby limit the amount of water recipients to investigate
- To provide background information on which chemicals to investigate and particularly in complex pollution situations, such as close to urban areas, waste water treatment plants (WWTPs), or dense industrial areas.
- To provide background information on which matrix to monitor in the area
- As a separate evaluation parameter and basis for classification of ecological status
- To trace the importance of potential sources of contaminants by analyzing effects upstream and downstream from a discharge and at the point of the discharge
- To predict which type of protective action that would be most cost-effective to undertake

Firstly, ecotoxicological assays provide information on the total effect of the complex mixture of pollutants present in the water recipient investigated. They also show if some of the pollutants are bioavailable, i.e. are taken up by the organisms, and exert some kind of physiological effects on the organisms. Secondly, effects on the ecological level, i.e. population and community level, may have several different causes of which environmental pollutants is one of them, and it may take many years before long-term effects on this level are observed. Biological assays can thus be applied to trace the causes of such ecological effects, or to provide an early warning in order to hinder these effects to occur in the future. Thirdly, ones an effect is observed it may also be possible to identify the compound or type of compound that has caused the effect by chemical analysis in combination with the ecotoxicological assays.

3.2 Typical pollutants analyzed

Steroids and xenosteroids in the environment

Endocrine disrupting chemicals (EDCs) are described as "exogenous agents that interfere with synthesis, secretion, transport, binding, action or elimination of natural hormones which are responsible for maintenance of homeostasis, reproduction, development and/or behaviour" in humans and animals (Kavlock et al. 1996). One of the most potent EDCs present in the environment is the chemical class of steroids, which are formed either naturally by humans, animals and plants, or are produced synthetically to be used in contraceptive pills or in other medications (Streck 2009). Steroids consist of a skeleton of three hexagonal and one pentagonal carbon rings and to these different functional groups and side chains are attached. All steroids stem from cholesterol and various types are depicted in appendix 1, figure 1 (see page 81). The three main natural estrogens are called estrone (E1), estradiol (E2), and estriol (E3). They are so called C18 steroids with different oxidation states on their carbon ring. Synthetic estrogens like 17α-ethinylestradiol (EE2) stem from estradiol (Streck 2009).

EE2 is the active substance in most contraceptive pills. Synthetic hormones are synthesized to resist the first pass metabolism in the liver in humans and animals, and are therefore fairly persistent in the the human body. A much lower oral dose of EE2 (40 μg) has the same estrogenic potency as an oral dose of 4 mg of E2 in a human which is partly explained by the fact that EE2 is more persistent than E2. The persistence of the synthetic hormones also makes them more endurable in the environment. For instance, the stability of EE2 results in longer life-times of this drug in sewage treatments plants (STPs) compared to that of natural hormones (Hallgren 2009).

Synthetic progestagens are also called progestins and they are C21 steroids. Progesterone is the only natural progestagen and it is secreted in the ovary by the *corpus luteum* or by the placenta. Contraceptive pills contain both synthetic progestagens and estrogens. Synthetic estrogens are also used to treat menopausal disorders, and progestagens are applied in the treatment of menstrual disorder, infertility, endometriosis etc. Androgens are C19 steroids (see figure 1, appendix 1). Synthetic and natural androgens are used as growth promoters in animal farming and in human and veterinary therapy (Streck 2009).

Xenoestrogens such as alkylphenols and bisphenol A may also to a smaller degree add to the estrogenic activity in surface waters; yet their estrogenic potencies have been found to be 4-5 orders of magnitude smaller than that of E2 (Gutendorf and Westendorf 2001; Kinnberg 2003). It is however the environmental stability and bioaccumulation potential of these lipophilic pollutants that can make them dangerous to the animals in the environment (Boelsterli, 2009).

Brominated Flame Retardants

Another important group of environmental pollutants that may have endocrine-disruptive (ED) potencies are the brominated flame retardants (BFRs). The ones that have the highest consumption in the world are tetrabromobisphenol (TBBPA), polybrominated diphenyl ethers (PBDEs) and hexabromocyclodecane (HBCD) (see appendix 1, figure 2). PBDEs are produced in three different commercial combinations based in the average level of bromination: penta-PBDE, octa-PBDE and deca-PDBE mixtures (Hamers et al. 2006). There are 209 different possible congeners of PBDEs, but the ones that are considered most problematic and are included as priority substances in the WFD are PBDEs number 28, 47, 99, 100, 153 and 154 (see table 1). PBDEs, HBCD and to a smaller degree TBBPA are found to be persistent organic pollutants that have potencies to bioaccumulate in the aquatic food chain.
In vitro screening of BFRs showed them to have various modes of action related to endocrine disruption: antiandrogenic, antiprostagenic, (anti-)estrogenic, T3-antagonistic, binding to human transthyretin (TTR), inhibition of the enzyme estradiol sulfotransferase (E2SULT) and potentiation of T3-mediated effects (Hamers et al. 2006).

Table 1: Priority substances relevant for Sweden and the eight additional pollutants added by the EU to WFD and their inland surface water EQS (SWECO 2009; SEPA Report 5801).

CAS-no	Name	Uses or emission	National regulation	AA-EQS (µg/l)	MAC-EQS (µg/l)	Log K_{ow}	Water solubility (mg/l)	Reference
71-43-2	**Benzene**	Incomplete combustion; component in petroleum products	Restricted use	10	50	2.13	1800 (25 °C)	SWECO 2009
32534-81-9	**PBDE (Pentabrominated diphenyl ethers) ## 28,47, 99, 100, 153, 154**	Flame retardant	Phased out, banned from Aug 2004	0.0005	n.a.	5.03- 8.09	<0.01 (20 °C)	SWECO 2009
7440-43-9	**Cadmium, Cd**	Numerous	Restricted use	0.08-0.25 (depending on H_2O hardness)	0.45-1.5	n.a.	Insoluble, some compounds are soluble	SWECO 2009
117-81-7	**DEHP (Di(2-ethylhexyl)-phthalate)**	Plasticiser	1999 restricted in children´s toys	1.3	n.a.	4.88-7.6	0.3-0.4, lower in salt water. DEHP will absorb to particles in water (especially salt water), even though solubility is low.	SWECO 2009
115-29-7	**Endosulfan**	Pesticide	Banned in 1996	0.005	0.01	3.5	0.32- 0.52	SWECO 2009
206-44-0	**Flouranthene**	Incomplete combustion		0.1	1	4.7	0.265 (20 °C)	SWECO 2009
7439-92-1	**Lead, Pb**	Numerous	Phase-out	7.2	n.a.	n.a.	Insoluble, some compounds may be soluble	SWECO 2009
7439-97-6	**Mercury, Hg**	Numerous	Phase-out	0.05	0.07	n.a.	Insoluble to 0.0639	SWECO 2009
7440-02-0	**Nickel, Ni**	Numerous		20	n.a.	n.a.	Insoluble (some compounds are soluble)	SWECO 2009
104-40-5	**Nonylphenol (NP), 4-para-nonylphenol**	Industrial chemical, forms through degradation of NP-ethoxylates	Phase-out	0.3	2.0	4.2- 4.7	3-11 (pH dependant)	SWECO 2009
140-66-9	**Octylphenol (OP), 4-tert-octylphenol**	Industrial chemical; forms through degradation of OP-ethoxylates	No restrictions	0.1	n.a.	5.28 (4-tert)	5 (25 °C)	SWECO 2009

CAS-no	Name	Uses or emission	National regulation	AA-EQS (µg/l)	MAC-EQS (µg/l)	Log K_{ow}	Water solubility (mg/l)	Reference
	Polycyclic aromatic hydrocarbons (PAH):							
50-32-8	**Benzo(a)pyrene**	Incomplete combustion		0.05	0.1	6.35	0.0016-0.0038 (25 °C)	SWECO 2009
205-99-2 **207-08-9**	**Benzo(b)flour-anthene** **Benzo(k)flour-anthene**	Incomplete combustion		Σ= 0.03	n.a.	6.6 6.84	0.0012 0.0008 (25 °C)	Toxnet 2011
191-24-2 **193-39-5**	**Benzo(g,h,i)-Perylene** **Indeno(1,2,3-cd)-pyrene**	Incomplete combustion		Σ= 0.002	n.a.	6.63 5.6-7.7	0.00026 (25 °C) 0.062 (20 °C)	Toxnet 2011
688-73-3	**Tributyl tin compounds (tributyl tin cation), TBT**	Antifoulant; preservative; stabilizer in plastics	1993: all ships under 25 m; no new use after 2003	0.0002	0.0015	3.19-3.84	n.f.	SWECO 2009
8 additionell pollutants								
309-00-2 **60-57-1** **72-20-8** **465-73-6**	**Aldrin** **Dieldrin** **Endrin** **Isodrin**	Cyclodiene pesticides		Σ= 0.01	Σ= 0.005	6.50 5.40 5.20 6.75	0.027 (27 °C) 0.195 (25 °C) 0.25 (25 °C) 0.014 (25 °C)	Toxnet 2011
n.a.	**DDT total**	Organochlorine insecticide		0.025	n.a.	n.a.	n.a.	SEPA Report 5801
50-29-3	**p´, p´-DDT**	Most important isomer of DDT		0.01	n.a.	6.91	0.0055 (25 °C)	Toxnet 2011
56-23-5	**Carbon tetrachloride**	Industrial solvent		12	n.a.	2.83	800 (20 °C)	Toxnet 2011
127-18-4	**Tetrachloro-ethylene**	Textile industrial chemical		10	n.a.	3.40	150 (25 °C)	Toxnet 2011
79-01-6	**Trichloro-ethylene**	Anesthetic; solvent		10	n.a.	2.61	1280 (25 °C)	Toxnet 2011

n.a. = not applicable
n.f. = information not found

Table 2: Potential river basin specific pollutants in Sweden and their proposed EQS values for inland surface water and other surface waters (SEPA report 5799).

CAS -no	Name	Type of substance	Mode of Action	EQS inland water (µg/l)	EQS other water (µg/l)	Log Kow	Water solubility (mg/l)	Reference
n.a.	**Chromium**	metal	Carcinogenicity, genotoxicity, cytotoxicity	3	3	n.a.	115-2355 g/L	Toxnet 2010
7440-66-6	**Zink**	metal	Induction of MT(metallothionein), stress response	8 at hardness > 24 mg $CaCO_3$/l 3 at hardness < 24 mg $CaCO_3$/l	8	n.a.	n.a.	Sanders 1993; Pedersen et al. 1997
7440-50-8	**Copper**	metal	Induction of MT, stress response	4		n.a.	0.8-10,000,000	Sanders 1993; Pedersen et al. 1997
52-51-7	**Bronopol**	biocide	radical oxide formation	0.7	0.3	0.18-1.5	240 000	Toxnet 2010
28159-98-0	**Irgarol 1051**	biocide	Inhibits photosystem 2	n.a.	0.003	3.95	7.0	SEPA report 5799
3380-34-5	**Triclosan**	Antifungal, antibacterial agent	Ah-receptor binding after photodegradation	0.05	0.005	4.8	12	Boelsterli 2009
85535-85-9	**MCCP (medium-chain chlorinated paraffin)**	secondary plasticiser in PVC etc.	n.f.	1	0.2	7	0.027	Toxnet 2010
n.a.	**Non-dioxin-like PCBs**		Endocrine disruption	30 µg total-PCB/kg[1]	20 µg total-PCB/kg[1]	n.a.	n.a.	Toxnet 2011
n.a.	**Dioxin-like PCBs, dioxins and furans**	n.a.	Ah-receptor binding; immunotoxicity; developmental and reproductive effects; neurotoxicity; carcinogenicity	0.9 ng WHO-TEQ_{fish} /kg	0.9 ng WHO-TEQ_{fish} /kg	n.a.	n.a.	Hurst et al. 2004

[1] EQS for sediment

CAS -no	Name	Type of substance	Mode of Action	EQS inland water (µg/l)	EQS other water (µg/l)	Log Kow	Water solubility (mg/l)	Reference
1763-23-1	**PFOS (Perflouro-octane sulfo-nates)**	Surfactant, emulsifier	Disruption of hepatocyte membrane integrity in fish	30	3	n.a.	570	Toxnet 2010
25637-99-4 and 3194-55-6	**HBCD (Hexabromo-cyclododecane)**	Flame retardant	Endocrine disruption	0.3	0.03	5.625	0.066	Hamers et al. 2006
80-05-7	**Bisfenol A**	Intermediate in polycarbonate, epoxy resin production	Endocrine disruption	1.5	0.15	3.4	120-301	Toxnet 2011
n.a.	**Nonylphenol ethoxylates (NPE)**	Non-ionic surfactant; stabilizer and antioxidant in plastic	Breaks down to persistent metabolite NP during sewage treatment ; estrogenicity	0.3 NP-TEQ	0.3 NP-TEQ	n.a.	n.a.	Toxnet 2011
74070-46-5	**Aclonifen**	Diphenyl ether herbicide	Inhibits protoporphyrinogen oxidase	0.2	*	4.37	1.4	SEPA report 5799
25057-89-0	**Bentazone**	herbicide	Inhibits photosynthesis electron transfer	30	*	-0.46 (pH 7, 22°C)	570 (20°C)	SEPA report 5799
21725-46-2	**Cyanazine**	herbicide	Inhibits photosynthesis	1	*	2.1	171 (25°C)	SEPA report 5799
83164-33-4	**Diflufenican**	herbicide	indirect interference with plant photo-synthesis	0.005	*	4.9	<0.05 (25°C)	SEPA report 5799
15165-67-0	**Diklorprop-p**	herbicide	Auxinic mode of action	10	*	-0.25 (pH 7, 25°C)	590 (20°C)	SEPA report 5799
60-51-5	**Dimethoate**	Organo-phosphorous insecticide	Inhibition of acetylcholinesterase (AChE)	0.7	*	0.704	23800 (pH 7, 20°C)	SEPA report 5799
67564-91-4	**Fenpropimorph**	fungicide	Inhibibition of sterol biosynthesis	0.2	*	4.1 (pH 7)	4.32 (pH 7)	SEPA report 5799
1071-83-6	**Glyphosate**	herbicide	Interfering with the biosynthesis of aromatic amino acids in plants	100	*	-3.2 (25°C)	10500 (20°C, pH 2)	Toxnet 2011

CAS -no	Name	Type of substance	Mode of Action	EQS inland water (µg/l)	EQS other water (µg/l)	Log Kow	Water solubility (mg/l)	Reference
1698-60-8	**Chloridazon**	herbicide	Inhibition of photosynthesis and the Hill reaction	10	*	1.19 (pH 7)	340 (20°C)	Homepage of KEMI 2011; Toxnet 2011
94-74-6	**MCPA** (2-methyl-4-chlorophenoxy-acetic acid)	Phenoxy herbicide	Auxinic mode of action	1	*	-1.07-0.59 (pH 9-5)	294 000 (pH 7, 25°C)	KEMI 2008
7085-19-0, 16484-77-8	**Mekoprop, Mekoprop p**	herbicide	Auxinic mode of action	20	*	1.18 -0.18-1.43 (pH 5, 20°C)	734 (25°C) 860 (20°C)	SEPA report 5799
41394-08-2	**Metamitron**	herbicide	Inhibibition of photosynthesis	10	*	0.83	1700 (20°C)	SEPA report 5799
21087-64-9	**Metribuzin**	herbicide	Inhibition of photosynthesis	0.08	*	1.6 (pH 5.6, 20°C)	1050 (20°C)	Toxnet 2011
74223-64-6 74223-64-6	**Metsulfuron metyl**	herbicide	Inhibits acetolactate synthase (ALS)	0.02	*	-1.7	2790 (pH 7)	SEPA report 5799
23103-98-2	**Pirimicarb**	Carbamate insecticide	Inhibits Cholinesterase	0.09	*	1.7 (20°C)	3060 (pH 7.4)	Toxnet 2011
141776-32-1	**Sulfosulfuron**	Sulphonyl urea herbicide	Inhibits acetolactate synthase (ALS)	0.05	*	-0.44 -0.73 (pH 9-5)	1627 (pH 7)	SEPA report 5799
79277-27-3	**Thifensulfuronmethyl**	Sulphonyl urea herbicide	Inhibits acetolactate synthase (ALS)	0.05	*	1.7 (pH 7)	2240 (pH 7)	SEPA report 5799
101200-48-0	**Tribenuron methyl**	Sulphonyl urea herbicide	Inhibits acetolactate synthase (ALS)	0.1	*	0.78 (pH 7)	2040 (pH 7, 20°C)	SEPA report 5799

n.a. = not applicable
n.f. = information not found
* =not determined

4. Inventory of ecotoxicological methods and assays for analysis of water and sediment samples

Ecotoxicological test methods can be used as a complement to chemical analysis of compounds in order to analyze complex environmental mixtures. Tens of thousands of chemicals are presently in circulation and use in the European Union, but only a few (less than 100) have reliably been risk assessed with respect to their toxic effects on humans and on the environment (SEPA Report 5596). A chemical analysis measure only selected priority pollutants and therefore ignores other possible pollutants in a given sample. It also ignores the additive, synergistic and antagonistic effects of combinations of pollutants in the environment. The environmental sample typically contains a complex mixture of organic and inorganic pollutants (Kwan 1995).

Ecotoxicological assays or bioassays as they are named when applied on environmental samples look at specific biological endpoints. Examples of these are abnormal behavior, mortality, fertility, growth inhibition, genotoxicity, carcinogenicity, endocrine disruption and these give a reliable estimation on toxicological effects in the organisms. Whole organism tests or *in vivo* test employ living animals in the field or in a laboratory, whereas *in vitro* or mechanistic tests employ cell cultures in a laboratory from the species of interest.

A method called the Triad approach combines three assessment tools: bioassay, chemical and ecological methods, in order to make a more complete risk assessment of complex environmental mixtures. There is a need to understand the causal link between chemical and ecological status of water recipients. For this purpose, combined biological and chemical techniques have been developed, and are now more frequently being employed by environmental scientists. Mass balance analysis, Toxicity identification and evaluation (TIE) and effect-directed analysis (EDA) are examples of such techniques (Streck 2009; EC guidance document no. 25).

Mass balance analysis (also called potency balance analysis) means that targeted substances in the sample are quantified by chemical analysis, and an investigation is made to answer whether the known composition of the sample accounts for the entire magnitude of biological response or not. In order to do this, multiple dilutions of sample are tested (in order to make dose-response curves), and e.g. bioassay-derived dioxin equivalents (TEQs) and estrogen equivalents (EEQs), (see section 4.3.1), are calculated. Bioassay-derived values are then compared TEQ and EEQ values derived from chemical analysis (Khim el al. 2001).

In the other two approaches, TIE and EDA, pollutants are identified without targeting specific compounds. Instead, bioassay and chemical analysis are combined with physio-chemical manipulation and fractionation methods which permit the identification of pollutants in different matrices, and for different biological endpoints (Streck 2009; EC Guidance document no. 25). The process is bioassay-directed, i.e. the bioassays will direct the manipulations and fractionations until the complexity of the sample extracts have been reduced, and it's possible to identify the pollutants by chemical analysis (Grung et al. 2007; Houtman et al. 2006). It is thus possible to identify both unknown and known pollutants in the samples.

The octanol-water partition coefficient (K_{ow}) of a substance is a measure of its hydrophobicity and a good indicator of its partitioning potential in the organic fraction of the

sediment (K_{oc}) in aquatic environments. According to the EC guidance document on monitoring of sediment and biota, a rule of thumb is that compounds with a log K_{ow} >5 should primarily be found in sediments, while compounds with a log K_{ow} <3 should primarily be found in the water matrix. For substances with a log K_{ow} between 3 and 5, the sediment matrix is an optional matrix to analyze depending on the degree of contamination (EC guidance document no. 25).

In Swedish environmental monitoring of water courses bioassays have so far been used sparingly and there are only a few standardized test methods developed. In the monitoring of industrial waste water and landfill leachate water however, short term tests that investigate acute toxicity on the three trophic levels (fish, crustacea and algae), have been employed, but the long term sublethal effects on development and reproduction in the organisms have not been tested. These long term effects however are a greater environmental problem than the short term effects (SEPA Report 5596).

In the evaluation of toxicity of sediments and dredged materials, a tiered testing approach has been suggested by the OSPAR (The Convention for the Protection of the Marine Environment of the North-East Atlantic) and Helsinki Conventions (convention for the Protection of The Marine Environment of the Baltic Sea area). The toxicological importance and complexity of the bioassays increase with the tiers. It is thus a hierarchal approach that covers all levels, from cellular, whole organism, population to community (Nendza 2002):

- Tier 1 testing consists of screening and detection of toxic impacts. If no toxic effects are observed in this step but other chemical analysis or physio-chemical investigations indicate that there is a problem, tier 2 testing may be employed.

- Tier 2 testing consists of the characterization of toxic impacts, and it looks at a wide range of biological endpoints, including sublethal effects after long-term exposure. It also covers a broad range of species and matrices.

- Tier 3 testing may be employed if the results from tier 1 and 2 testing are not clear. It involves a verification of the *in situ* changes, either by using biomarkers for specific exposure in the field, or by verification of laboratory tests in the field.

4.1 Extraction procedures in preparation of samples for ecotoxicological assays

The sediment compartment can be divided into three phases: solid, water soluble and organic soluble phases (Gagné et al. 1996). The sampling matrices such as sediment, sediment particulate matter (SPM), fresh water and sea water are many times not appropriate for direct bioassay testing and therefore the samples must first be pre-concentrated (ICES WGBEC Report 2005). Extraction methods for both water and sediment samples exist and are described below. The extraction procedure is a way to get rid of chemicals that are of lesser environmental concern and that might interfere with the bioassay. Either solid phase or liquid phase extraction methods can be applied. The extraction of sediment or water samples is usually divided into three steps: (1) liquid-solid phase or liquid-liquid phase extraction into a crude extract, (2) clean-up in order to remove interfering compounds, and/or fractionation in order to isolate pollutants into reduced less complex mixtures, and (3) solvent-exchange,

which means that the solvent extract is exchanged into a water miscible, non-toxic solvent such as dimethylsulfoxide (DMSO), that can be added to the ecotoxicological assay.

4.1.1 Sediment extracts

There are two types of methods used for solid material (i.e. sediment, SPM, sludge or soil) and both are liquid-solid phase extractions. Liquid-solid phase extraction means that particles which are soluble in the solvent used will be extracted out from the solid sample: (A) Equilibrium sampling which means shaking, swirling or tumbling with a polar or non-polar organic solvent. It is usually aided by ultrasound and/or pressure, and it is a very fast method which takes less than one hour to perform. (B) The soxhlet extraction method, which takes 24 hours or more to perform, but can be done overnight and therefore the time aspect is not a limiting factor (ICES WGBEC Report 2005).

Polar pollutants from municipal or domestic waste waters in sediments may be extracted using polar organic solvents, such as DMSO or N,N´-dimethylformamide (DMF), and the method used is usually method (A). More hydrophobic organic pollutants are extracted with non-polar solvents such as hexane, or dichloromethane (DCM), and the method used is usually method (B). They can also be extracted with solvent mixtures such as DCM/methanol or hexane/acetone. Which solvent to use depends on the physiochemical properties of both the sediment and the contaminants (Chen and White, 2004). Sediment extract is the most widely used matrix applied to *in vitro* assays.

4.1.2 Water extracts

There are three types of methods used for water samples:
(A) Liquid-liquid phase extraction, and the type of solvent used depends on which group of pollutants that are targeted. DCM and cyclohexane have the capacity to extract the most common pollutants of interest. If no filtering of particulate matter in the water sample is done before hand, the technique will reveal the total presence of contaminants in the sample.

(B) Solid phase extraction, in which pollutants become bound to a solid phase material and then are eluted out. The sorbents used are general broad specificity sorbents such as silica-linked long chained alkanes (C8 or C18), resin based polymers (e.g. XAD), and several other sorbents like Tenax and blue rayon.

(C) Partition controlled sampling (also called passive sampling) and extraction. The devices used are based on a liquid or solid phase matrix that is encircled by a semipermeable membrane fitting, which is placed out in the field. These membrane devices collect the bioavailable fraction of pollutants in water during a prolonged time period and can therefore assess the time-weighted average exposure, or pick up periodic pollution events (ICES WGBEC Report 2005).

4.1.3 Sediment elutriates

Pollutants that have been accumulated in the sediments for a long time may suddenly become bioavailable due to chemical (pH, salinity fluctuations), physical (e.g. dredging in harbours, flood events) or biological (bioturbation) changes, and once again enter pore water or surface waters. By preparing elutriates (i.e. ”flushing” the sediment) it is possible to get out the water-extractable pollutants in the sediments and make an assessment of which risk they would pose

to the organisms living in the water column (Davoren et al. 2005b; Strmac and Braunback 2000).

Sediment elutriates are prepared by adding sea water to 10 gram sub-samples of sediment. The slurry is shaken for one hour at 240 rpm. It is then centrifuged at 1200 x g for 30 minutes at 4 ºC. The supernatant is collected and filtered through a 0.2 µm filter and after that conductivity, salinity and pH of elutriate are determined (Davoren et al. 2005b).

For *in vitro* testing one method is to prepare aqueous elutriate extracts that may be used in cell culture bioassays. These aqueous elutriates from whole sediment can then be used to reconstitute powdered cell media, allowing the cells to be exposed to concentration of contaminants (that are bio-available in the aqueous phase) at almost the same concentration as in the original sediment sample. Sediment elutriate testing was originally invented to make a risk assessment of pollutants that leached from dredged material (Davoren et al. 2005a).

4.1.4 Whole Sediment

So called direct testing employs whole sediment which means that the sediment sample is not manipulated in any way before testing. It measures the total toxic activity of the sediment and therefore gives a very realistic picture without the interferences of solvents etc. It also shows if any pollutants in the sediment sample are bioavailable, i.e. are taken up by the organisms and exerting some kind of effect. Direct testing thus measures the effects of both soluble/insoluble, organic/inorganic and volatile/non-volatile pollutants. Direct testing has been shown to be very sensitive and can detect low levels of toxicants in the sample (Kwan 1995).

4.1.5 Pore water extracts

Pore water extracts are simpler to prepare than sediment extracts, and no additional clean-up procedures are needed that might have detrimental effects on the cell cultures. The pore water represents the water-soluble and bioavailable fraction of the sediment, and may indicate a major route of exposure to benthic organism that burrow into and live in the bottom deposits of the sea (e.g. clams, tube worms, crabs). Bioassays employing pore water extracts have been shown to be as sensitive as or even more sensitive than whole sediment tests (Davoren et al. 2005b).

Pore water extracts are prepared by taking 25 ml sub-samples of sediment, and centrifuging them at 1200 x g for 30 minutes at 4 ºC. The supernatant which is the pore water is collected, and then filtered through a 0.2 µm filter. At last, conductivity, salinity and pH of the pore water sample are determined (Davoren et al. 2005b).

4.2 Whole organism (in vivo) tests

In the whole animal tests it is hard to deduce the mechanism of action of the chemical since it may act on several targets, and because of the complexity of the regulatory processes in the whole organism (Sohoni and Sumpter 1998). *In vivo* assays are more time-consuming, work intensive, complex and expensive to perform than *in vitro* assays.

Detoxifying enzymes, DNA repair, storage of pollutants in fat deposits and other protective functions may hinder the spread of the toxic response in the organism. There is a relationship between a chemical´s primary interaction with its site of action, which may lead to a localized cellular disturbance, and the responses at higher levels of organism and community. The localized response may in turn lead to a physiological, biochemical or behavioral changes at the whole organism level, which may affect such vital functions as the growth or reproduction of the organisms. This in turn may eventually lead to effects on the ecosystem level (Walker et al. 2006). Field biomarkers are a way to investigate how this relationship works.

Changes at the whole organism level as measured by *in vivo* assays usually occur at higher concentrations of pollutants than changes at the subcellular and cellular levels as measured by *in vitro* assays, and therefore they are less sensitive. Whole organism or *in vivo* assays to test water toxicity have been developed in six categories of organisms: invertebrates, plants, algae, fish, amphibians and microorganisms (Tothill and Turner 1996). Primarily these test have been developed to test individual chemicals for risk assessment purposes but they have also been applied in the characterization of industrial waste water and landfill leachate water in Sweden (Home page of Toxic on AB 2010). There are some additional types of *in vivo* assays:

- Field biomarkers that measure effects on biochemical, physiological, histopathological levels.
- *In situ* tests where organisms are placed in a cage in the field and monitored for specific biological endpoints.
- Microcosm and mesocosm tests which are a semi-field tests that study community structures of organisms.

Toxicity tests on vertebrates such as fish and amphibians should not be conducted routinely due to ethical considerations. Only if absolutely necessary and scientifically justified, acute (96 h LC_{50}) or chronic fish early life stage test (FELS) may be employed (Nendza 2002).

Toxicon AB in Sweden performs toxicity testing using organisms (algae, crustacea, fish) from three trophic levels on environmental fresh water or sediment samples (using organic extracts or pore water from sediments). These 3 trophic-level investigations take three to four weeks to perform. Usually five concentrations/dilutions per sample are used in each test. Due to ethical concerns, however, only one concentration is prepared in the fish test, making use of the lowest EC_{50} (concentration at which 50 % effect is observed in the test population) value from the tests on the other two trophic levels. If it is found that the fish was the most sensitive species in the initial test, five additional concentrations may be tested. Tests on marine water species are only available on two trophic levels (algae, crustaceans) by Toxicon AB however.

A *static test* means that the test material is added once to the test system and the test medium is not exchanged during the entire period of the experiment, and no flow occurs. In a

semistatic test, the test medium and the test material are replaced at intervals. In a *flow through test*, the test medium and the test material are added at a constant rate and concentration to the test organism (OECD 2006).

4.2.1 Marine algal growth Inhibition test

The marine algal growth test based on the ISO 10253 standard procedure (SS-EN ISO 10253, 2006) with *Skeletonema costatum* has been applied on sediment porewater and elutriates from estuarine sediments (Davoren et al. 2005b).
The *Skeletonema costatum* alga is first precultured in a laboratory for three days in Algal growth medium. Nutrient stock solutions are added to the samples in order to make sure that nutrients are not a limiting factor in the assay. The pH is held at 8.0 ± 0.2. The samples are diluted in five test concentrations (20, 40, 60, 80 and 100%) in the Algal growth medium. The alga is exposed for 72 hours at 20 ± 1 ºC with continuous shaking at 100 rpm and at an illumination of 10,000 lux. After the exposure, the cell density is measured in a Neubauer Improved chamber and the average specific growth rate (μ) and percentage inhibition of average specific growth rate (% Ir) relative to the control are calculated (Davoren et al. 2005b).

The marine algal growth test was found to be very appropriate in the testing of sediment elutriates and porewater, and responded with great sensitivity, and the authors recommend it for test batteries in the assessment of dredged sediments.

4.2.2 Daphnia magna Reproduction test

The advantages of using daphnids in toxicity testing are that they are highly sensitive to a broad range of pollutants, have short reproductive cycles and a nonsexual reproduction (Tothill and Turner 1996). They can easily be cultured in a laboratory. They are also an important connection in the aquatic food chain, providing food for small fish. Daphnids belong to the order *Cladocera* and the subphylum of Crustaceans. The family name is *Daphniidae* which includes both *Daphnia* sp. and *Ceriodaphnia* sp. They can be found everywhere in temperate fresh waters, and are most abundant in lakes, ponds and calm sections of streams and rivers (Environment Canada 2007).

In the *Daphnia magna* reproduction test (OECD nr 211, 2008) young female neonates less than 24 hours old are exposed to the environmental sample diluted in test medium at a range of five different concentrations. One young female neonate is placed in 60-80 ml of test water in a 100 ml beaker. Ten replicates are used for each concentration plus a control. This is a description of a semistatic test. The test is performed at 18-22 ºC and the exposure time is 21 days which corresponds to five broods. The daphnia are fed with microalgae (*Chlorella, Pseudokirchneriella* or *Scenedesmus*) and the food supply should be based on the amount of organic carbon given to each parent animal per day. The ration levels should be between 0.1 to 0.2 mg C/daphnia/ day. Every two to three days the number of surviving organisms and the number of living offspring produced per parent animal are counted. The adults are then moved to fresh test medium and the offspring are discarded. The endpoint in the test is the percent reduction of reproductive output of the adult daphnids compared to the control (Walker et al. 2006; OECD nr 211, 2008).

A confounding factor in the assay is that there is no standardization of the food supply and the variation in quantity and quality of food supply can have an influence on the results (Walker et al. 2006). The daphnia clone should also be standardized.

4.2.3 Fish Short-term Toxicity test on Embryo and Sac-fry Stages

The short-term (subchronic) toxicity test on the embryo-larval stage of the fresh water fish *danio rerio* (zebra fish) based on the Swedish Standard (SS 02 81 93, 1988), which also exists as an OECD standard (OECD nr 212, 1998), was applied in the characterization of waste water influents and effluents from pulp mill industries in Sweden between the years 2001-2007 (SEPA Report 6304).

The zebra fish is a tropical freshwater fish that originates from the Indian subcontinent. It has become a model organism for toxicity testing and it has many advantages: Its small size (3-5 cm) makes it easy to manage in large quantities in a laboratory which reduces husbandry costs and space. The small size of its eggs and juveniles makes it possible to test it in a high-throughput screening format (with multi-well plates) and from this a satisfactory database can be created for statistical evaluation and validation of results. The embryonic development of zebra fish is very fast: In 24 hours after fertilization all major organs are developed, and after three to five days the fish has hatched and begun to look for food. After a successful fertilization the eggs and sac-fry survive well, normally over 90 percent (OECD nr 212, 1998). The generation time is approximately 3-5 months. A female can lay up to 200 eggs per week. Since the zebra fish develops outside the mother´s body and the eggs are transparent, the developmental stages can easily be observed in a microscope (Bopp et al. 2006).

In this test the early life stage, i.e. the embryo and sac-fry stages, of the zebra fish are exposed to a range of concentrations of the environmental sample for 14 days at 25 ºC. No food is provided in the assay and it is terminated while the sac-fry are still nourished from the yolk sac. The assay starts with placing fertilized eggs in the test chambers and is ended just before the yolk sac of any larvae has been fully absorbed, or before mortalities due to starvation begin in the controls. Lethal and sublethal endpoints such as frequency of hatching of embryos, median hatching time, median survival of embryo/fry stages, and frequency of malformation of embryos are assessed (OECD nr 212, 1998; SEPA Report 6304). The embryo and sac-fry test is viewed as a less sensitive test than the Full Early Life Stage test (FELS), (OECD nr 210, 1992) which lasts longer (35 days), particularly with pollutants that are highly lipophilic (log K_{ow} > 4) and those with a specific mode of action. According to the OECD standard nr 212 this test can form a bridge between sublethal and lethal tests, or be used as a screening test for chronic toxicity. One advantage of this test is that food supply is not a confounding factor.

A drawback with the zebra fish as a test animal is that is not ecologically relevant for Sweden although it belongs to the same family (*Cyprinidae*) as the Swedish roach (*Rutilus rutilus)* and bleak ((*Alburnus alburnus*) SEPA Report 6304). The three-spined stickleback (*Gasterosteus aculeatus L.*)[2] is becoming more and more popular as a test organism, however, and it is ecologically relevant for Sweden. Another popular test organism is the rainbow trout (*Oncorhynchus mykiss*)[3] which belongs to the family of salmonid fish (*Salmoninae*). Full life

[2] *Gasterosteus aculeatus L*. is a small fish (maximum 10 cm), belonging to the family *Gasterosteidae*, that live in both marine and fresh waters, along the entire Swedish coast as well as in larger lakes (Nationalencyklopedin 2011a) .

[3] *Oncorhynchus mykiss* originates from North America and north-east Asia, but has been transplanted to Sweden (among other countries) since the 19th century. It is used for food production and angling. It does not reproduce

cycle tests with salmonid fish are very impractical however, since this fish reaches maturity in as long as 2-5 years (Environment Canada 1998).

4.2.4 Nitocra spinipes Development and Reproduction test

A chronic test called "Harpacticoid and calanoid copepod development and reproduction test" for the salt water and brackish water species *Nitocra spinipes* is presently under validation by the OECD (Homepage of Toxicon AB).

Nitocra spinipes belongs to the order of Harpacticoids, the class of Copepods and the subphylum of Crustaceans. *N. Spinipes* along with all other harpacticoid copepods consist of 475 species in Scandinavia and more than 3000 species in the world. They exist both in marine, fresh water and ground water environments but most of them are free-living benthic organisms, and they are the second most common group of animals (next to nematodes) in marine benthic communities (Nationalencyclopedin 2010a; SEPA Report 5596). Copepods are important components in the marine food web and serve as source of nutrition for many significant fish species (Encyclopedia Britannica 2010a). *N. Spinipes* is very common in brackish environments, on shallow soft bottoms along the entire Swedish coast. It is very small (0.5-0.8 mm), and has sexual reproduction. It also has a short generation time (15-20 days at 20 ºC) and is easy to cultivate in a laboratory. It has been used since the 1970´s in the study of sublethal and lethal effects of chemicals, complex waste waters and landfill leachate waters in Sweden (SEPA Report 6304; SEPA Report 5596).

N. Spinipes has 11 juvenile stages in its development, divided into 6 nauplii and 5 copepodite stages. The 6^{th} nauplii-stadium and the 1^{st} copepodite stadium are easily discernible morphological stages. In the test 10-15 newly hatched animals (within 24-36 hours) are placed in the test water of each replicate, and 8 replicates are used for each concentration of environmental sample at the beginning of the test. The *N. Spinipes* are fed with the red microalga *Rhodomonas salina* on days 0, 2 or 3 and 5. The incubation time is around 8 days in order to measure the larval development ratio. After 6-8 days exposure, approximately 50 percent of the control animals have been transformed to the copepodite stage. The main sublethal endpoints applied are: larval development ratio (LDR), which was previously named larval development rate, and larval mortality. LDR is calculated by dividing the number of emerged copepodites to the number of animals alive at the end of the test in each replicate (SEPA Report 6304; Eklund et al. 2010).

4.2.5 Growth Inhibition test of the Marine macroalga Ceramium tenuicorne

A new ISO standard has recently been issued for a growth inhibition test with the red macroalga *Ceramium tenuicorne* which belongs to the family *Ceramiaceae* (ISO 10710, 2010). This test has now been applied both to whole sediments and to salt and brackish water samples (Eklund et al. 2010).

Red algeas are members of the phylum *Rodophyta* which consist of about 4100 species of mostly marine algae that grow on rocks, cliffs or on other large aquatic plants along the shore lines. The multicellular form of *Rodophyta* forms branched flattened thalli or filaments. *Ceramium tenuicorne* doesn´t get longer than 1 dm and it is a primary producer which

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very well in Swedish waters however. It´s related to the ecologically relevant Swedish brown trout (*Salmo trutta*) (Nationalencyclopedin 2011b).

supplies food and living space for many invertebrates and fish fry. It is a cosmopolitan species that exist in both the southern and northern hemisphere, and it growths along the entire Swedish coast (SEPA Report 6304; Dictionary of Biology 2008; Encyclopedia Britannica 2010b). Salinity along the entire Swedish coast varies between 4 and 32 ‰ and the two clones of *Ceramium tenuicorne* cover this entire range (see appendix 2, page 89).

The growth inhibition test works for both the marine and brackish water clones of the *Ceramium* that exist in Sweden. 2 -3.5 mm tips of the algae are cut off from growing cultures of *C. tenuicorne*. Two algae tips are added to each replicate and four replicates are exposed to various concentrations of the environmental sample for 7 days. The length of the algae from first forking to the most distant tip is measured, and growth rate at the different concentrations is recorded. Percent inhibition of growth compared to controls and EC_{50} are calculated (SEPA Report 6304; Eklund et al. 2010).

4.2.6 Whole Sediment Toxicity tests with Fresh water Invertebrates

Whole sediment toxicity tests can be performed using artificially made sediments or natural sediments. Artificial sediments can be used when testing chemicals whereas natural sediments (collected in the field) are normally used in order to determine a temporal or spacial distribution of sediment toxicity in the place of investigation. *Spiking* means that a test material, such as a chemical, mixture of chemicals, sewage sludge, oil, particulate matter or a highly contaminated sediment is added to a clean negative control or reference sediment in order to assess the toxicity of this particular test material (ASTM Standard, 1994). The reference sediment is taken from a clean area in the field and must have very similar sediment characteristics (i.e. total organic carbon content (TOC), particle size distribution, pH etc) as the test sediment. Most of the standards found were only developed for the testing of chemicals; yet the ASTM standard mentioned above describes the alternative applications.

Natural sediments used in the test must be characterized. This involves at a minimum a determination of pH and ammonia concentration of pore water, organic carbon content (total organic carbon, TOC), particle size distribution (percent sand, silt, clay) and percent water content. Other analysis of sediments may include: biological oxygen demand (BOD), chemical oxygen demand (COD), cation exchange capacity, total inorganic carbon, total volatile solids, acid volatile sulfides etc. Also, a pore water analysis may be performed, and qualitative aspects such as color and texture of sediment and the presence of macrophytes or animals should be investigated.

In the technical guidance document (TGD) on risk assessment from 2003, chapter 3, from the European Commission (ECB 2003), toxicity test methodologies in fresh water sediments are recommended for the following species: Amphipods: *Hyalella azteca, Gammarus sp.,* and *Diporeia sp*.; Insects: *Chironomus sp.* and *Hexagenia sp*.; Oligochaetes: *Tubifex tubifex* and *Lumbriculus* (earth worm*) variegates*; Nematodes: *Caenorhabditis elegans,* and references to these methods are given. Some of these methods are listed in appendix 2 (see page 88) and a few are described below. According to the TGD only whole sediment assays testing long term sublethal endpoints, such as reproduction and development, are applicable to marine sediment risk assessment. The reason for this is that the sediments act as a permanent sink to highly hydrophobic substances that accumulate steadily and expose benthic organisms during their entire life cycles.

4.2.6.1 Whole Sediment Acute Toxicity tests

An US-EPA (United States Environmental Protection Agency) standard (US-EPA Ecological Effects Test Guideline, 1996c) exists for testing whole sediment acute toxicity in invertebrates in fresh water sediments. This test guideline employs the amphipod *Hyalella azteca* and the insect larva *Chironomus tentans*. Acute or short term test last for maximum 10 days whereas a long term test last up to 20-65 days depending on species (ASTM standard, 1994; OECD nr 218, 2004). The primary endpoint in the 10-day sediment toxicity test with *H. azteca* is survival, and with *C. tentans* dry weight and survival.

Hyalella azteca lives in North and South America in lakes, ponds and streams. Individuals of *H. azteca* are often found in mesotrophic and eutrophic lakes, but can also be found in brackish waters with a salinity below 29 ppt (parts per thousand). They are epibenthic detritivores, i.e they feed on decomposing organic matter, and burrow in the sediments. Individuals of *H. azteca* are however sensitive to the hardness of the water and they are not found in waters with a $CaCO_3$ concentration above 7 mg/l. *H. azteca* complete a life-cycle in 27 days, or longer, depending on the temperature. The development goes through a minimum of 9 instars: Instars 1-5 form the juvenile stage, instars 6-7 form the adolescent stage, and instar 8 and older form the adult stage. They reproduce sexually with an average of 18 eggs per brood and 15 broods every 152 days. Advantages with this test species are: Short generation time, easily collected in the field or cultured in the laboratory in large quantities, a wide tolerance to sediment grain size, tolerance to a wide range of temperatures, and reference data on survival, growth and reproduction can be obtained (ASTM Standard, 1994).

The test starts with 7-14-day-old amphipods. The test is conducted at 23 ºC with an illumination of 500-1000 lux. The amphipods are fed with a mixture of yeast, Cerphyl and trout chow (YCT) at a rate of 1.5 ml per day and test chamber. Surviving organisms are isolated and preserved in 8 % sugar formalin at the end of test, and growth is measured.

The larval stages of *Chironomus tentans* are often found in eutrophic lakes and ponds and they usually penetrate a few centimeters into the sediments. In both lentic and lotic water with soft bottoms, approximately 95 percent of the chironomid larva are located in the upper 10 cm of the sediment. The larvae are found in the field in a temperature range between 0 and 35 ºC. The life-cycle of *C. tentans* is divided into three stages: The first one is the larval stage, consisting of 4 instars. The second one is the pupal stage, and the third one is the adult stage. Under optimal conditions the larvae will pupate and emerge as adults after 24-28 days at 20 ºC (ASTM Standard, 1994). Advantages with this test species are: short generation time, a tolerance to a wide range of sediment grain size and a wide range of organic matter content.

In the short term test, third instar chironomids are employed at the start of the test. The midges are fed daily with 1.5 ml of a 4 g/l suspension Tetrafin™ daily (ground tropical fish food flakes). Larval survival and growth can be assessed after 10 days, and growth is preferably determined using dry weight.

4.2.6.2 Whole sediment Long term Toxicity tests

Additional endpoints for *H. azteca* on reproduction can be obtained with a long term test lasting up to 30 days. These are: reproductive behavior, secondary sex characteristics, egg production and the number of young produced (ASTM Standard, 1994). This long term test is recommended by the European commission.

Two OECD standards (OECD nr 218 and nr 219) from 2004 are available for testing long term toxicity to the larvae of the fresh water dipteran *Chironomous sp*. in sediment and sediment pore water, respectively. The two tests are similar with the only difference that the test material is spiked into the sediment in nr 218, and spiked into the overlaying water in nr 219. The test duration for *C. tentans* is 28-65 days and for *C. riparius* 20-28 days. In this long term test first instar chironomids are employed at the start of the test. The endpoints are development time and total number of emerged adult female and male midges. Larval survival and growth may also be determined in a short term test after 10 days if needed. The purpose with this long term test is to investigate how different exposure routes for *Chironomous sp*. may contribute to the overall toxicity of the sediments. For this purpose food is added to the clean negative control sediment before the test material is added. For highly lipophilic pollutants (with a log K_{ow} >5) which absorb strongly to the organic material in the sediment or for those which bind covalently to it, ingestion of contaminated food may be a major exposure route.

An OECD standard (OECD nr 225) from 2007 is also available för testing long term toxicity to the fresh water endobenthic oligochaete *Lumbriculus variegates*. Endobenthic organisms burrow in the sediments and take in sediments particles below the sediment surface. The exposure routes for these organisms are thus several: Via contact with sediment, ingestion of contaminated sediments particles, via pore water or overlying water. The exposure time of this assay is 28 days and the endpoints are reproduction and biomass (growth). This test is recommended by the European commission.

Table 3: Examples of standardized Whole organism *in vivo* tests investigated.

Test ref.	Organism	Endpoint	Matrix	Exposure time	Price range	Performer
OECD 201 (2006)	Microalgae and Cyanobacteria	Growth inhibition	FW, X	72 hours	7	Toxicon AB, Pelagia Miljökonsult AB, Sweden
ISO 10253 (2006)	Microalgae: *Phaeodactylum tricornutum*	Growth inhibition	SW, P, SE, X	72 hours	7	Toxicon AB, Sweden
OECD 202 (2004), ISO 6341	*Daphnia magna* (Crustacea)	Immobilization	FW, X	48 hours	6	Toxicon AB, Pelagia Miljökonsult AB, Sweden
OECD 211 (2008), ISO 10706	*Daphnia Magna* (Crustacea)	Reproduction	FW, X	21 days	7-10	Toxicon AB, Pelagia Miljökonsult AB, Sweden
OECD (modified draft)	*Nitocra spinipes Boeck* (Crustacea)	Larval development ratio (LDR)	WW, BW, X	10 days	n.f.	Toxicon AB, Sweden
SS 02 81 06 (1991)	*Nitocra spinipes Boeck* (Crustacea)	Mortality	WW, BW, X	48 hours, alt. 96 hours	6	Toxicon AB, Sweden
SS 28214 (1996)	*Ceriodaphnia dubia* (Crustacea)	Immobilization	LL, URW, WW, X	48 hours	6	Toxicon AB, Sweden
OECD 203 (1992), SS 02 81 62	Zebra fish and salmon fish	Mortalitiy, Acute toxicity	FW, X	96 hours	7 or 8	Toxicon AB, Sweden
OECD 210 (1992)	Zebra fish, Rainbow trout, and others	Lethal and sublethal effects on eggs, embryo, larvae and juvenile fish	FW, SW	30-60 days	n.f.	n.f.
OECD 212 (1998), SS 02 81 93	*Brachydanio rerio* , Zebra fish	Lethal and sublethal effects on eggs, embryo and larvae	FW, WW, X	14 days	9-10	Toxicon AB, IVL (Swedish Environmental Research Institute), Pelagia Miljökonsult AB, Sweden
OECD 204 (1984)	*Brachydanio rerio*, Zebra fish and others	Mortality, behaviour	FW	14 days	n.f	n.f.
OECD 221 (2006)	*Lemna minor* (duckweed)	Growth inhibition	LL	7 days	7	Toxicon AB, Pelagia Miljökonsult AB, Sweden

n.f. = information not found

Price ranges: 1= 0-50 € (Euro)/ sample, 2= 51-100 € / sample, 3= 101-200 € / sample, 4= 201-400 € / sample, 5= 401-600 € /sample, 6= 601-1000 € / sample, 7= 1001-2000 € / sample, 8= 2001- 3000 € / sample, 9= 3001-4000 € / sample, 10= 4001- € / sample

Matrices: S=whole sediment, SE=sediment elutriate, P= Porewater, X=Sediment extract, FW= Fresh Water, BW= Brackish water, SW= Salt water, SS= sewage sludge, WW= WWTP influent and effluent, LL=Landfill leachate water, URW= Urban runoff water

4.2.7 Field Biomarkers applied in the Biomonitoring of Marine Coastal Ecosystems

In the biological effect monitoring of coastal fish in Sweden, a battery of field biomarkers are applied twice a year to measure biochemical, physiological, histological and pathological changes in the fish. The aim with these investigations is to explain how subcellular and cellular changes induced by environmental contaminants will cause disturbance on the whole organism level and eventually on the ecosystem level. This "bioeffect approach" has been employed since the 1980´s in Sweden on complex emissions of pollutants into water recipients from metal and papermill industries as well as from other industries and WWTPs and has been proven sensitive (Guidance document for environmental screening, 2006; Lethonen et al. 2006). By using a broad battery covering early sublethal changes on immune system, liver function, reproduction, ion balance, metabolism of pollutants, etc. early warning signals to environmental pollutant exposure can be provided. Examples of test batteries of biomarkers used in different research projects in Sweden and in the Baltic sea are listed in appendix 4 (see page 108). Field biomarkers can be divided into general stress biomarkers, semispecific biomarkers and specific biomarkers, and examples of these are given below (Lehtonen et al. 2006).

The physiological status of wild fish are also affected by environmental conditions. Abiotic factors such as climate, hydrography, oxygen levels and salinity will therefore have an impact on the biomarker measurements. Biotic factors such as age, size of animals, sexual maturity, nutrient supply and presence of parasites will also have a bearing on the results. To avoid these confounding factors, fish of the same age, size and sex are employed in the experiments and they are conducted at the same time, season and location every year.

The BEEP project (Biological Effects of Environmental pollution in Marine Coastal Ecosystems) was a EU-funded project that was carried out during 2001-2004. Its goal was to validate and intercalibrate a battery of biomarkers in selected so called bioindicator species in the Mediterranean, the North Atlantic and the Baltic Sea (Lehtonen et al. 2006). Appendix 4 lists the biomarkers employed in this project in the Baltic Sea, and conclusions and observations made. The bioindicator species used in this project, living in the different subregions of the Baltic sea, were (1) Baltic clam (*Macoma balthica*), a deposit/ filter feeder that has a widespread distribution in the Baltic Sea in soft bottom sediments, (2) the blue mussel (*Mytilus spp*.), a filter feeder that is also widespread and lives in rocky and sandy areas near the coast up to mid-gulf of Bothnia, (3) eelpout (*Zoarces viviparous*), a benthic, stationary, viviparous predator fish that lives in near-coastal areas in the entire Baltic sea, (4) the European flounder (*Platichthys flesus*), a benthic predator fish living in estuaries, coastal and offshore areas in the entire Baltic sea, and lives in close contact with sediments directly taking up pollutants, and (5) perch (*Perca fluviatilis*), a relatively stable pelagic predator fish (that is originally a fresh water species) that lives in estuaries and river mouths and is restricted to low-salinity areas, and is not common in the south-west Baltic Sea (Lehtonen et al. 2006).

4.2.7.1 General stress field Biomarkers

General stress biomarkers are caused by exposure to various contaminants. Examples of these are Lysosomal membrane stability (LMS) which provides information on the start and progression of liver/digestive gland pathology and similar dysfunctions, Macrophage activity,

MA-AP (enzyme activity of acid phosphatase (AP) in liver macrophage aggregates (MA)) which is an implication of immunotoxicity, Micronuclei frequency (MN) implying genotoxicity, expression of Stress proteins due to oxidative stress or damage. Other examples are externally visible fish diseases, and general histopathology of fish liver and gonads, such as LSI (liver somatic index) and GSI (gonadsomatic index) (Lethonen et al. 2006).

4.2.7.2 Semi-specific field Biomarkers

Examples of semi-specific biomarkers are the neurotoxicity biomarker Acetylcholinesterase (AChE) inhibition which is caused by organophosphates and carbamate insecticides, and the induction of detoxification enzymes such as 7-Ethoxyresorufin-O-deethylase (EROD), Catalase or glutathione-S-transferase (GST).

General stress biomarkers and semi-specific biomarkers can be applied in the screening of new chemicals, as well as provide an early warning or indication of severe health effects of contaminants (Lethonen et al. 2006).

4.2.7.3 Specific field Biomarkers

Examples of specific biomarkers are the detection of PAH metabolites in bile and DNA adducts (genotoxicity) which are mostly caused by polyaromatic hydrocarbons (PAHs) in the fish environment. Other examples are the biomarkers of endocrine disruption such as imposex caused by organotin compounds (tributyl tin (TBT)) in gastropods, intersex (ovotestis) in male fish or the induction of vitellogenin in male fish caused by xenoestrogens in the environment. Still other examples are the inhibition of the heme pathway aminolaevulinic acid dehydratase (ALAD) caused by lead, the induction of metallothionein caused by heavy metals, and specific gonad and liver histopathology (liver nodules, neoplastic lesions etc.).

Specific biomarkers reveal exposure of organisms to specific chemicals or class of chemicals. The biological response here is specific, which then can direct the government environmental inspector to a more detailed chemical analysis of the exposed biota or sediments (Lethonen et al. 2006).

4.3 Mechanistic (In Vitro) Tests

In vitro assays can provide information on the mechanism of action of the pollutants but they are limited their ability to inform anything about the absorption, biotransformation, distribution and excretion of the compound in the whole organism (Sohoni and Sumpter 1998). The most significant advantage of *in vitro* systems is that they are appropriate for large-scale screening of environmental pollutants. This advantage in turn is due to lower costs, shorter duration of tests and the sparing of experimental animals such as fish in the field (Kinnberg 2003). Other advantages are that the environmental conditions (temperature, pH, etc) can be controlled, the results are highly reproducible, and very small amount of sample is needed for each test.

Disadvantages of mechanistic assays are that the cell lines used usually have minimal metabolic capacity and therefore cannot show the effects of bioactivation of toxicants in the animal or if the compound would be detoxified or degraded *in vivo*. *In vivo* assays must thus normally accompany *in vitro* assays to confirm their results. Results from *in vitro* assays can in turn help in the design and interpretation of the whole organism assays.

The existence of inhibitory or cytotoxic substances in environmental samples that affect cell viability or growth is a problem with *in vitro* assays. Therefore a cytotoxicity test is most often accompanying the assay. In this case yeast cells are more robust and less sensitive to cytotoxicity than animal cells.

4.3.1 In vitro assays for monitoring of endocrine disruptive chemicals

In vitro assays that are applied in monitoring endocrine disruption in the environment are divided into three categories: The first one is the ligand binding assay (example is the enzyme-linked receptor assay (ELRA)) and it can be used for high throughput screening. It does not measure if the receptor is activated but only the binding affinity of ligand to receptor. Consequently, this assay cannot discern between agonistic and antagonistic mechanisms of the substance and they can neither show that the effect truely takes place *in vivo*, or that the pollutant in the end would have an adverse effect on the organisms. These assays can therefore best be applied for the screening of potential endocrine-disrupting compounds (Streck 2009).

The second type of assay is the recombinant receptor-reporter assay and this is the most commonly used in detecting EDCs in the environment. Examples are the YES and YAS assays, CALUX (Chemically Activated LUciferase eXpression) assays, and other reporter gene assays. These employ yeast, fish or mammalian cell lines that have been transfected with DNA sequences from estrogen, progesterone or androgen receptors and with their respective steroid response element (SRE), that is fused into the promoter region of a reporter gene. The translation product of the reporter gene thus becomes a way to measure the transactivation of the steroid receptor by the EDC. This occurs via a cascade of events in which the receptor changes its conformation after the binding of the EDC and heatshock proteins are released from it. The receptor usually then translocates into the nucleus, dimerizes and then binds to steroid response elements on the DNA. These assays measure the activation of the receptor and thereby the toxic potency of the substance (Streck 2009).

The receptors belong to a superfamily of nuclear transcription factors/receptors that includes receptors for steroid hormones, vitamin D3, thyroid hormones and retinoids (Chatterjee et al. 2008), and between these receptors rersearchers have found there to be crosstalk, meaning that a compound that normally activates one receptor may instead activate or block another receptor in the superfamily.

The third type of assays are the cell proliferation assays (examples E-Screen and A-Screen) that measure pro- or anti-proliferative effects of androgens, estrogens or thyroidogens on their target cells.

4.3.1.1 Yeast-based reporter gene essays

In vitro assays for endocrine disruption measure the total androgenic, estrogenic or progestogenic activity of the environmental water sample without concern for which substances are the cause of the activity. Yeast cells do not naturally possess estrogen or androgen receptors but these receptors have been found to function well in yeast (Kinnberg 2003). The advantage is that there are no endogenous hormone receptors that may interfere with the results in the yeast-based reporter gene assay. The reporter gene assays can be applied in measuring both agonistic and antagonistic activities of pollutants. In the latter case a reference steroid compound is added to the assay at a concentration that gives a submaximal response. The ability of the test compound to inhibit the colour-change induced by the reference estrogen, androgen or progesterone is then measured.

YES assay

The yeast estrogen screen (YES) assay employs yeast cells (*Saccharomyces cerevisiae*) where DNA sequences from the human estrogen receptors (hERα or hERβ) have been stably incorporated into the genome (Streck 2009). The cells have then been transfected with expression plasmids containing estrogen response elements (ERE) that regulates transcription of a reporter gene called *lacZ* which encodes the enzyme ß-galactosidase. When an estrogenic compound binds to the receptor, ß-galactosidase is synthesized and sectreted into the medium (see figure 1). Consequently, a chromogenic substrate present called chlorophenol red ß-D-galactopyranoside (CPRG) undergoes a colour transformation from yellow to red and the absorbance of the end product can be measured at 540 nm (Sohoni and Sumpter 1998). The total estrogenic activity of the sample is compared to the activity of the most potent natural estrogen 17β-estradiol (E2) and can be expressed as percent of activity induced by the positive control, E2. Alternatively, it can be expressed in estradiol equivalents (EEQ). For single substances, EEQ is calculated as the ratio between the EC_{50} of E2 and that of the substance: EEQ = EC_{50} of E2/ EC_{50} of substance, and for environmental samples or extracts with unknown composition EEQ is calculated as the ratio between EC_{50} of E2 and the dilution of the sample/extract giving a response equal to 50 percent of the maximum response caused by E2: EEQ = EC_{50} of E2/1/dilution factor. Thus, for environmental samples EEQ becomes the product of the dilution factor and the EC_{50} of E2. This allows for a quantification of estrogenic activity without knowledge of the individual chemical components responsible for the activity in the sample (Kinnberg 2003). If chemical analysis of the individual pollutants in the sample is performed, an instrumentally derived EEQ can also be calculated by the equation:

$$EEQ = \Sigma x_i c_i$$

where x_i represent the relative potency of the of the individual pollutant and c_i represents the concentration of this pollutant in the mixture (Gutendorf and Westendorf 2001).

The YES assay is cost and time-saving because it is performed in 96-well microtiter plates that can analyze large amount of samples at many different concentrations and the results can be acquired in 3 to 4 days. There is no need for cell harvesting, counting and the results can be observed visually which allows for both qualitative and quantitive interpretations (Routledge and Sumpter 1996).

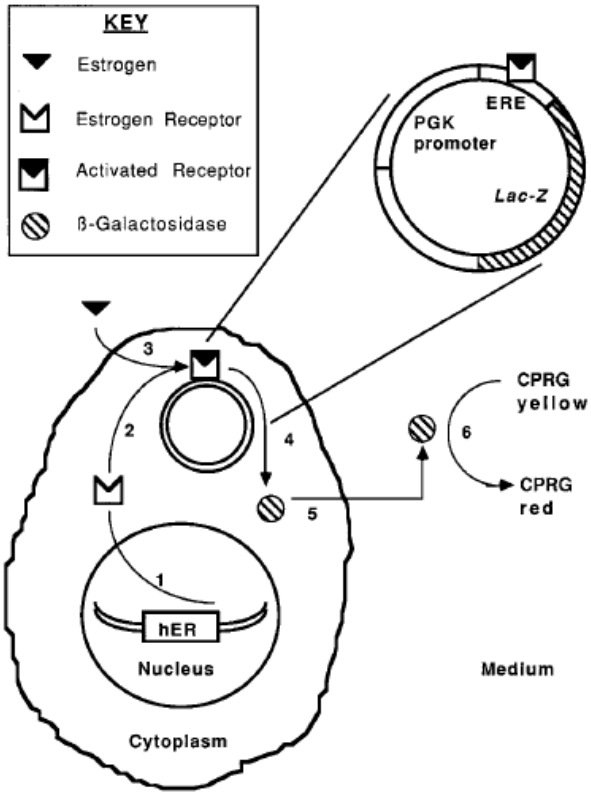

Figure 1: Visual representation of the Yeast Estrogen Screen (YES) assay (Routledge and Sumpter 1996).

YAS assay

The Yeast androgen screen (YAS) assay works by similar principles as the YES assay except that the human androgen receptor (hARα) has been stably incorporated into the genome of the *Saccharomyces cerevisiae* cells and they have then been transfected with expression plasmids containing the androgen response elements (ARE) fused to the promoter region of the reporter gene *lacZ*. The reference androgen in the YAS assay is dihydro-testosterone (DHT) and the androgenic potency may be expressed as dihydtotestosterone equivalents (DEQ).

The YAS assay was however found to be ten times less sensitive then the YES assay and also somewhat less specific. Estrogenic compounds such as 17β-estradiol also showed some activity although with much less (1/30) potency than DHT or testosterone. Progesterone and estrone showed low activities but only at concentrations above 10^{-8} M (Sohoni and Sumpter 1998).

Progesterone receptor transactivation assay

The progesterone receptor transactivation assay, designed by Chatterjee et al., works by similar principles as the YES and YAS assays, except that instead of using *lacZ* as a reporter gene it employs an optimized green fluorescent protein (yEGFP). A yeast YPH 499 strain is cotransformed with two plasmids, one containing the human progesterone receptor (hPR), and the other one containing progesterone response elements (PRE) fused to a minimal yeast based promoter (CYC1) and the reporter gene yEGFP. The activity of the hPR is monitored by measuring fluorescence in a spectroflourimeter with an excitation wavelength 485 nm and an emission wavelength of 530 nm. Progesterone is used as the positive reference compound in the assay. The mean flourescence measured at 1 nM (nanomolar) progesterone is appointed the value of 100 procent and the other data are compared to this value (Chatterjee et al. 2008).

4.3.1.2 Mammalian-based reporter gene assays

Due to the present increase in discharge of unknown potentially endocrine disrupting chemicals such as medicines and personal care-products into the aquatic environment there has been a call to develop more assays to test for effects on hormone dependant physiological functions in humans and wildlife (Schriks et al. 2009). CALUX (Chemically Activated LUciferase eXpression) assays are rapid, sensitive tools for assessing toxic potency of samples from river and estuarine sediments caused by PHAHs (polyhalogenated aromatic hydrocarbons) and other endocrine disrupting substances.

Biodetection Systems has developed a series of CALUX assays from stable human cell lines that specifically detect compounds that interacts with androgen (AR), progestagen (PR), glucocorticoid (GR), thyroid hormone (TR) or estrogen (ER) receptors (Sonneveld et al. 2005; Schricks et al. 2009). The advantage with the human U2-OS osteosarcoma cell line is that the receptors whose activities we wish to monitor (e.g. ER, PR, GR and AR) are not endogenously present in the cells at detectable levels, which makes the cross reactivity unlikely. The assays thus are highly specific and selective, and the receptors are highly inducible, with a 30 fold induction with the reference hormone compounds (Van der Linden et al. 2008). Because this CALUX series uses the same human cell line in all assays, they are easily comparable, and they can be applied in the monitoring of hormonal activity in extracts of surface water, ground water, municipal or industrial waste water, as well as of sewage sludge (Homepage of Biodetection Systems, 2010). The CALUX assays are performed on 96 well cell culture plates.

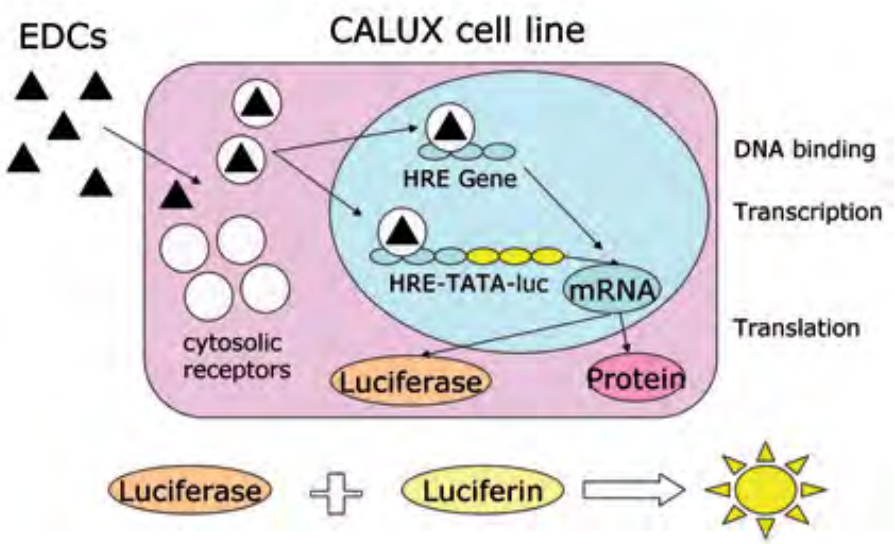

Figure 2: Visual representation of the principles of the CALUX assay. A mammalian cell-line has been transfected with a construct consisting of hormone response elements (HRE) fused to a minimal TATA promoter and a luciferase reporter gene (*luc*). The endocrine disrupting chemicals (EDC) under investigation binds to the hormone receptor and stimulates transcription of *luc.* Luciferase acitivity is then quantified by adding the substrate luciferine and measuring light production (Schriks et al. 2009).

DR-CALUX assay

DR-CALUX (Dioxin Responsive Chemically Activated LUciferase eXpression) assay is applied on complex environmental samples and is a good screening tool. Instead of identifying individual congeners, it analyzes the total toxic equivalent, i.e. the TEF equivalent of chemical mixtures in the sample. Dioxin-like compounds are coplanar and the mechanism of action is via the aryl hydrocarbon receptor (AhR). They are classified as PHAHs and include congeners of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs) and polychlorinated biphenyls (PCBs). They are persistent in the environment, tend to bioaccumulate and due to their hydrophobic nature, sediments are often the ultimate compartment (or sink) for these compounds where they are bound to organic particles or bioaccumulated in benthic organisms (Hurst et al. 2004). Via the food-web they biomagnify to higher organisms.

The dioxin responsive chemically activated luciferase gene expression (DR-CALUX) bioassay uses a rat hepatoma (H4I1E) cell line that has been stably transfected with a construct consisting of the dioxin-responsive element (DRE) sequence and a luciferase reporter gene from the firefly *Photinus pyralis*. Luciferase activity is then quantified by adding the substrate luciferin and measuring light production. The induction of luciferase activity is dose-dependent upon exposure to PHAHs and is comparable to the natural activity of the CYP-1A enzyme. The relative potency of the PHAHs in the CALUX assay is compared to the most potent agonist of the Ah receptor, 2,3,7,8,-tetrachlorodibenzo-*p*-dioxine (TCDD) and is measured in so called TCDD equivalents (CALUX-TEQs) (Murk et al. 1996). When calculating TEQ (TCDD equivalent, measures total toxicity in the sample) values, analytical quantifications of various congeners of the PCDDs, PCDFs and planar PCBs are multiplied

with toxicity equivalency factors (TEFs) for each of the congeners. TEFs are internationally agreed dioxin potency values based on large amount of toxicity data for each congener (Hurst et al. 2004).

PAHs and their metabolites have also been found to contribute to dioxin-like or Ah receptor mediated toxicity in sediments. Machala et al. (2001) found that the azaarene dibenzo[*a,h*]acridine (a derivative of the PAH dibenzo[*a,h*]anthracene) added significantly to the overall Ah receptor mediated toxicity of river sediments in the Czech Republic in the CALUX assay. PAHs are not as persistent as PHAHs but they should also be considered in this assay since they are major pollutants in many environmental samples, and the peak of activity of PAHs are found after 6 hours in the assay (Machala et al. 2001; Vondráĉek et al. 2001). This is because less persistent AhR agonists are broken down in the *in vitro* cell system during the complete 24 hour exposure period. Another problem is that clean-up procedures eradicate PAHs from the sample which is why crude sediment extracts must be used to detect their dioxin-like activity. The relative potency of the PAHs in the CALUX assay is compared to a most potent PAH inducer Benzo[*a*]pyrene (B*a*P) and is measured in B*a*P equivalents (CALUX-BEQs) (Vondráĉek et al. 2001).

ER-CALUX assay

The estrogen responsive chemically activated luciferase gene expression (ER-CALUX) assay uses T47-D human breast adenocarcinoma cells that express endogenous human estrogen receptors hERα and hERß and have been safely transfected with a construct consisting of three estrogen responsive elements (ERE) fused to a TATA box and a luciferase reporter gene. It works by similar principles as the DR-CALUX assay (Legler et al. 1999). CALUX assays based on a human U2-OS osteosarcoma cell line have also been developed that are more specific for certain ERα and ERß interacting ligands (ERα-CALUX and ERβ-CALUX), (Sonneveld et al. 2005). To rule out the influence of confounding factors such as toxicity of water or sediment extracts, cells are investigated for signs of cytotoxicity by light microscopy or other methods.

The EC_{50} value for the positive control agonist is often used to measure the sensitivity of bioassays. This EC_{50} value (also called EC_{50} detection level) shows the concentration of reference agonist needed to give a response equal to 50 percent of the maximum signal in the dose-response curve in the assay (Hallgren 2009). The EC_{50} detection levels for various bioassays are shown in table 4. The EC_{50} value for the reference estrogen receptor agonist 17ß-estradiol in the ER-CALUX assay is in the range of 2-5 pM which makes the assay very sensitive.

AR-CALUX assay

The androgen responsive chemically activated luciferase gene expression (AR-CALUX) bioassay developed by Biodetection Systems in the Netherlands consists of the human U2-OS osteosarcoma cell line that has been stably transfected with the human androgen receptor and a luciferase reporter construct consisting of three androgen response elements (ARE) coupled to a minimal TATA promoter. This will prevent the interference of signal transduction pathways other than AR-mediated signals (such as via glucocorticoid receptor (GC), progesterone receptor (PR) and mineralcorticoid receptor (MR), classified as the 3C group) and thereby make the cell line highly sensitive and specific for (anti-)androgens (Sonneveld et al. 2005). EC_{50} detection levels for various androgen receptor agonists are in the nM -scale.

PR-CALUX assay

The progestagen responsive chemically activated luciferase gene expression (PR-CALUX) bioassay developed by Biodetection Systems in the Netherlands consists also of the human U2-OS osteosarcoma cell line that has been stably transfected with the human progesterone receptor (hPR) and a luciferase reporter construct, and works by the same principles as the AR-CALUX assay. The reference compound used is the synthetic progestin Org2058 (Schrick et al. 2009). EC_{50} detection levels for various progestins are in the range of 50-125 pM.

MVLN bioassay

The MVLN bioassay works by similar principles as the ER-CALUX assay, except that a different cell line, the MVLN cell line derived from MCF-7 (mammary carcinoma fibroblast) cells, is employed. The cells have been stably transfected with a plasmid (pVit-tk-Luc) containing the luciferase gene under the control of estrogen response elements (ERE) from 5´ flanking region of the *Xenopus* Vitellogenin A2 gene, and a herpes simplex virus promoter for thymidine kinase (tk) (Demirpence et al. 1993). Luciferase activity is measured in cell lysates after the addition of luciferine with a microplate luminometer Luminoscan (Machala et al. 2001). This bioassay can detect both estrogenic and anti-estrogenic potency in test samples. Maximum induction is obtained with 10^{-9}M of the reference ER agonist, 17ß-estradiol. Sample values are then expressed as mean percentages of maximum induction (Demirpence et al. 1993).

4.3.1.3 Fish-based reporter gene assays

The advantage with using fish cell lines is that it provides taxon-specific data, which is ecologically relevant if the purpose is to study effects on fish populations. For instance it would be more relevant to estimate dioxin equivalents (TCDD equivalents, TEQs) for fish cells rather than for mammalian cells in this situation. This could be done by using the PLHC-1 cell line from hepatocellular carcinoma of topminnow (*Poeciliopsis lucida*) instead of rat hepatoma cells (Huuskonen et al. 2000).

Similarly, steroid receptors from different species have been shown to have different binding affinities for environmental steroids/xenosteroids. For example the rainbow trout ER (rtER) binds with lower affinity to E2 than the human ER, and in the RTG-2 reporter gene assay described below, nonylphenol, octylphenol, bisphenol A and o,p´-DDD were shown to have higher affinity for rtER than in assays employing mammalian ER (Ackermann et al. 2002). Thus these differences can be important for the calculation of estradiol equivalents (EEQ) of the sediment or water samples.

RTG-2 Reporter gene assay

The RTG-2 reporter gene assay is a fish-specific assay based on a rainbow trout (*Oncorhynchus mykiss*) gonad cell line (RTG-2) that has been transiently contransfected with an expression vector containing rtERα complementary DNA, a mammalian neomycin phosphotransferase gene (pCL-neo-rtERα), and an estrogen-responsive firefly luciferase reporter vector (pERE-TK-luc) as well as an internal control reporter vector (pRL-TK, containing *Renilla* luciferase and *Herpes simplex* virus TK promoter) which is employed in order to get minimal experimental variability. The assay has an 3-4 -fold induction with the reference positive agonist E2 compared to control cells, and is more sensitive in detecting estrogenic activity in environmental samples than the *in vitro* vitellogenin assay described under the

section 5.3.4.2. It has been applied on WWTP effluent extracts from Switzerland (Ackerman et al. 2002).

4.3.1.4 Cell Proliferation Assays

Androgens and estrogens have been found to regulate cell numbers in their respective targets tissues (epithelial cells in prostate, mammary gland, endometrium etc.) via three different mechanisms: (1) increased cell proliferation, (2) inhibition of cell death, and (3) inhibition of cell proliferation (Szelei et al. 1997). The mechanism of cell proliferation regulation in target sex organs is not completely understood but sex steroid receptors are believed to play an important role. It has been observed that the proliferation of androgen and estrogen target cells is controlled by sex hormones in two alternative steps: In a step 1 process, sex steroids induce cell proliferation by cancelling the inhibition by specific serum-borne proteins, and the result is cell proliferation of target cells. In a step 2 process, sex steroids directly trigger the expression of non-identified endogenous inhibitors of the proliferation of target cells, i.e. the result is inhibition of cell proliferation (Szelei et al. 1997). The E-screen method (see below) employes the first of these mechanisms whereas the A-screen method (see below) employs the second mechanism.

Thyroid hormones in turn have been found to play a significant role in foetal and prenatal development in vertebrates, such as in the development of sexual organs and the central nervous system in mammals, the metamorphosis of amphibians and of flounder (Gutleb et al. 2005). Several classes of pollutants were found to interfere with the thyroid hormone signal transduction pathways and thereby disturbing thyroid hormone homeostasis. The T-screen assay is founded on thyroid hormone dependant cell proliferation and investigates the disturbance of xenobiotics with T_3-receptor (T3R) interaction in the cells (Gutleb et al. 2005). The structural similarity between thyroxine (T_4), the precursor to the active thyroid hormone 3,3´,5-triiodothyronine (T_3), and the flame retardants TBBPA and PBDE, may provide the explanation why these BFRs disturb the thyroid axis. Previous research has shown that TBBPA and HBCD have T3-like activity *in vitro.* Other studies have indicated that TBBPA and hydroxylated PBDEs competed *in vitro* with T_4 for binding to human transthyretin (TTR) which is a protein that transports T_4 in the blood (Hamers et al. 2006).

E-Screen

In the E-Screen assay proliferation of MCF-7 (mammary carcinoma fibroblast) cells in response to estrogens is measured. The E-screen assay is based on the three following assumptions: (1) mammalian serum-born molecules inhibit the proliferation of human MCF-7 cells, (2) estrogens induce cell proliferation by annulling this inhibitory effect by the serum-born molecules, and (3) non-estrogenic steroids and growth factors do not abolish the inhibitory effect on cell proliferation by mammalian serum (Soto et al. 1995; Kinnberg 2003).

The MCF-7 cell line was developed at the Michigan Cancer Foundation in the 1970s and it stems from a woman in the late phases of metastatic mammary carcinoma. It is often applied in studies of estrogenic chemicals since these cells have been shown to have an estrogen-responsive cell growth (Kinnberg 2003).

The cells are allowed to proliferate for 4-6 days in a medium that contains serum which has been made free of estrogens (and other sex steroids) by charcoal-dextran adsorption, together with the environmental sample in a range of different dilutions/concentrations. In the end of

the incubation period the cells are lysed and nuclei are counted on a Coulter Counter (Kinnberg 2003). The proliferative effect (PE) is defined as the ratio between the highest cell yield achieved with the environmental sample and that with the hormone-free control. The estrogenic effects of the pollutants is assessed by (a) calculating its relative proliferative potency (RPP) which means the ratio between the minimum concentration of estradiol needed for maximal cell yield and the minimum dose of the environmental sample needed to get the same effect, or by (b) calculating its relative proliferative effect (RPE), which is 100 times the ratio between the highest cell yield achieved with the environmental sample and that with E2. A full agonist thus has an RPE =100 (same proliferative response as E2) and a partial agonist has an RPE between 0 and 100 (Soto et al. 1995).

The E-screen assay has been applied in the analysis of effluents from WWTPs in southern Germany as well as river water samples upstream and downstream from a WWTP in Italy (Körner et al. 1999, 2000, 2001; Bicchi et al. 2009).

A-Screen

Szelei et al. (1997) designed a MCF-7 cell line that responds to androgens with inhibition of cell proliferation. A full human androgen receptor (AR) vector was transfected into MCF-7 cells. The resulting cell line, called MCF-7-AR1 cell line, contained five times more AR than the wildtype MCF-7 cells. When grown in serum-free medium with added physiological concentration of androgens (testosterone, DHT or methyltrienolone (R1881)) proliferation of the MCF-7-AR1 cells was inhibited. The cells were arrested in the G_0/G_1 phase within 24 hours. When estrogens were added to these cells cell proliferation was induced as normal. The conclusion was that the androgen–induced inhibition of cell proliferation was AR-mediated (Szelei et al. 1997).

No application on environmental samples have yet been performed or reported with this assay.

T-Screen

The T-Screen is a mammalian cell-based assay that was created to bridge the gap between the limitations of *in vitro* assays such as the T4-TTR- binding competition assay, which uses only isolated molecules (enzymes, transport proteins), and the more complex, expensive and time –consuming *in vivo* assays. The T-Screen assay is founded on the thyroid hormone dependant cell proliferation of a rat pituitary tumour cell line (GH3) in serum-free medium. In the serum-free medium the cells cannot grow without the addition of T_3. The cells are seeded into 96-well microplates and the environmental samples incubated for 96 hours. The reduction of AlamarBlue™ as a result of metabolic activity is included in the assay to measure cell growth. In the AlamarBlue™ test, resazurin is reduced from its non-flourescent oxidized form to its highly fluorescent reduced form resorufin. Environmental samples are tested both in the presence and absence of thyroid hormone (T_3), at EC_{50} concentration of T_3, in order to test both for agonistic and antagonistic activities. Cell proliferation is expressed relative to the maximum response attained with 10 nM of T_3 which is set as 100 % induction (Gutleb et al. 2005).

4.3.1.5 Ligand binding assays

The enzyme-linked Receptor assay

The enzyme-linked receptor assay (ELRA) is founded on the same principle as the competitive enzyme-linked immunoassay (ELISA), with ligand–protein interactions. The only difference is that instead of using an antibody as linking protein it uses the relevant human estrogen receptor (hERα). The ELRA investigates the competition of environmental sample estrogens and anti-estrogens toward estradiol. The principles of the assay are depicted in figure 3: (1) An antigen, in this case 17-β-estradiol, is coupled to a coating protein, BSA (bovine serum albumin) and coated on a solid phase in a 96-well microplate. (2) hERα and environmental samples of different dilutions are added to the wells. The estrogens and antiestrogens of the sample then compete with 17-β-estradiol for the restricted number of binding sites to hER. The inbubation period is ended with washing. (3) A biotinylated mouse anti-ER antibody is added to each well for quantification of ER-binding to 17-β-estradiol or xeno-estrogens. (4) After washing, the addition of a substrate and chromogen, in this case a streptavidin-POD-biotin complex, allows for measurement of peroxidase activity at the wave length of 450 nm. This is done on a commercial ELISA multiplate reader. It gives an estimate of agonistic and antagonistic receptor binding of the environmental samples (Meulenberg et al. 1995; Kase et al. 2008, 2009).

The ELRA can be applied on freshwater samples, as well as marine and brackish water samples up to a salinity of 20.5 ‰. The salinity of the sample is very important when testing waste water samples and sediment samples from the tidal areas of a river. In these areas of rivers the sediment changes its salinity many times a day (Kase et al. 2009).

The advantages of the ELRA are that it is fast, reliable, inexpensive, highly reproducible and it can be applied for high-throughput screening. It has great resistance toward cytotoxicity and microbial contamination. It is less sensitive than the YES assay and E-screen in terms of detection levels of estradiol, but it is regarded as a good pre-screening tool. It has been previously applied in the assessment of wastewater, sludge and soil (Kase et al. 2009).

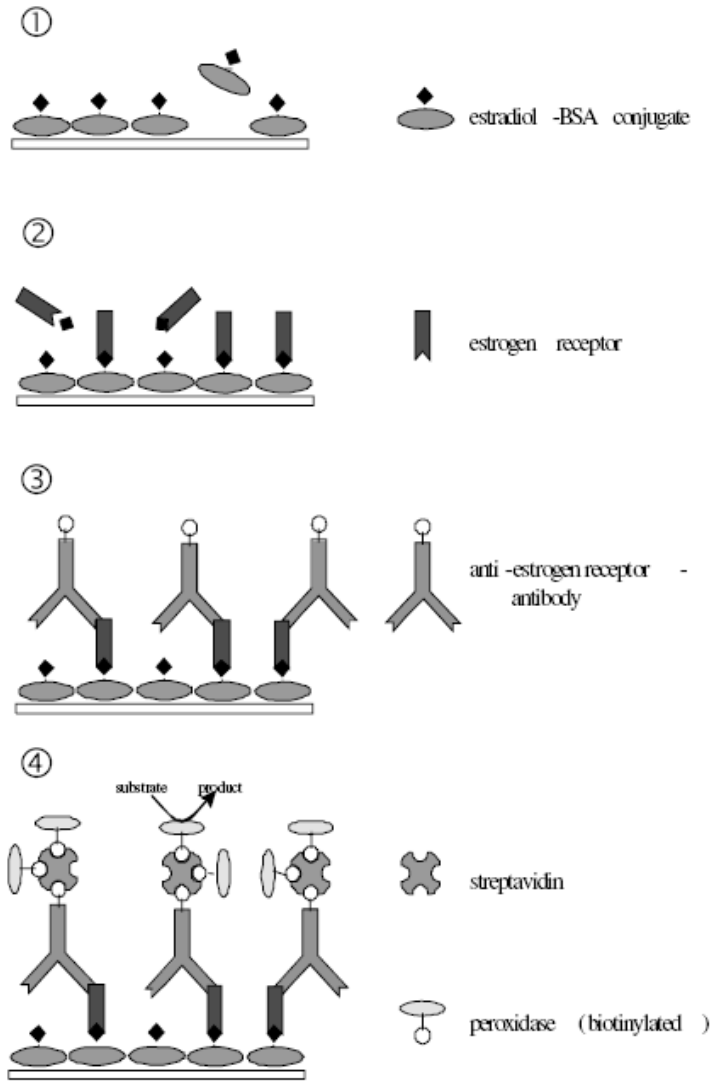

Figure 3: The principles behind the enzyme-linked receptor assay (ELRA) (Kase et al. 2008).

The T4-transthyretin (TTR) binding competition assay

In the T4-TTR binding assay purified human TTR is incubated with a displacable radioligand [^{125}I]-labeled thyroxine and unlabeled T4 as well as environmental samples as competitors at various concentrations in Tris-HCl buffer. The incubation mixtures are allowed to reach binding equilibirium overnight at 4 °C. Then protein-bound and free [^{125}I]-T4 are separated on 1 ml Biogel P-6DG columns and spin-force eluted with additional 200 µl Tris HCl buffer. The radioactivity of the TTR-bound [^{125}I]-T4 containing eluate is measured in a gamma counter. Free T4 is bound to the Biogel matrix and is not contaminating the eluate fractions. Competition binding curves are drawn. Binding affinity is expressed as a relative potency, i.e. the ratio between inhibitor concentration at 50% inhibition (IC_{50}) of environmental sample and the IC_{50} of unlabeled T4 [IC_{50}(competitor)/ IC_{50}(T4)] (Lans et al. 1993; Hamers et al. 2006).

The highest binding affinities for TTR has been observed for hydroxy metabolites of various PCB congeners (PCB-OHs), with hydroxy-groups situated on *meta* or *para* positions and one or more chlorine atoms situated next to the hydroxy group on either or both aromatic rings. Hydroxy metabolites of of PCDDs and PCDFs with the same structural constellations of hydroxy groups and halogens also showed high binding affinity for TTR. All of these compounds fulfil the structural requirements similar to that of the physiological thyroxine or the active thyroid hormone T3 for binding to TTR (Lans et al. 1993).

E2 SULT inhibition assay

Kester et al.(2000) found that hydroxylated PCB metabolites have the capacity to inhibit estradiol metabolism by inhibiting the enzyme estrogen sulfotransferase (EST). This is an alternative mechanism of indirect estrogenic activity which may explain the estrogenicity of PCBs and possibly also that of polyhalogenated aromatic hydrocarbons (PHAHs) (Kester et al. 2002). The result is increased cellular bioavailability of E2. Maximum EST inhibitory activity was found with PCB-OHs with an OH group in the *para* position with two neighbouring Cl substituents on either side on one ring.

In this assay the EST inhibition potency of the environmental sample is investigated by incubating tritium-labeled estradiol (^{3}H-E2) for 30 minutes at 37°C with recombinant human sulfotransferase (SULT1E1), the cofactor 3´-phosphoadenosine-5´-phosphosulfate (PAPS) and in the presence or absence of the environmental sample. Unreacted ^{3}H-E2 is removed by extraction with dichloromethane. The water soluble fraction of ^{3}H-E2-sulfate formed in the assay is quantified in a beta counter (Kester el al 2000; Hamers et al. 2006). The IC_{50} values of several PCB-OHs were found to be in the subnanomolar range.

4.3.1.6 Conclusion

The potential hazard of the pollutants under investigation cannot be fully assessed with *in vitro* assays alone since they only look at specific mechanisms and not at the whole picture. For instance cross-talk between biological pathways and bioavailability of toxicants are not addressed. In the case of endocrine disruption, there may be many possible modes of action whereas most *in vitro* assays only look at the general receptor-binding mechanism or at cell proliferation. Chemicals may instead have endocrine disrupting effects by inhibiting or stimulating enzymes responsible for steroid biosynthesis or steroid metabolism, thereby raising or lowering the level of endogenous hormones in the body. Estrogenic, progestagenic, thyroidogenic or androgenic activity may also be stimulated by other signalling pathways, such as via growth factors (Sohoni and Sumpter 1998; Kinnberg 2003). Examples of assays testing these alternative mechanisms are the two last ligand binding assays described. A combination of *in vivo* assays and *in vitro* assays can thus help discern other mechanisms than Ah/ steroid receptor agonism/antagonism.

Table 4: : Summary of *In vitro* tests for endocrine disruption and Ah-receptor activation investigated.

Test	Mechanism measured	Detection limits	EC_{50} detection level	Exposure time	Matrix	Price range	Performers	Reference
YES (Yeast estrogen screen)	(anti-)estrogenicity	3.7 -11 pM E2 or 1-3 ng E2/L or 0.03 ng EEQ/L	100 pM E2	72 hours	X, FW, LL, WW	6	IVL (Swedish Environmental Research Institute).	Legler et al.(2002);Kinnberg 2003
YAS (Yeast androgen screen)	(anti-)androgenicity	n.f.	n.f.	72 hours	X, P, FW, LL, WW	6	IVL (Swedish Environmental Research Institute).	Sohoni and Sumpter (1998)
Progesterone transactivation assay	(anti-)progestagenicity	0.1 nM progesterone	1 nM progesterone	24 hours	WW	n.f.	n.f.	Chatterjee et al.(2008)
XDS-CALUX® Assay	Ah-receptor agonism			24 hours		4	Xenobiotic Detection Systems, Inc. USA	
DR-CALUX	Ah-receptor agonism	< 1 pM TCDD	10 pM TCDD	6 and 24 hours	X, P	3-4	Biodetection systems (BDS), The Netherlands.	Murk et al. (1996)
EROD- activity assay	Ah-receptor agonism	n.f.	0.186 nM TCDD	4 and 24 hours	X			Huuskonen et al. (2000)
ER-CALUX	(anti-)estrogenicity	0.4 pM E2 or 0.1 ng E2/L or 0.001 ng EEQ/L	6 pM E2	24 hours	X	3-4	BDS, The Netherlands	Legler et al. (2002); Kinnberg 2003
ERα-CALUX	(anti-)estrogenicity	0.8 pM E2	20 pM E2	24 hours	FW, WW	3-4	BDS, The Netherlands.	Sonneveld et al. (2005)
AR-CALUX	(anti-)androgenicity	3.6 pM DHT	0.13 nM DHT	24 hours	FW, WW	3-4	BDS, The Netherlands.	Sonneveld et al. (2005)
AR-LUX	(anti-)androgenicity	46 pM R1881	86 pM R1881	24 hours	X, FW, WW			Blankvoort et al. (2001)
PR-CALUX	(anti-)progestagenicity	1.3 pM Org 2058	0.09 nM Org 2058	24 hours	FW, WW	3-4	BDS, The Netherlands.	Sonneveld et al. (2005)
GR-CALUX	(anti-) glucocorticogenicity	0.2 pM dexamethasone	0.37 ± 0.08 nM dexamethasone	24 hours	FW, WW	3-4	BDS, The Netherlands.	Sonneveld et al. (2005)
TR-CALUX	(anti-)thyroidogenicity	n.f.	0.8 pM T3	24 hours	FW	3-4	BDS, The Netherlands.	Schriks et al. (2009)

Test	Mechanism measured	Detection limits	EC_{50} detection level	Exposure time	Matrix	Price range	Performers	Reference
MVLN bioassay	(anti-)estrogenicity	2 pM E2	5 pM E2	24-72 hours	X,P, FW,SW	n.f.	n.f.	Gutendorf and Westendorf (2001); Vondráček et al.(2001)
LUMI-CELL® ER bioassay	(anti-)estrogenicity	0.1 pM E2	14.3 pM E2	24 hours	n.f.		Xenobiotic Detection Systems, Inc. USA	Gordon et al. (2003)
RTG-2 reporter gene assay	(anti-)estrogenicity	0.05 nM E2	0.33 nM E2	48 hours	WW	n.f.	n.f.	Ackerman et al. (2002)
E-screen	(anti-)estrogenicity via increased cell proliferation	1.0 pM E2 or 0.27 ng E2/L or 0.07-0.14 ng EEQ/L	5 pM E2	6 days	WW, SS, FW			Gutendorf and Westendorf (2001); Kinnberg (2003)
A-screen	Androgenicity via decreased cell proliferation	n.f.	n.f.	24 hours	n.f.	n.f.	n.f.	Szelei et al. (1997)
T-screen	(anti-)thyroidogenicity via regulation of cell proliferation	n.f.	0.191 nM T3	4 days	X			Gutleb et al.(2005)
ELRA	Binding affinity to ER	0.327nM E2 or 89 ng/L E2		1.5 hours	FW, SW, BW, SE, P, WW, SS			Kase et al. 2008; 2009.
TRR-binding comp. assay	Interference with T4 binding to plasma transport protein TRR	16.2 ±0.3 pmol T4 equivalents/g dw	55 nM [a] T4	overnight	X, WW, FW			Houtman et al. (2004); Hamers et al. (2006)
E2 SULT inhib. assay	Estrogenicity via alternative pathway		150 ± 60 nM[a] pentachlorophenol (PCP)	30 min				Hamers et al. (2006)

n.f.= information not found
[a]= IC_{50} detection level

<u>Price ranges:</u> 1= 0-50 € (Euro)/ sample, 2= 51-100 €/ sample, 3= 101-200 €/ sample, 4= 201-400 €/ sample, 5= 401-600 €/ sample, 6= 601-1000 €/ sample, 7= 1001-2000 €/ sample, 8=2001- €/sample

<u>Matrices</u>: S=whole sediment, SE=sediment elutriate, P= Porewater, X=Sediment extract, FW= Fresh Water, BW= Brackish water, SW= Salt water, SS= sewage sludge, WW= WWTP influent and effluent, LL=Landfill leachate water

4.3.1 In vitro assays for testing Acute Toxicity

4.3.2.1 The Microtox assay

The Microtox® Acute Toxicity test is a prime example of minimalistic microscale toxicity tests that have been developed in the last thirty years in the field of environmental screening. It can be applied in the assessment of acute toxicity of surface waters, ground waters, waste waters, landfill leachates, sediment elutriates and pore water as well as organic and aqueous sediment extracts (Johnson 2005).

The Microtox assay has been applied frequently in the toxicity screening of marine, coastal and estuarine sediments for testing general acute toxicity of various pollutants such as insecticides, petroleum products, PCBs and PAHs (Johnson and Long 1997). The assay detects acute toxicity (physiological dysfunctions and lethality) in the marine bioluminescent bacteria *Vibrio fischeri*[4]. The assay uses freeze-dried test organisms which avoids the time and expense of using continuous cell cultures. Decrease in luminescence is a stress response in the bacteria to chemical pollutants. The environmental sample is incubated with the bacteria for 20 minutes at 17 ºC and then light emissions are measured in a luminometer. The results from the test can be attained within 24 hours (Johnson and Long 1997; Naracci et al. 2009).

The Microtox assay also has been adjusted to a Solid Phase Test where the toxicity to organisms in direct contact with sediments is measured. In this test a suspension of sediment sample is incubated with *V. fischeri* for 20 minutes. The pore water is then extracted by pressure and the light emission of the bacteria is measured in the pore water after 5 and 15 minutes (Davoren et al. 2005b). The results are usually expressed as Sediment Toxicity Index (S.T.I.), which means that a positive control sediment, that has been spiked with a known toxic substance (such as phenol), is used as a reference. The EC_{50} value of this positive control sediment is given the number one, and used as the baseline for the toxicity reference index. This positive control sediment EC_{50} value is then divided by each of the samples´ EC_{50} values and then placed in the toxicity index (Johnson and Long 1997).

Strategic diagnostics Incorporated (SDI) in the UK sells equipment such as the Microtox M500 analyzer, and the test kit and reagents in order to perform this assay.

4.3.2.2 The Toxi-chromo test

The Toxi-chromo test can be applied in the environmental screening of surface and ground water, industrial effluents, municipal disharges etc. for the presence of toxic pollutants. The test uses a highly permeable mutant of *E. coli* that has been genetically engineered to be responsive to a wide spectrum of pollutants including mycotoxins, pesticides and heavy metals (Kwan 1995). The test also exist as direct sediment toxicity procedure (DSTTP) that can screen toxicants in solid samples such as aquatic (bottom or suspended) sediments, sludge, municipal soils, industrial solid effluents and other solid waste materials. This sediment test is called the Toxi-chromo pad test (Homepage of Environmental Biodetection products Inc., 2010).

[4] *V. fischeri* belongs to a genus of bacteria called *Vibrio* and the family name is *Vibrionaceae*. They are gram-negative, comma-shaped, motile, mostly aerobic bacteria that live in aquatic environments, and are chemo-organotrophic (Dictionary of Plant Sciences 2006; Dictionary of Biology 2008b).

The test uses freeze-dried bacteria as in the Microtox test, and it is based on the pollutants´ ability to inhibit the production of an inducible enzyme, β-galactosidase, in the sample. The environmental sample is mixed with stressed bacteria and a mixture containing the specific inducer for the chromotogenic enzyme and other essential ingredients, and incubated for 90 minutes (Kwan 1995). The results can be determined qualitatively with the eyes by the reduction of blue colour or quantitatively by spectrophotometry using a microplate reader (Homepage of Environmental Biodetection products Inc., 2010). It exists as a testkit and the test can be completed in 5-6 hours.

4.3.3 In vitro assays for testing Genotoxicity

Each animal species cell type has a different capability to metabolize pro-mutagens and pre-carcinogens to their active, toxic form. By employing different cell lines it is therefore possible to investigate species-specific metabolism and toxicity of environmental samples with *in vitro* systems without the cost of using living animals (Kocan et al. 1985).

The endpoints used for sediment genotoxicity assessment can be divided into four categories: (1) gene mutations, (2) chromosomal damage, (3) DNA damage and (4) cancer. Prokaryotic genotoxicity assays investigate gene mutations or induction of the SOS response[5], and they account for approximately 58 percent of all sediments assessments performed. These assays are good for screening purposes and for processing large number of samples. However the cells do not have cell nuclei and their DNA is not organized into chromosomes. Thus this type of assays cannot detect effects such as chromosomal aberrations, micronuclei, aneuploidy or sister chromatid exchanges. Consequently assays employing eukaryotic cells from fish, amphibians and mammals have been developed in order to measure such chromosomal damages. These assays are more complex and expensive than prokaryotic assays but they are less demanding than *in vivo* animal assays (Chen and White 2004).

Another sensitive biomarker for genotoxicity is DNA strand breakage. Many substances can cause single strand breaks even if they don´t induce cytochrome P-450 enzymes or form DNA adducts. These lesions can create mutant cells (Kammann et al. 2000). Examples of assays that detect DNA damage (such as single strand breaks, alkali labile sites and cross-links) are the Comet assay (also called the Single cell gel electrophoresis (SCGE) assay), the Alkaline precipitation assay (APA) and the Alkaline unwinding assay. All of these assays have been applied to fish cells (such as primary rainbow trout hepatocytes (PRTH)) in both marine and freshwater sediment testing (Chen and White 2004). Other examples are the Unscheduled DNA synthesis (UDS) assay and the Nick translation assay (NTA), which measure the degree of excision DNA repair, and have been applied to Brown Bullhead fish cells in fresh water sediment testing, and to primary rainbow trout hepatocytes (PRTH) in salt water sediment testing, respectively (Ali et al. 1993; Gagne and Blaise 1995). Appendix 3 lists some of the applications of these genotoxicity assays mentioned.

[5] The SOS response is described as "a cellular response to extensive DNA damage in which certain genes, called SOS genes, are sequentially activated in order to repair the damaged DNA" (Dictionary of Genetics, 2007).

4.3.3.1 Prokaryotic genotoxicity bioassays

The Ames/microsome bacterial test

The Salmonella/microsome test, also called the Ames test after the person who invented it, employes a mutant strain or several strains of *Salmonella typhimurium* that have a mutation in the operon coding for histidine biosynthesis (Maron and Ames 1983). These strains lack the ability to grow in the absence of histidine and they are unable to perform excision DNA repair. After the addition of different doses of the environmental sample, reversion to a histidine-positive phenotype will confirm a positive result in the assay (Maron and Ames 1983; Pitot III and Dragan, 2001). It may or may not use exogenous rat hepatic postmitochondrial supernatant fraction (S9) for metabolic activation, since bacteria differ in their metabolic capacity compared to mammals. Metabolic activation means the biotransformation of progenotoxins to genotoxins.

For the analysis of complex environmental samples, strains TA98 and TA100 are mostly used for the detection of frameshift and base-pair mutations, respectively. More recently, metabolically enhanced versions of TA98 and TA 100 (ex. YG1021, YG1024 and YG1026, YG1029) have been used for the analysis of environmental samples (Chen and White 2004), (see appendix 3, page 103).

Environmental Biodetection Products Inc. in Canada provides test kits called Muta-chromoPlate kits that are 96-well versions of the Ames/microsome test, and it takes 3-5 days to complete this test. The assay is conducted in liquid culture, as a fluctuation test, with multiple colour yes/no endpoints, in accordance with a modification of the original agar pour-plate assay (Homepage of Environmental Biodetection products Inc., 2010). It can be applied to the analysis of mutagenic potential of water soluble extracts of sediment, surface and ground water, municipal discharges, and industrial effluents etc.

The Umu-test

This test uses the bacterium *Salmonella choleraesius subsp. Chol.* (strain TA1535/pSK1002) which has been transfected with a plasmid (pSK1002) carrying a gene fusion of *umuC* and *lacZ* (Oda et al. 1985). The *umu* operon is induced by DNA damaging agents. It is responsible for the error prone repair process and is one of the SOS response genes. Through the reporter gene construct, the β-galactosidase activity becomes a measure of the activation of the SOS response in the sample. The response is expressed as induction rate (IR) and is calculated from growth factor of bacteria and β-galactosidase units in the assay. According to the ISO 13829 standard a sample is determined as genotoxic in the Umu assay if the IR is above 1.5 (Brinkman and Eisentraeger 2008).

The Umu-test has been shown to be as good, sensitive and applicable as the Salmonella-/microsome-test in the environmental screening of carcinogenic and mutagenic substances. It is a short-term assay that may or may not use exogenous rat hepatic postmitochondrial supernatant fraction (S9) for metabolic activation. It can be applied to the assessement of genotoxic potential of complex environmental water samples such as river water extracts, industrial waste water but also of sediments, contaminated soils or industrial sludge (Brinkman and Eisentraeger 2008).

The Umu test has been developed by Brinkman and Eisentraeger into a miniaturized and automated, robotized bioassay that can be used to analyze large amounts of environmental

samples and chemicals in a cost- and time-saving manner. It works well with nonvolatile compounds that are moderately to strongly genotoxic (Brinkman and Eisentraeger 2008).

The Mutatox assay

The mutatox assay is an initial screening assay to detect DNA-damaging substances in waters, sediments, waste waters, and leachates. It can be applied on an organic or aqueous extract from contaminated surface waters, from sediment or pore water, or from domestic or industrial waste water. It is a short-term *in vitro* prokaryotic assay that uses exogenous rat hepatic postmitochondrial supernatant fraction (S9) for metabolic activation. It uses the bioluminescent marine bacteria *Vibrio fischeri* where the genotoxic response is a measure of the sample extract´s ability to revert a dark mutant of the bacteria (M169) into its luminescent wildtype condition (Johnson 1993). The strength of the luminescent signal, measured in a luminometer, is directly related to the the reversion frequency and therefore the mutagenic activity of the sample (Chen and White 2004).

The mutatox test responds to a variety of modes of action including base-pair and frameshift mutations, cross-links, intercalation of DNA molecules, and DNA synthesis inhibition. A major disadvantage with the assay is that there is a lack of clear understanding of the molecular mechanisms behind the reversion of the dark mutant strain of *V. fischeri.* It is also not possible to compare the luminescent signals between samples in the same assay, and therefore this assay is only apropriate for screening purposes. Advantages with the assay are: It does not require aseptic technique, the exposure time is shorter and the sample size is smaller than the Salmonella-/microsome assay (Chen and White 2004). The assay is no longer available comercially from Strategic diagnostics Incorporated (SDI) in the UK.

SOS-Chromo test

The second most common prokaryotic genotoxicity test employed in sediment assessments is the SOS-Chromo test. The test employes a variant of *E. coli* (PQ37) strain, that contains a gene fusion between the reporter gene *lacZ* and one of the SOS genes called *sfiA*. This gene is involved in cell division inhibition which is a part of the SOS response to DNA damage. SOS induction in the assay thus causes the transcription and synthesis of β-galactosidase which can be monitored colourimetrically. Sample potency is defined as SOS inducing potency (SOSIP) which is equivalent to the initial linear slope of the concentration response curve in the assay (Quillardet et al. 1982; Chen and White 2004; Homepage of Vito 2010). A bacterial viability test is performed simultaneously in the assay by measuring the activity of alkaline phosphatase which is monitored colourimetrically as well. The results can be obtained qualitatively by the color changes observed visually, or quantitatively by spectrophotometry measurements in a microplate reader (Homepage of Environmental Biodetection products Inc., 2010).

Environmental Biodetection Products Inc. provides test kits that are 96-well versions of the test, and it takes 4 hours to complete this test. The assay can be applied to the analysis of genotoxic potential of industrial effluents, municipal discharges, surface and ground water (Homepage of Environmental Biodetection products Inc., 2010).

Advantages with the SOS-chromo test are its fastness, ease of use, automation, its lack of need for sterility or survival of the tester strain and its ability to correct for bacteriostatic

effects. It is often used as a screening tool for genotoxicity, but it cannot differentiate between different modes of action the way that the Ames/microsome test can (framshift mutation versus basepair substitution etc.). Another disadvantage is that turbid or coloured samples adversely influence the colourimetric endpoint (Chen and White 2004).

Table 5: Summary of *In vitro* tests for Acute toxicity and Genotoxicity investigated.

Test	Endpoint	Exposure time	Matrix	Price range	Performers
Toxi-ChromoTest™ Kit	Non-specific toxicity (EC_{50})	90 min	FW, WW	1 tk	Environmental Biodetection Products Inc. (EBPI), Canada.
Toxi-Chromo Pad™bacterial assay	Non-specific toxicity (EC_{50})	90 min	S, SS	1 tk	EBPI, Canada.
Microtox Solid phase Test	Non-specific toxicity (EC_{50})	20 min	S	4	Toxicon AB, , Pelagia Miljökonsult AB, Sweden
Microtox acute toxicity basic test	Non-specific toxicity (EC_{50})	20 min	X, P, SE, FW, SW, WW, LL	4	Toxicon AB, Pelagia Miljökonsult AB, Sweden
The Ames-/microsome bacterial test, OECD nr 471 (1997)	Mutagenicity	2 days	X, FW, WW	1 tk, 7	EBPI, Canada, Pelagia Miljökonsult, Sweden
The Umu-test, ISO 13829 (2002)	Induction of SOS-repair system (Primary DNA damage)	2 hours	X,FW, WW,LL,SS	1 tk, 7	EBPI, Canada, Pelagia Miljökonsult AB, Sweden.
The Mutatox assay	Induction of the SOS-repair system	16 -24 hours	X, S, P, WW, LL	n.f.	n.f.
SOS-chromotest	Induction of the SOS-repair system	2 hours	X, FW, WW	1 tk	EBPI, Canada.

n.f.= information not found
tk = test kit, including several samples per kit

Price ranges: 1= 0-50 € (Euro)/ sample, 2= 51-100 € /sample, 3= 101-200 €/ sample, 4= 201-400 €/ sample, 5= 401-600 €/ sample, 6= 601-1000 €/ sample, 7= 1001-2000 €/ sample, 8=2001- €/ sample

Matrices: S=Whole sediment, SE=Sediment elutriate, P= Porewater, X=Sediment extract, FW= Fresh Water, BW= Brackish water, SW= Salt water, SS= sewage sludge, WW= WWTP influent and effluent, LL=Landfill leachate water

4.3.4 In vitro Biomarker assays

4.3.4.1 EROD-activity assay

Induction of *CYP1A1* (gene coding for the cytochrome P450 1A1 enzyme) has been observed in fish and other vertebrates with various PAHs, PCBs, PCDDs and other halogenated compounds. This induction can activate PAHs into mutagenic compounds and may result in carcinogenicity (Arinc et al. 2000).

The ethoxyresorufin-O-deethylase (EROD) assay is founded on the biological reaction of the aryl hydrogen receptor (AhR) to certain planar halogenated hydrocarbons, PAHs and other structurally similar compounds. The ligand-activated AhR initiates a cascade of reactions, including the induction of *CYP1A1*. This increases the *O*-deethylation of 7-ethoxyresorufin and can be measured by flourescence spectroscopy (Whyte and Tillitt, 2011). The reaction is as follows:

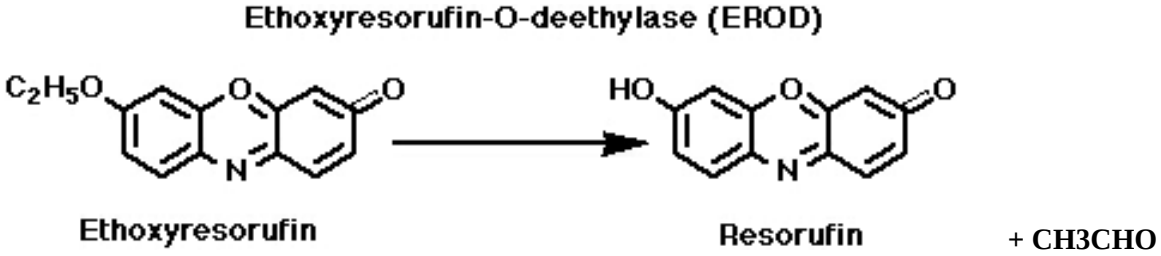

Figure 4: Reaction of EROD assay (Miami University 2011)

The EROD-activity assay described by Laville et al. in 2004 employes a fish cell line, PLHC-1, that originates from hepatocellular carcinoma of topminnow (*Poeciliopsis lucida*), and another cell line created from primary rainbow trout hepatocytes (PRTH) which is described under section 4.3.4.2 The cell lines are seeded on 96-well (or 48-well) microplates and the cells are exposed to environmental extracts in cell culture medium for 24 hours at 30 °C for PLHC-1 cells, and at room temperature for PRTH:s. After cell medium is removed, it is replaced with culture medium containing 2 μM 7-ethoxyresorufin and the production of resorufin is monitored in a microplate flourimeter for 15 minutes, at 530 nm excitation wavelength and 590 nm emission wavelength. Total cellular protein content is also determined. The positive control for EROD induction in the assay is 1 μM benzo(a)pyrene. EROD activity is expresses as picomol of resorufin/min/mg protein (Huuskonen et al. 2000; Laville et al. 2004).

The EROD assay was compared with the DR-CALUX assay in rat hepatoma (H4IIE) cells by Murk et al. in 1996, and it was found that the DR-CALUX assay is somewhat more sensitive and has a threefold greater induction than the EROD activity assay. The DR-CALUX assay also has a greater capacity to detect less potent agonists, since the activity of the EROD enzyme is inhibited at substrate concentrations of 50 nM or more; yet this concentration is needed for the induction by less potent pollutants. The CALUX assay also had a two- to threefold lower detection limit than the EROD assay.

4.3.4.2 Vitellogenin induction

Vitellogenin (vtg) is a large calcium-rich phopsholipoglycoprotein. It is produced in the liver of female oviparous vertebrates in response to a rise in circulating endogenous estrogens. It is carried with the blood stream to the ovary, where it is taken up by developing oocytes and is modified to form egg yolk. It there becomes the major supply of amino acids and lipids to the developing embryo and a food reserve for hatched larvae. The extent to which an estrogenic compound is able to induce vitellogenin synthesis in a fish is determined by its ability to activate the hepatic estrogen receptor alpha (ERα). This in turn will cause the transcription of E2-inducible genes, including vtg (OSPAR Commission 2007).

The recommended *in vivo* method by the OSPAR Commission for the monitoring of exposure to xenoestrogens, is the detection of vitellogenin in the bood plasma of male fish. Male fish that are exposed to (xeno)estrogens produce vtg in the liver just like the female fish, but they have no ovaries to take it up and therefore it builds up in the blood. Vtg concentrations can rise a million-fold in blood plasma in response to estrogens, which makes this assay very sensitive and a highly specific biomarker for estrogenic exposure (OSPAR Commission 2007).

An *in vitro* assay to detect vitellogenin induction has been developed using primary rainbow trout hepatocytes (PRTH). PRTHs are prepared from livers of 2-year-old male fish. PRTH cells can maintain *in vivo*-like enzymatic activity during 3-8 days which means that they are able to bioactivate and biotransform contaminants (Laville et al. 2004). These cells abound in metabolizing enzymes and specifically in the cytochrome P-450 enzymes (Gagné and Blaise 1995).

After 96 hours exposure to environmental sample, total RNA is prepared. The amount of VTG-mRNA produced is quantified in a nonradioactive dot blot/RNase protection assay. The results are compared to E2 induction rates and expressed as estradiol equivalents (EEQ) (Pawlowski et al. 2003).

Pawlowski et al. (2003) made a comparative study between different *in vitro* bioassays in order to detect (xeno)estrogens in STP (municipal sewage treatment plants) effluents and water samples from the river Rhine. The vitellogenin mRNA induction assay was found to be more sensitive in detecting estrogenic activity than the YES assay and a luciferase reporter gene assay based on transfected human embryonic kidney cells (HEK 293), (see appendix 3, page 99). This is thought to be due to the known metabolic capacity of the PRTH cells, which makes it possible for them to detect proestrogens. This assay is thus able to identify important estrogenic metabolites.

5. Assessment Criteria

Assessment of water or sediment samples using ecotoxicological assays should consist of a battery of tests, which includes a recommended minimum of three or more tests. The ideal set of tests should be representative of all the animals and plants, and all trophic levels of the ecosystem under investigation. It should also consist of the most sensitive species of that ecosystem. According to the OSPAR Commission (2007), all of the following criteria ought to be taken into account in the choice of a test battery:

- *Ecologically and/or toxicologically relevant tests*
- *The test battery is representative of all organisms and trophic levels in the ecosystem under investigation*
- *It encompasses all effects of all possible substances and action mechanisms, both acute and chronic effects*
- *The tests are sufficiently sensitive, specific and discriminatory to predict effects*
- *The tests are reliable and reproducible*
- *Availability of test species*
- *Financial aspect*
- *Laboratory availability*
- *Ethical concerns in the use of test animals*
- *Availability of standardized tests that can be incorporated into a metric*

The OSPAR Commission has proposed an assessment tool based on a system developed in the Netherlands that employs so called environmental risk limits (ERLs). (This assessment tool is based on the so called the environmental risk limit system for individual substances issued by the EU). The proposed ERLs are:

I. NE = negligible effect
II. MPE = maximum permissible effect
III. SE = serious effect

The proposed metric used for *in vivo* bioassays for (1) surface water samples or (2) whole sediment or porewater samples are described below in tables 6, 7 and 8 (OSPAR Commission 2007, chapter 7). The ideal battery of tests should contain a minimum of three acute or chronic *in vivo* bioassays on three different species, families or orders (so called *taxonomic groups*), and it should preferably not include vertebrate species. It should also include one or more *in vitro* assays. This is because certain action mechanisms, such as genotoxicity, immunotoxicity, endocrine-disrupting effects, dioxin-like toxicity and the intial stages of neurotoxicity, are more fully elucidated with *in vitro* assays (OSPAR Commission 2007).

5.1 Water assessment

The most appropriate strategy for identification and evaluation of environmental pollutants ought to consist of a combination of *in vitro* and *in vivo* bioassays constituting a series of endpoints and test organisms (Ackerman et al. 2002). This would give a greater certainty as to the ecological relevance of the results (OSPAR Commission 2007). It is recommended that *in vivo* test such as vitellogenin induction or gonadal effects are used in combination with *in vitro* tests in order to determine which type of mechanisms are responsible for the effects

observed (Kinnberg 2003). In addition, it may be difficult to discern between androgenic, anti-estrogenic and anti-androgenic effects of pollutants by using *in vitro* assays alone (Pawlowski et al. 2003). By using a battery of *in vitro* test that investigate a series of steroid receptor agonisms and antagonisms, however, it would be possible to discern the mechanisms. The standard metric suggested below based on three basic tests is only for *in vivo* assays however.

5.1.1 Standard for preliminary effect assessment of water samples

The battery of tests are performed on three trophic levels in order be representative of the entire ecosystem in question. The original untreated water sample can be concentrated by means of solid phase extraction as described in section 4.1.2 before it is being applied in the bioassays. The concentration factor (cf) describes the degree of concentration of the original water sample. In order to determine the NE and MPE results from at least three acute or chronic *in vivo* tests need to be accessible.

Table 6: Standard for *in vivo* acute tests on water samples.

ERL	No of tests	Effect (E)	Concentration factor (cf)
NE	3	0 (in practice < EC_{50})	100
MPE	3	0 (in practice < EC_{50})	10
SE	1	≥ EC_{50}	10
SE	2	EC_{20} < **Effect** < EC_{50}	10

cf = concentration factor compared to the untreated (original) water sample.
ERL= Environmental risk limit
NE= negligible effect, MPE =maximum permissibe effect, SE= serious effect

Table 7: Standard for *in vivo* chronic tests on water samples.

ERL	No of tests	Effect (E)	Concentration factor (cf)
NE	3	0	10
MPE	3	0	1
SE	1	≥ EC_{50}	1
SE	2	NOEC < **Effect** < EC_{50}	1

cf = concentration factor compared to the untreated (original) water sample.
ERL= Environmental risk limit
NE= negligible effect, MPE =maximum permissibe effect, SE= serious effect

5.2 Sediment assessment

In tier 1 testing of sediments (screening and detection of toxic impacts), test species from different taxa at different trophic levels are used in order to cover various exposure routes, behavior and feeding modes in different matrices (unmodified whole sediment, porewater, sediment elutriate). This multitrophic test battery with a variety taxa and end-point effects also represent different habitats as well as different sensitivity to toxicants, ensuring an

accurate assessment of sediment toxicity. In the test battery, the endpoint effect should include both acute (lethal) and prolonged (sub)lethal effects. Employing test species that are native to the particular region of interest improves the ecological relevance of the tests (Nendza 2002; Narracci et al. 2009).

In tier 2 testing of sediments (characterization of toxic impact), a broad range of endpoints, test species and matrices are used. It includes short term acute effects and long term subchronic effects in bivalves, echinoids, crustaceans, amphipods, polychaetes, fish and bacteria (Nendza 2002). It also could include *in vitro* tests for specific mechanisms such as genotoxicity, endocrine effects, dioxine-like activity, neurotoxicity, immunotoxicity in order to identify type of pollutants present.

Tier 3 testing involves the measurement of biomarkers in fish and bivalves of specific exposures in the field, and also to verify results from laboratory whole organism tests or *in vitro* test, in the field (by so called *in situ* testing). The specific exposures could be based on bioaccumulation tests that proved the accumulation of specific pollutants in the organisms, and tests on long term effects on epibenthic and endobenthic community structure and function. These tests are more expensive and time-consuming (Nendza 2002).

5.2.1 Standard for effect assessment of sediment samples

The battery of tests are performed on three trophic levels in order to be representative of the entire ecosystem in question. In order to determine the MPE the results from at least three chronic tests need to be accessible, comprising *at least two whole sediment tests.* Whole sediment or pore water samples cannot be concentrated like surface water samples can so the NE (negligible effect) standard is not used. Results from acute tests can also be used but they are converted to chronic values by employing an acute-chronic ratio of 10.

Table 8: Standard for *in vivo* chronic tests on sediment samples.

ERL	No of tests	Effect (E)	Concentration factor (cf)
MPE	3	0	1
SE	1	≥ EC_{50}	1
SE	2	NOEC < **Effect** < EC_{50}	1

cf = concentration factor compared to the untreated (original) water sample.
ERL= Environmental risk limit
NE= negligible effect, MPE =maximum permissibe effect, SE= serious effect

6. Results from Inventory

6.1 Evaluation of and comments on In vitro tests

Crosstalk has been found between nuclear receptors which explains why for instance dioxine-like compounds which usually bind to the Ah receptor may instead bind to or in some cases block the activation of steroid receptors. One example is TCDD which has been found to activate the ER in the absence of an ER ligand and thereby activate estrogen responsive genes. Oppositely, in the presence of an ER ligand, TCDD has been found to block the expression of estrogen-responsive genes (Boelsterli 2009). This may explain why some dioxin-like active compounds also have endocrine disrupting effects.

Another example is the o,p´-isomer of DDT which has a weak binding affinity to ER and is therefore weakly estrogenic. The major metabolite of p,p´-DDT however, called p,p´-DDE, has been shown to have anti-androgenic potency. It has affinity for the AR and hinders androgen ligands from binding and thereby blocks the transactivation of AR-meditated genes (Boelsterli 2009).

Mammalian-based reporter gene assays have lower detection limits than yeast-based reporter gene assays. For example the ER-CALUX assay was found to be approximately a factor of 6-20 times more sensitive than the YES assay in detecting estradiol and other xenoestrogens (Legler et al. 2002). The DR-CALUX and ER-CALUX can detect femtograms of TEQs or EEQs and are therefore sensitive enough to be applied in TIE analysis.

Mammalian cells are harder and more expensive to cultivate and are more sensitive to cytotoxic damage than Yeast cells. Yeast cells are less sensitive to non-sterile conditions and therefore may perform better with environmental monitoring. In addition they are more resistant to environmental pollutants such as heavy metals and bacterial endotoxins. Other advantages are low cost in cultivation, robustness and lack of endogenous receptors that could interfere with the results.Yeast-based reporter gene assays are simpler because no cell lysis is required and the colorimetric response is secreted directly into the cell medium (Kinnberg 2003). They are appropriate for high thtoughput screening of more contaminated samples requiring little or no sample clean-up (Chatterjee et al. 2008). It is not obvious if the YES system can metabolize prosteroids into steroids which is a disadvantage, whereas mammalian cells are able to do it. Hence mammalian reporter gene assays may be used to confirm the result from yeast-based reporter gene assays (Arnold et al. 1996). In addition there is difficulty in consistency in detecting and anti-estrogenic responses with the YES assay but not with the ER-CALUX and other mammalian-based assays.

Human MCF-7 breast cancer cells are employed in the E-screen assay and the endpoint is cell proliferation. It has been found however that the E-screen assay is not as estrogen-specific in its response as presumed since non-estrogenic substances such as progesterone, androstenediol, insulin-like growth factors, epidermal growth factor, caffeine and ethanol have been observed to influence the proliferation of MCF-7 cells. The E-screen assay also has been found to have problems with a significant inter-laboratory variability in test results and a very great sensitivity to variations in cell culture conditions (Kinnberg 2003). The E-screen has however been used successfully in the determination of total estrogenic activity in extracts from municipal STPs in Germany. No cytotoxicity was observed and no clean-up steps of environmental samples were needed.

The human breast carcinoma cell line T47D expresses endogenously the AR as well as other members of the nuclear receptor family; ER, PR, retinoc acid (RAR) and retinoid X (RXR) receptors. This cell line is employed in the ER-CALUX and AR-LUX assays (see appendix 3, page 95). These assays will thus also mirror any indirect effects on the ER- (or AR-) mediated response via other signal transduction routes or biochemical pathways (Blankvoort et al. 2005).

Salinity has been shown to have an effect on both the bioavailability of pollutants as well as the cytotoxicity of the environmental sample in a bioassay. It also has an effect on the conformation and solubility of proteins and therefore will influence binding capacity of receptors to agonists and antagonists in the environmental sample. Salinity thus will affect the results of the bioassay. Kase et al. (2008) found that the YES and the E-screen are not appropriate for water phase testing at higher salinity conditions. According to them it would be more appropriate to use the ELRA, which can be applied on native water of more or less brackish character and on high-salinity waste water effluents. In the E-screen sodium choride inhibit cell proliferation by having a cytotoxic effect. YES and YAS assays were found however to be markedly more tolerant to salinity than the E-screen.

Comparisons have been made between *in vivo* assays and *in vitro* assays with regards to measuring estrogenic activity in environmental samples, and the discrepancies were rather great (approximatley 10-fold). This is thought to be explained by a greater bioavailability and absorption of chemicals by living animals as compared to *in vitro* cells. Another explanation is bioaccumulation *in vivo* and the effects of indirect mechanisms via non-ER ligands. An observation made is that the synthetic estrogen EE2 was found to have 10-100 times stronger estrogenic potency than E2 in *in vivo* fish assays such as vitellogenin synthesis in male zebrafish or ovo-testis induction in japanese medaka during early life stage exposures. In *in vitro* assays however, EE2 is only somewhat more potent (1.2 times) than E2. The explanation for this is probably the greater persistence in the enviroment and in animals of EE2 mentioned in section 4.2 (Kinnberg 2003).

6.2 Evaluation of and comments on In vivo tests

In vivo assays lack specificity but are highly relevant; i.e. certain action mechanisms cannot be detected in a complex environmental sample due to a lack of sensitivity in the assay or because the effects will occur only after a very long exposure period (OSPAR Commission 2007). The only exception to this is field biomarker assays which have been been shown to be rather specific and sensitive; yet they are rather expensive, work intensive and require the use of living animals in the field.

Chronic (long term) *in vivo* bioassays have many disadvantages: They are more expensive, complex and take more time, space and manpower to execute. They should only be used for site-specific assessment and for comparison with field situation (OSPAR Commission 2007). Subchronic *in vivo* tests are a good alternative. They are shorter and therefore less expensive; yet relevant enough to imply effects on development, reproduction or growth of the organisms. They cover a critical sensitive life-stage of the organism and therefore are very sensitive and good predictors of environmental effects.

Acute (short term) *in vivo* tests are good for screening purposes. They show crude effects (such as mortality), are shorter in duration and therefore less expensive to perform. Acute effects can be observed near point sources or after unpredicted accidental events; however they are otherwise not observed under normal conditions in salt or fresh surface water recipients. But if water concentrates (water extracts, see section 4.1.2) are used in the assays it would be possible to perform acute assays on the surface water samples. It is easier to perform several acute tests concurrently, and the shorter test duration means that the water medium can be kept more constant than if chronic testing is applied (OSPAR Commission 2007).

The results from the *in vivo* bioassays can be expressed as EC_{50} value (concentration at which 50 % effect is observed in the test population) but is also expressed as ED_{50} value (dilution at which 50 % effect is observed in the test population) in the case of an environmental sample, NOEC (no observable effect concentration), LOEC (lowest observable effect concentration) or toxic units (TU). ED_x (dilution causing a x% effect in the endpoint measured) is calculated by making a dilution series and plotting a dose-response curve. Toxic units are calculated as follows: $TU= 100/ED_x$ or 100/LOEC.

6.3 Evaluation of and comments on Extraction procedures

There is a need to develop and validate standardized protocols for bioassay extraction methods that could be applied in all member states of the European union for the implementation of the WFD. This will ensure consistency of application between laboratories and comparability of recorded data (OSPAR Commission 2007).

When applying the sediment elutriate matrix the following confounding factors should be taken into consideration: volume of sediment-water ratio, ammonia concentraion, sulphide concentration, and particle size distribution. When applying the sediment pore water matrix the following confounding factors should be taken into account: pH, salinity, dissolved oxygen concentraition (DO), ammonium and sulphide concentration. Sediment elutriates or pore water samples can be diluted if needed (OSPAR Commission 2007).

Water extraction has several advantages: The surface water samples can be concentrated up to a 1000-fold and then be applied in acute *in vivo* tests or *in vitro* tests. Without pre-concentration there may not be any effects in the assays. Confounding factors such as salinity, pH fluctuations, high ammonium content, ion imbalance or hardness are avoided with water (and sediment) extraction. After water and sediments extraction it is also possible to proceed with bioassay-directed TIE or EDA procedures. The disadvantage with extraction methods is that some pollutants, especially metals, may get lost in the process and therefore a complete toxicity evaluation is not achieved (OSPAR Commission 2007).

Passive samplers should be considered as an alternative to regular water grab samples, and extracts from passive samplers could be applied to *in vivo* and *in vitro* bioassays (OSPAR Commission 2007).

6.4 Examples of test batteries employed in research projects

The OSPAR Commission (2007) recommends the following species to be included in a test battery for marine water samples: (1) Copepods (such as *Tisbe battaglii*, *Acartia* sp.): 48 hour exposure, endpoint mortality, (2) Bivalves (*Crassostrea gigas*, *Mytilus* spp): 24 hour embryo exposure, endpoint percent net response, (3) Sea urchin (*Paracentrotus lividus*): 24 hour embryo exposure, endpoint percent normal development and larval length. Table 9 lists test batteries that have been employed in various research projects, matrices used and some results/ comments from these projects.

Table 9: Examples of test batteries employed in research projects

Test Battery	Reference	Matrix	Results/Comments
***Ceramium tenuicorne*-growth inhibiton, *Nitocra spinipes*- larval development ratio and mortality, Microtox solid phase test**	Eklund et al. 2010	**Whole sediment** in salt and brackish water of pleasure boat harbours.	All three organisms were very appropriate for evaluating the toxicity of contaminated sediments
1. ER-CALUX, AR-CALUX, PR-CALUX, GR-CALUX, TR-CALUX, 2. all of the above tests except TR-CALUX	**1**.Schriks et al. 2009, **2**. Van der Linden et al. 2008	**1.Surface water** from a river basin, **2**. **Raw industrial and hospital effluents, WWTP effluents from municipal area and paper mill industry**	Detected multiple types of hormonal activity in all samples. The assays showed high specificity and senstitivity to known agonists.
1. Microtox solid phase test, Toxi-chromo pad test, 2. Microtox acute toxicity test, MetPAD (bacterial) test, Marine algal growth inhibition test, Brine shrimp *A. Salina* (Crustacia) larvae (acute) assay	Davoren et al. 2005	**1. Whole sediment** (surface sediments) and **2**. **Sediment pore water and sediment elutriates** from estuarine sediments	The Microtox assay and the Algal growth inhibition assay were the most sensitive and apropriate to include in Tier 1 screening of sediments
DR-CALUX, ER-CALUX, T4-transthyretin (TTR) binding competition assay, the Umu-test, Microtox acute toxicity test	Houtman et al. 2004	Nonpolar total and acid-treated **sediment extracts** from an estuary	Endocrine disrupting potencies were more prevalent than genotoxic and general toxic potencies. ER- and DR-CALUX, and T4-TTR binding competition assays were all sensitive enough to be used in TIE analysis.
1. Microtox acute toxicity test, Mutatox test, the Umu test, 2. DR-CALUX, ER-CALUX	Klamer et al. 2005	**1. Crude sediment extracts, 2. Cleaned-up sediment extracts** by (a) a multi-layer silica column or by (b) a PL-Gel GPC column from coastal waters	The Umu-test was less sensitive than the Mutatox test to sediment extracts.The non-destrucitve GPC clean-up method gave significantly higher DR-CALUX signals than the other method which indicates a broader spectrum of environmental pollutants were detected.
1. *In situ* hepatic vitellogenin measurements in caged rainbow trout, 2. *In vitro* tests: YES assay, ER luciferase assay with HEK 293 cells, Vitellogenin-mRNA assay with PRTH cells	Pawlowski et al. 2003	**1.Water column** of a river, **2. Water extracts from two STP municipal effluents and from surface water downstream** in a river	A good correlation was found between *in situ* and *in vitro* measurements in the detection of estrogenic activity. The vitellogenin-mRNA assay was the most sensitive assay followed by the YES and the ER luciferase assays.

7. Conclusion and recommendations

- A *Ceriodaphnia* reproduction and survival standard test issued by Environment Canada in 2007 (see appendix 2, page 84) is recommended for use by Swedish water authorities. This three-brood chronic toxicity test can be completed in 5-8 days. It would reduce costs, labour and sample volumes needed to perform the otherwise standardized *Daphnia magna* reproduction test (which takes 14-21 days).
- The Toxi-chromo test uses the *Escherichia coli*, an intestinal bacterium in mammals and birds, and is therefore not an ecologically relevant species for environmental samples. *Vibrio fischeri* used in the Microtox test, however is a marine chemo-organotrophic bacteria which is ecologically relevant and more representative of the ecosystem in question.
- The Microtox test can be applied to both fresh water and marine water samples, since the osmotic pressure can be adjusted in the assay procedures. Obviously the test is widely used in both systems (see appendix 3, page 94).
- Bacteria represent a third trophic level (decomposers) as an alternative to vertebrate species, i.e. fish and amphibians (secondary consumers). Test using microbial organisms are rapid and can be completed in a few hours up to 24 hours and are less expensive and labour-intensive than whole organism tests employing fish or invertebrate species.
- Test kits are available for prokaryotic genotoxicity assays, and they are a cheaper alternative that can be utilized in non-specialized laboratories.
- Such low-cost microbiotests (test kits) involving miniaturisation and microscale prodecures have also been developed in other trophic level organisms such as invertebrates, plants and algaes but they are not covered in this report. Instead I recommend an article by Wadhia and Thompson from 2007.
- The acute fish embryo toxicity (FET) test (employing Zebra fish) is an alternative to acute tests with adult or juvenile fish. It tests a sensitive life stage (embryonic development), is fast (48 hours) and will reduce the suffering of living animals. It is recommended for use in frequent, routine monitoring of waste water and fresh water recipients. An ISO standard from 2007 exists for waste water and an OECD draft from 2006 (see appendix 2, page 87) for fresh water.
- A standard for testing toxicity to the early life stages of Salmonid fish (rainbow trout) was issued by Environment Canada in 1998 (see appendix 2, page 87). The shortest one of these assays (the embryo or E-test) lasts for 7 days, and measures acute toxicity to the fish embryos (the first part of the early life stages, transition from egg to embryo) [6]. It can be used for routine and frequent screening of WWTP effluents, landfill leachate water, sediment elutriates, and receiving fresh waters. This standard is also recommended for Swedish monitoring of water recipients.
- Tests are performed on three trophic levels in order to be representative of the entire ecosystem under investigation. Test batteries for fresh water, salt or brackish water systems (adjusted to Swedish regional conditions) are suggested in section 7.1 (tables 10-13). Test batteries should contain a combination of short term acute and prolonged (sub)lethal tests in tier 1 screening of water systems. Evaluation criteria described

[6] The other tests in the standard (transition from embryo to fish larva, and transition from fish larva to early juvenile stage) last very long, from 30 up to 120 days and would thus be very expensive to perform.

under sections 5.1.1 and 5.2.1 are recommended to be applied by Swedish water authorities.

- One or more mechanistic (*in vitro*) tests may be added to the test batteries in order to identify specific pollutants.

7.1 Recommended test batteries for Swedish water recipients

Table 10: Recommended test batteries for fresh water recipients

	Test organism	Test durarion	Endpoints	Matrix	Reference
Option 1	**Microalgae or cyanobacteria**	72 hours	Growth inhibiton	Water extract	OECD 201(2006), SS-EN ISO 8692 (2005)
	Daphnia magna* or *Ceriodaphnia dubia	48 hours	Immobilization	Water extract	OECD 202 (2004), ISO 6341; SS 28214 (1996)
	***Brachydanio rerio*, zebra fish**	48 hours	Teratogenicity (fish embryo toxicity)	Water extract	OECD draft (2006), DIN 38 415-T6(2001)
	or				
	***Oncorhynchus mykiss*, Rainbow trout**	7 days	Teratogenicity (fish embryo toxicity)	Water extract	Environment Canada (1998) EPS1/RM/28
Option 2	**Microalgae or cyanobacteria**	72 hours	Growth inhibition	Water extract	OECD 201(2006)
	Ceriodaphnia dubia	5-8 days	Reproduction and survival	Water extract	Evironment Canada (2007) EPS 1/RM/21
	Vibrio fischeri	1-30 minutes	Inhibition of bioluminescence	Water extract	SS-EN ISO 11348-3 (2008)

Table 11: Recommended test battery for fresh water sediments

Test organism	Test duration	Endpoints	Matrix	Reference
Microalgae or cyanobacteria	72 hours	Growth inhibition	Sediment pore water or elutriate	OECD 201(2006), SS-EN ISO 8692 (2005)
Daphnia magna* or *Ceriodaphnia dubia	7 days	Survival, reproduction	Whole sediment	ASTM standard (1994)
or				
Hyalella azteca* and *Chironomus tentans	10 days	Survival, growth	Whole sediment	US-EPA (1996c)
Vibrio fischeri	20 minutes	Inhibition of bioluminescence	Whole sediment	Brouwer et al. 1990

Table 12: Recommended test batteries for coastal salt and brackish water recipients

	Test organism	Test durarion	Endpoints	Matrix	Reference
Option 1	***Skeletonema costatum* or *Phaeodactylum tricornutum***	72 hours	Growth inhibition	Water extract	SS-EN ISO 10253 (2006)
	Nitocra spinipes	48 or 96 hours	Mortality	Water extract	SS 02 81 06 (1991)
	Vibrio fischeri	1-30 minutes	Inhibition of bioluminescence	Water extract	SS-EN ISO 11348-3 (2008)
Option 2	***Ceramium tenuicorne***	7 days	Growth inhibibition	Water extract	ISO 10710 (2010)
	Nitocra Spinipes	6-8 days	Larval development ratio(LDR)	Water extract	OECD modified draft
	or				
	***Mytilus edulis* embryos**	24-48 hours	Mortality, abnormal development	Water extract	US-EPA(1996a)
	or				
	Echinoid embryos*	24-48 hours	Abnormal develop-ment, larval length	Water extract	ASTM (1995)
	Vibrio fischeri	1-30 minutes	Inhibition of luminescence	Water extract	SS-EN ISO 11348-3 (2008)

*Echinoid species only exist on the West coast of Sweden

Table 13: Recommended test battery for coastal marine and estuarine water sediments

	Test organism	Test durarion	Endpoints	Matrix	Reference
Option 1	***Skeletonema costatum* or *Phaeodactylum tricornutum***	72 hours	Growth inhibition	Sediment pore water or elutriate	SS-EN ISO 10253 (2006)
	***Corophium* spp. or *Gammarus* spp.**	10 days	Mortality	Whole sediment	ASTM (1993), OSPAR(2007)
	Vibrio fischeri	20 minutes	Inhibition of luminescence	Whole sediment	Brouwer et al. 1990
Option 2	***Ceramium tenuicorne***	7 days	Growth inhibition	Whole sediment	Eklund et al. 2010
	***Mytilus edulis* embryos**	24-48 hours	Mortality, abnormal development	Sediment pore water or elutriate	US-EPA (1996a); OSPAR(2007)
	or				
	Echinoid embryos*	24-48 hours	Abnormal develop-ment, larval length	Sediment pore watera or elutriate	ASTM (1995); OSPAR (2007)
	Vibrio fischeri	20 minutes	Inhibition of luminescence	Whole sediment	Brouwer et al. 1990

*Echinoid species only exist on the West coast of Sweden

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Appendix 1

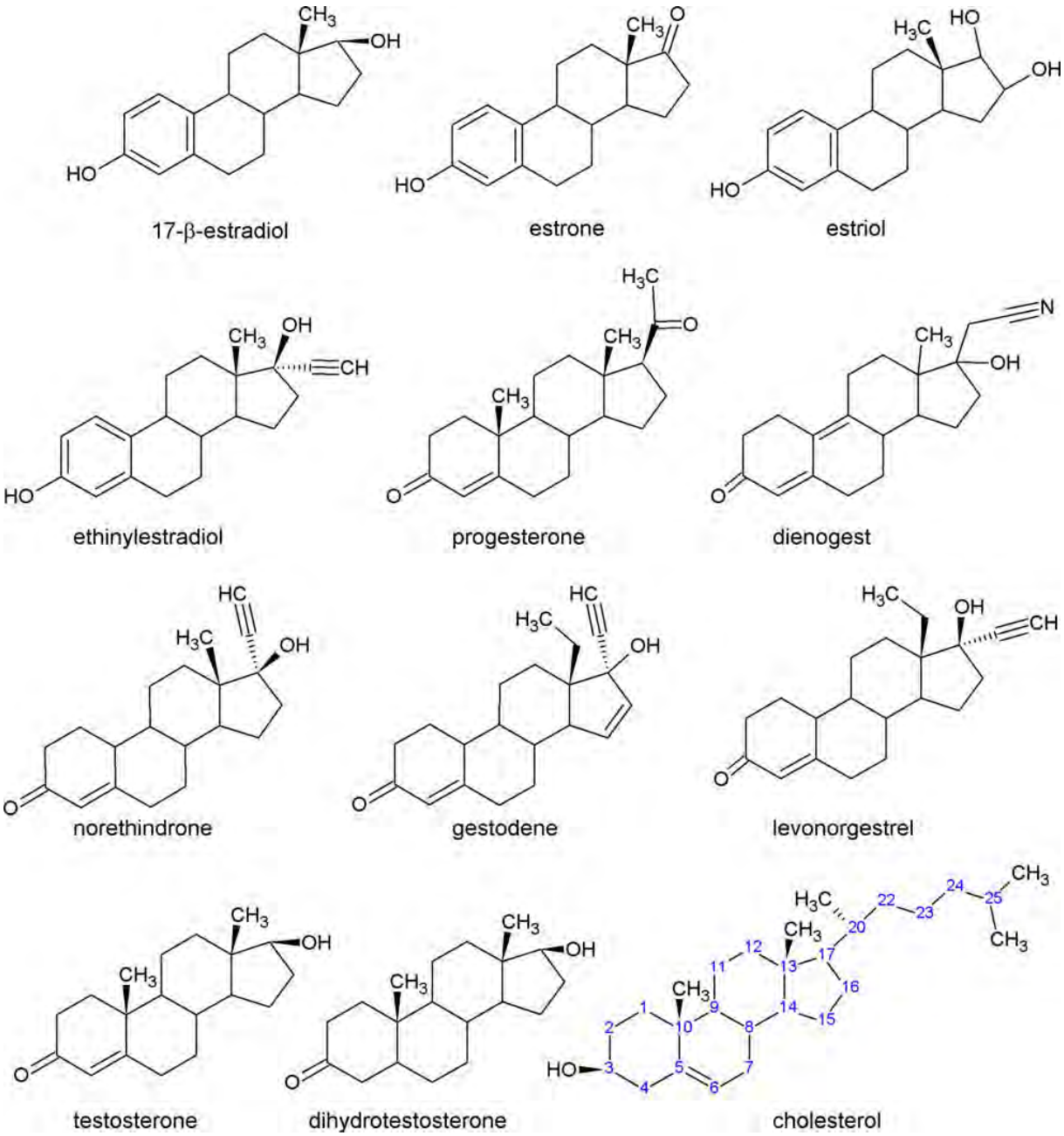

Figure 1: Examples of estrogens, progestagens, androgens and the parent compound cholesterol (Streck 2009)

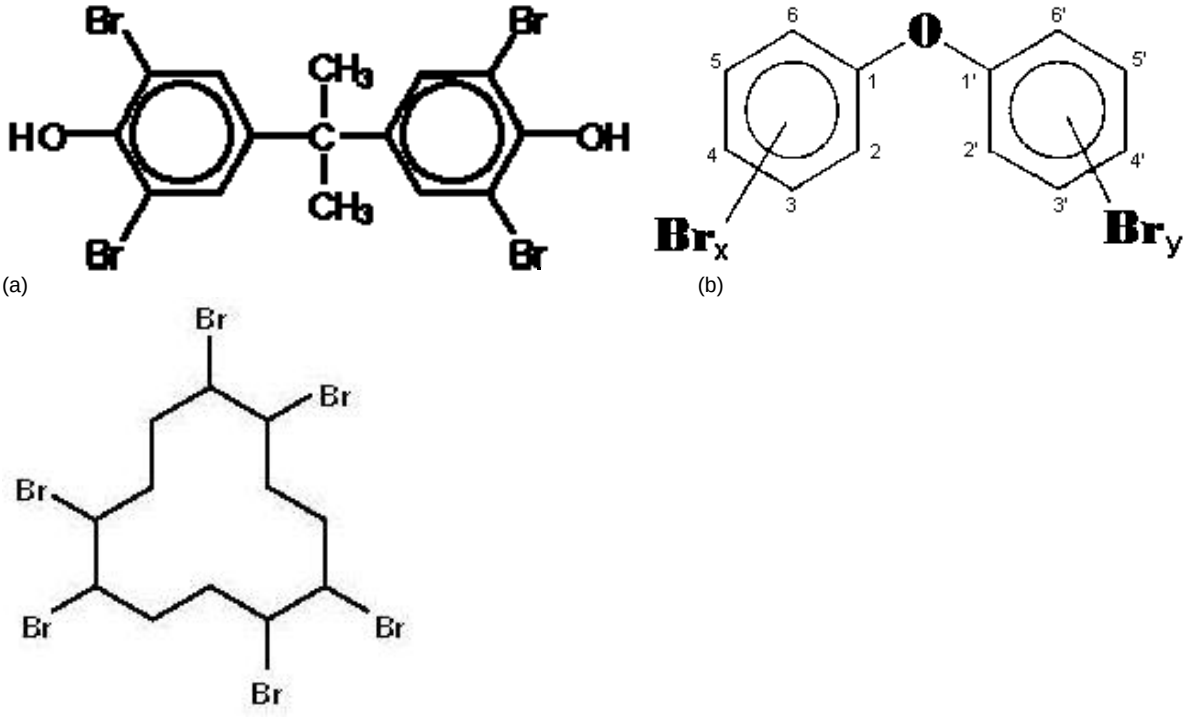

Figure 2 : Chemical structures of the brominated flame retardants (BFRs) (a) tetrabromobisphenol (TBBPA), (b) polybrominated diphenyl ethers (PBDEs) and (c) hexabromocyclodecane (HBCD).

Appendix 2: In Vivo Assays

Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute/ chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
OECD nr 201(2006), *Freshwater Alga and Cyanobacteria, Growth Inhibition Test;* SS-EN ISO 8692 (2005)		Algae, cyanobacteria	Microalgae and/or cyanobacteria	Growth inhibition	Fresh water	Laboratory animals	Acute toxicity	72 hours		The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period.	pH
SS-EN ISO 10253 (2006), *Marine algal growth inhibition test with Skeletonema costatum and Phaeodactylum tricornutum*	1.Davoren et al. 2005 b: *A test battery approach for the ecotoxicological evaluation of Estuarine sediments*	Algae	Microalgae: *Skeletonema costatum and Phaeodactylum tricornutum*	Growth inhibition	Salt water, 1: Sediment pore water and sediment elutriate (from estuarine sediments from the coast of Ireland)	Laboratory animals	Long term toxicity	72 hours		The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. pH should not vary by more than ±1 unit during the test.	1. Salinity of pore water samples had a major effect on the growth of the algae. High salinity enhances the extraction of NH_4^+ from sediments (into pore water) and causes inhibition of algal growth. Eutrophication in lake/sea increase algal growth due to elevated level of nutrients
OECD nr 202 (2004) *Daphnia sp., Acute Immobilisation test;* SS-EN ISO 6341 (2005)		Crustacea	*Daphnia magna* and other *daphnia* sp.	Immobilization	Surface water, ground water	Laboratory animals (less than 24 hours old at start of test)	Acute toxicity	48 hours		In the control, not more that 10 percent of the daphnids should have been immobilised; The dissolved oxygen concentration at the end of the test should be 3 mg/l in control and test vessels.	pH, temperature, total organic carbon conc.(TOC), chemical oxygen demand (COD), water hardness, light intensity and periodicity, water conductivity etc.

Appendix 2: In Vivo Assays

Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
OECD nr 211 (2008), *Daphnia magna Reproduction Test;* ISO 10706 (2000)		Crustacea	*Daphnia magna* (no more than 24 hours old at the start of test).Other Daphnia species may be used provided they meet the validity criteria in protocol.	Reproduction (total number of offspring/parent)	Fresh water, ground water	Laboratory animals	Long term toxicity	21 days	NOEC, LOEC, % reduction in reproductive output, EC_x	The mortality of the parent animals (female Daphnia) does not exceed 20% at the end of the test, the mean number of live offspring produced per parent animal surviving at the end of the test is > 60.	The dissolved oxygen concentration , pH, Hardness above 140 mg/l (as $CaCO_3$) is recommended, TOC and/or COD.
SS (Swedish Standard) 28214 (1996), *Determination of acute toxicity to the crustacean Ceriodaphnia dubia*	SNV rapport 4035	Crustacea	*Ceriodaphnia dubia*	Immobilization	Landfill leachate water	Laboratory animals	Acute toxicity	48 hours			
Environment Canada (2007), EPS 1/RM/21, *Test of Reproduction and Survival using Cladoceran Ceriodaphnia dubia*		Crustacea	*Ceriodaphnia dubia* (water flea)	Reproduction, mortality	Fresh water; WWTP effluents, landfill leachate water, sediment elutriates, and receiving fresh waters.	Laboratory animals	Chronic toxicity	5-8 days			
SS 28106 (1991), *Determination of toxicity of chemical products and waste water with the crustacea Nitocra spinipes Boeck*		Crustacea	*Nitocra spinipes*	Mortality	Salt water (brackish water from 0 ‰ to 35 ‰ salt conc.)	Laboratory animals	Acute toxicity	48 h, alt. 96 hours			

Appendix 2: In Vivo Assays

Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
Development test. OECD modified draft		Crustacea	*Nitocra spinipes*	Development and Reproduction: Larval development rate (LDR)	Salt water (brackish water from 0 ‰ to 35 ‰ salt conc.)	Laboratory animals	Long term toxicity	10 days			
OECD nr 203 (1992) Fish, Acute Toxicity Test; SS-EN ISO 7346		Fish	Zebra-fish, Fathead Minnow, common carp, Ricefish, Guppy, Bluegill, Rainbow trout	Mortality	Surface water and ground water	Fish farmed or laboratory animals	Acute toxicity	96 hours		The mortality of controls should not exceed 10 percent at the end of test. The dissolved oxygen conc. must have been at least 60 percent of the air saturation value throughout the test.	pH, temperature, dissolved oxygen concentration, water hardness etc.
OECD nr 204 (1984) *Fish, Prolonged ToxicityTest: 14-day Study*		Fish	*Brachydanio rerio*, *Pimephales promelas*, *Cyprinus carpio*, *Oryzias latipes*, *Poecilia reticulata*, *Lepomis macrochirus*, *Oncorhynchus mykiss*	Mortality, Behavioral observations	Fresh water	Fish farmed or laboratory animals	Long-term toxicity	14 days		The mortality in the controls should not exceed 10 per cent at the end of the test. The dissolved oxygen concentration should be at least 60 per cent of the air saturation value throughout the test.	pH, temperature, dissolved oxygen concentration, water hardness etc.
OECD nr 210 (1992), *Fish, Early-life Stage Toxicity Test*		Fish	*Oncorhynchus mykiss*, Rainbow trout, *Pimephales promelas*, Fathead minnow, *Brachydanio rerio*, Zebra fish, *Oryzias latipes*, Ricefish. (**Other possible species**, see test guideline)	*Lethal and sublethal effects*: Stage of embryonic development, Hatching and survival (of eggs, embryo, larvae, and juvenile fish), abnormal behavior, abnormal appearance, weight and length.	Fresh water	Laboratory animals	Subchronic test: (lethal and sublethal effects)	30-60 days (Depends on species) From eggs are fertilized until control fish are free feeding.		The dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation value throughout the test.	pH, hardness, temperature, dissolved oxygen concentration, total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made.

Appendix 2: In Vivo Assays

Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
OECD nr 210 (1992), *Fish, Early-life Stage Toxicity Test*		Fish	*Cyprinodon variegatus*, Sheephead minnow. **Other possible species**: *Menidia menidia*, Atlantic silverside, *Menidia peninsulae*, Tidewater silverside	*Lethal and sublethal effects*: Stage of embryonic development, Hatching and survival (of eggs, embryo, larvae, and juvenile fish), abnormal behavior, abnormal appearance, weight and length.	Salt water	Laboratory animals	Subchronic test: (lethal and sublethal effects)	30-60 days (Depends on species) From eggs are fertilized until control fish are free feeding.		The dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation value throughout the test	pH, hardness, temperature, dissolved oxygen concentration, total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made.
OECD nr 212 (1998), *Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages*		Fish	*Oncorhynchus mykiss*, Rainbow trout, *Oryzias latipes*, Japanese medaka, *Pimephales promelas*, fathead minnow, *Cyprinus carpio*, Common Carp, *Brachydanio rerio*, Zebra fish and other possible species.	*Lethal and sublethal effects*: Stage of embryonic development, Hatching and survival (of eggs, embryo, larvae), abnormal behavior, abnormal appearance, weight and length.	Fresh water	Laboratory animals	Subchronic test: (lethal and sublethal effects)	Zebra fish: 14 days		The dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation value throughout the test.	pH, temperature, dissolved oxygen concentration, conc. of heavy metals, major anions and cations, total organic carbon and suspended solids.
SS 28193 (1988), *Determination of embryo-larval toxicity to freshwater fish - Semistatic procedure*	1.SEPA Report 6304 (2009)	Fish	*Brachydanio rerio,* Zebra-fish	*Lethal and sublethal effects*:Frequency of hatching of embryos, median hatching time, median survival of embryo/fry stages, frequency of malformation of hatched embryos	Fresh water, 1. WWTP influents and effluents from pulp mill industries, Sweden	Laboratory animals	Subchronic test: (lethal and sublethal effects)	14 days	1.Effects expressed as toxic units =100/ LOEC		pH, temperature, dissolved oxygen concentration

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
Environment Canada (1998), EPS1/RM/28, *Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)*		Fish	*Oncorhynchus mykiss,* Rainbow trout, and other possible salmon species.	*Lethal and sublethal effects:* The E-test, short term acute test (7 days) tests number or percentage of non-viable embryos (teratogenicity).	Fresh water: WWTP effluents, landfill leachate water, sediment elutriates, and receiving fresh waters.	Laboratory animals	Acute test	7 days	EC_{25} , EC_{50}		
OECD nr 229 (2009), *Fish Short Term Reproduction Assay*		Fish	*Oryzias latipes*, Japanese medaka, *Pimephales promelas*, fathead minnow, *Brachydanio rerio*, Zebra fish	*Androgenicity, Oestrogenicity and aromatase inhibition via the biomarkers* vitellogenin production, secondary sexual characteristics, *Fecundity, Histopathology of gonads (optional), Survival, behaviour, appearance.*	Fresh water	Laboratory animals		21 days			pH, temperature, dissolved oxygen concentration, conc. of heavy metals, major anions and cations, total organic carbon and suspended solids.
OECD nr 230 (2009), *21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition*		Fish	*Oryzias latipes*, Japanese medaka, *Pimephales promelas*, fathead minnow, *Brachydanio rerio*, Zebra fish	*Androgenicity, Oestrogenicity and aromatase inhibition via the biomarkers* vitellogenin production, secondary sexual characteristics, *Survival, behaviour, appearance.*	Fresh water	Laboratory animals		21 days		Dissolved oxygen >60% of saturation; mean temperature of 24 ± 2°C, 90% survival of fish in the controls.	pH, temperature, dissolved oxygen concentration, conc. of heavy metals, major anions and cations, total organic carbon and suspended solids.
OECD draft (2006), *Fish Embryo Toxicity (FET) Test*		Fish	Zebra fish (*Danio rerio),* Japanese Medaka *(Oryzias Latipes),* Fathead minnow *(Pimephales promelas)*	*Lethal effects on embryonic stages,* teratogenicity*:* 4 apical endpoints:(1) coagulation of fertilized eggs, (2) lack of somite formation, (3) lack of detachment of the tail-bud from the yolk sack, and (4) lack of heart beat	Fresh water	Laboratory animals		24 hours, 48 hours	LC_{50}, LOEC, NOEC		

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
Zebra fish embryo test (DIN 38 415-T6, 2001)	1.Kammann et al. 2004	Fish	Zebra fish (*Danio rerio)*	Teratogenicity: Detects mortality rate (coagulation of the egg or lethal malformations) and non-lethal malformations of eggs.	1: Marine sediments (organic sediment extracts from North Sea and the Baltic Sea)	Laboratory animals		24 hours, 48 hours			
ISO 15088 (2007), *Vattenundersökningar - Bestämning av avloppsvattens akuta toxicitet på ägg från Zebrafisk (Danio rerio)*		Fish	Zebra fish (*Danio rerio)*	Teratogenicity: Acute toxicity to zebra fish eggs (see standard for further information)	WWTP influents and effluents, industrial eflfuents	Laboratory animals	acute test	48 hours			
OECD nr 221(2006), *Lemna* sp. Growth Inhibition Test			*Lemna gibba, Lemna minor (duckweed)*	Growth inhibition (frond number, total frond area, dry, wet weight, yield)	Landfill leachate water	Laboratory plant		7 days	x % inhibition of growth	The doubling time of frond number in the control must be less than 2.5 days (60 h), corresponding to appr.a 7-fold increase in seven days and an average specific growth rate or 0.275 d^{-1}	light intensity, temperature, pH,
	Granmo et al. 1989	Bivalve	*Mytilis edulis*, Blue mussel	Fertilization, early development	WWTP effluents in marine waters	*In situ* test		72 hours			
1. ASTM (1995): E 1563-95. *Standard guide for conducting static acute toxicity test with echinoid embryos*	2.OSPAR Commission 2007:*Backround document on Biological effects monitoring, ch 8.*	Echinoids	*2. Paracentrotus lividus, Strongylo-centrotus droebachiensis (sea urchin)*	2. Percentage morphologically abnormal larvae, Larval length, size inrease of larvae	1.Salt water, 2. Sediment seawater elutriate and pore water	Laboratory organisms or collected from field	Acute test	24-48 hours	Toxic units (TU)=100/ED_{50} or 100/ED_{20} , ED= effective dilution		

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
1.US-EPA Ecological Effects Test Guideline (1996a): *OPPTS 850.1055: Bivalve Acute Toxicity Test (Embryo-Larval)*	2.OSPAR Commission 2007:*Backround document on Biological effects monitoring, ch 8.*	Bivalve	*1.Crassostrea virginica (*Eastern oysters), *Crassostrea gigas* (Pacific oysters), *Mercenaria mercenaria* (quahogs), *Mytilis edulis* (bay mussels), 2.*Crassostrea gigas, Mytilus edulis, Mytilus galloprovincialis*	Mortality (lethal) or abnormal development (sublethal).	1.Salt water, 2. Sediment seawater elutriate and pore water	Laboratory organisms or collected from field	Acute test	24-48 hours	$EC_{50,}$ LOEC, NOEC, 2.Toxic units (TU)= $100/ED_{50}$	Mortality or aberrant development in the controls should not exceed 30 % for oysters or 40 % for clams at the end of each test. Embryos should not be more than 4 hours old from fertilization at the beginning of the test.	salinity, pH, temperature, the dissolved oxygen concentration
ISO 10710 (2010), *Water quality - growth inhibition test with the marine and brackish water macroalga Cera-mium tenuicorne; Whole sediment and leachate tests with Ceramium tenuicorne – Growth inhibition; ITM*	1. Eklund et al. 2010, 2.SEPA Report 6304 (2009)	Algae	Macroalgae: *Ceramium tenuicorne*	Growth inhibition	1.whole sediments, whole sediment leachate, salt and brackish waters (4-32 ‰), 2.WWTP influents and effluents from pulp mill industries, Sweden	Laboratory organisms	sublethal test	7 days	1. Effects expressed as toxic units =100/EC50		temperature, light, salinity
Whole sediment test and leachate tests with Nitocra spinipes – Larval development ratio and mortality; ITM	1. Eklund et al. 2010, 2. SEPA Report 6304 (2009)	Crustacea	*Nitocra spinipes*	Larval development ratio, larval mortality.	1. Whole sedi-ments, whole sediment leachate, salt and brackish waters (0 ‰ to 35 ‰ salt conc.), 2.WWTP influents and effluents from pulp mill industries.	Laboratory organisms	sublethal and lethal test	6-8 days	1. Effects expressed as toxic units= 100/LOEC		

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
ASTM (1993). *Standard guide for conducting 10-d static sediment toxicity tests with marine and estuarine amphipods*	1. Cleveland et al. 1997, 2. Narracci et al. 2009. *A test battery approach for ecotoxicological characterization of Mar Piccolo sediments in Taranto (Ionian Sea, Southern Italy),* 3. OSPAR Commission 2007, ch 10.	Amphipods	1. *L. plumulous,* 2. Amphipods: *Corophium insidiosum and Gammarus aequicauda, 3. Corophium spp.*	Mortality	1. Whole sediment extracts and SPMD dialysates (from Antarctica marine sediments) , 2. Sediments from a marine sea (from brackish, estuarine water of the infralittoral zone, an inner sea with restricted water circulation)	Field animals	Acute sediment toxicity test (and integrated toxicity battery)	10 days	3. Toxic units (TU) = 100/ LD_{50} (LD_{50} is the theoretical dilution with reference sediment that causes 50 % mortality in amphipods)	The mortality in the control sediment should be <20%, while the sediment is considered toxic, if it significantly differs from control mortality (t-test; p<0.05)	temperature, salinity, dissolved oxygen, ammonium content and pH of the overlying water
US-EPA Ecological Effects Test Guideline (1996b): *OPPTS 850.1740*, *Whole sediment Acute Toxicity Invertebrates, Marine*		Amphipods	Estuarine and marine amphipods: *Ampelisca abdita, Eohaustorius estuarius, Rhepoxynius abronius, and Leptocheirus plumulosus*	Mortality	Estuarine and marine sediments	Field organisms (collected and added to test jars)	Acute	10-28 days		Recovery of organisms from control sediment should equal or exceed 90 percent in a 10–day test or 80 percent in a 28–day test.	Salinity, pH, and dissolved oxygen (DO), temperature, light intensity, periodicity, characterization of sediment.
ASTM (1994): E-1383-94a. *Standard guide for conducting sediment Toxicity Tests with Fresh water invertebrates*		Insect	*Chironomus sp.* (insect larva).	Larval emergence, survival, growth or adult emergence	(Fresh Water) Whole sediment	Brood stock of animals can be obtained from the field, another laboratory, or a commercial source.	≤ 10 days is short term test, >10 days is long term test	25-30 days (long term test for adult Emer-gence)		Average recovery of organisms from control sediments should equal or exceed 70 percent. Hydrogen sulfide should not be greater than 0.3 mg/L	Dissolved oxygen (DO), temperature, Conductivity, hardness, pH, alkalinity of overlying water
ASTM (1994): E-1383-94a. *Standard guide for conducting sediment Toxicity Tests with Fresh water invertebrates*		Insect	*Hexagenia* sp.(mayfly)	survival, growth	(Fresh Water) Whole sediment	Animals can be obtained in the field, from another laboratory or a commercial source in the form of nymphs or eggs.	Long term test	21 days		Average recovery of organisms from control sediments should equal or exceed 80 percent	Dissolved oxygen (DO), temperature. Conductivity, hardness, pH, alkalinity of overlying water

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
US-EPA Ecological Effects Test Guideline: *OPPTS 850.1735 (1996c), Whole sediment Acute Toxicity Invertebrates, Fresh water*		Amphipods, Insect	*A) Hyalella azteca* (amphipod), *B)Chironomus tentans* (insect larva)	A) survival, B) survival, growth and/or emergence	(Fresh Water) Whole sediment	Laboratory organisms	≤ 10 days is short term test, >10 days is long term test	10-28 days		*H. Azteca* are not found in waters with calcium at < 7 mg/L and DO at < 2 mg/L. Chironomid larvae are not found when hydrogen sulfide is greater than 0.3 mg/l.	Dissolved oxygen (DO), temperature. Conductivity, hardness, pH, alkalinity and ammonia conc. of overlying water, light intensity, periodicity; characterization of sediment.
ASTM (1994): E-1383-94a. *Standard guide for conducting sediment Toxicity Tests with Fresh water invertebrates*		Amphipod	*Hyalella azteca*	survival, growth, reproduction	(Fresh Water) Whole sediment	Brood stock of animals can be obtained from the field, another laboratory, or a commercial source.	Long term test	30 days		Average recovery of organisms from control sediments should equal or exceed 80 percent	Dissolved oxygen (DO), temperature. Conductivity, hardness, pH, alkalinity of overlying water
ASTM (1994): E-1383-94a. *Standard guide for conducting sediment Toxicity Tests with Fresh water invertebrates*		Crustacia	*Daphnia* sp. , *Ceriodaphnia* sp.	survival, reproduction	(Fresh Water) Whole sediment	Laboratory organisms	Short term chronic toxicity test	7 days		Controls must have 80 % survival, with *C .dubia* controls averaging 15 young per surviving female, and *D. magna* averaging 20 young per surviving female.	Dissolved oxygen (DO), temperature. Conductivity, hardness, pH, alkalinity of overlying water.
ASTM (1994): E-1383-94a. *Standard guide for conducting sediment Toxicity Tests with Fresh water invertebrates*		Oligochaeta	*Tubifex tubifex (sludge worms)*	survival, reproduction	(Fresh Water) Whole sediment	Organisms are collected in the field and cultured in a laboratory for 8 weeks before testing.	Long-term toxicity	28 days		Survival of original adults in control sediments should be 90-95 %. The coefficient of variation (COV) for production of total young and total cocoons should be less than 25 % in controls.	Temperature, population density, Dissolved oxygen (DO); Conductivity, hardness, pH, alkalinity of overlying water; Sediment characteristics.

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
OECD nr 225 (2007), *Sediment-Water Lumbriculus Toxicity test using spiked sediment.*		Oligochaeta	*Lumbriculus* (earth worm) *variegatus*	Reproduction, biomass	Whole sediment (Fresh water)	Laboratory organisms	Long-term toxicity	28 days	EC_{50}, EC_{25}, EC_{10} for reproduction, biomass compared to control, NOEC, LOEC.	The average number of living worms per replicate in the controls should have increased by a number of at least 1.8 at the end of the exposure. The pH of the overlying water should be between 6 and 9.	Dissolved oxygen (DO), Conductivity, ammonia concentration, temperature, light intensity. The total hardness of the water should be between 90 and 300 mg/l of $CaCO_3$ if natural water is used.
OECD nr 218 (2004), *Sediment-Water Chironomid Toxicity test using spiked sediment*		Insect	*Chironomus riparius, Chironomus tentans, Chironomus yohimatsui* and other *Chironomus* sp.	Number of emerged adults and time of emergence	(Fresh water) Whole sediments	Laboratory organisms	Long term toxicity (and short term toxicity)	20-28 days for *C. riparius,* and 28-65 days for *C. tentans*,	% reduction in emergence or larval survival or growth (EC_{15}, EC_{50} etc) compared to control sediment	The emergence in the controls must be at least 70% at the end of the test. Emergence to adults from control vessels should occur between 12 and 23 days for *C riparius* and *C yoshimasui* and between 20 to 65 days for *C tentans*.	Dissolved oxygen (DO), temperature, pH. The total hardness of the test water should not be higher than 400 mg/l as $CaCO_3$, *Characterization of sediment:* pH, TOC, C/N ratio, granulatory etc.
OECD nr 219 (2004), *Sediment-Water Chironomid Toxicity test using spiked water*		Insect	*Chironomus riparius, Chironomus tentans, Chironomus yohimatsui* and other *Chironomus* sp.	Number of emerged adults and time of emergence	(Fresh water) sediment pore water	Laboratory organisms	Long term toxicity (and short term toxicity)	20-28 days for *C. riparius,* and 28-65 days for *C. tentans*,	% reduction in emergence or larval survival or growth (EC_{15}, EC_{50} etc) compared to control sediment	The emergence in the controls must be at least 70% at the end of the test. Emergence to adults from control vessels should occur between 12 and 23 days for *C riparius* and *C yoshimasui* and between 20 to 65 days for *C tentans*.	Dissolved oxygen (DO), temperature, pH The total hardness of the test water should not be higher than 400 mg/l as $CaCO_3$, *Characterization of sediment:* pH, TOC, C/N ratio, granulatory etc.

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Test Guideline	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Lab-cultured organism/ Field organism	Acute /chronic test	Time until reading	Evaluation criteria	Validity criteria	Other environmental parameters
	Swedish Defense Research Agency (FOI) (2003)	Fish, Bivalves	*Mytilus Edulis*, blue mussel, *Macoma Baltica*, Baltic clam , *Perca fluviatilis*, Perch	Tissue analysis (chemical analysis) of PCB, organotin compounds, Metals (As, Cd, Cd, Cu, Cr, Hg, Mn, Ni, Pb, Zn, V)	Marine sea: Particle bound pollutants in water phase (3-10 m) and in bottom sediments (20-40 m)	Field (Baltic Sea, Stockholm archipelago)					
	Swedish Defense Research Agency (FOI) (2003)	Crustacea	*Ceriodaphnia dubia (*water flea)	Immobilization	Marin sea: Sediment (1-15 cm)	Laboratory animals	Acute toxicity	48 hours, 72 hours			
	Swedish Defense Research Agency (FOI) (2003)		*L-929 cell culture (mouse)*	cell growth inhibition *(in vitro* assay)	Marin sea: Sediment (1-15 cm)	Laboratory animals	Acute toxicity				

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Toxi-ChromoTest™ Kit	Acute and Chronic Toxicity (EC_{50})	1.Rodgers et al. 1996, 2. Cheung et al. 1996	Intestinal Bacterium *Escherichia coli (E.coli)*	Surface and ground water, industrial effluents, municipal discharges, 1. Effluents from an industrial nuclear, fossil and hydroelectric energy generating facility, Ontario, Canada, 2. Sediment elutriates from coastal water sediments of Hong Kong.	Wide spectrum of pollutants		90 min.		1.The Toxi-chromo test did not correlate well with other toxicity tests on ecologically relevant species such as *Daphnia Magna* and rainbow trout on the industrial effluents.
Toxi-Chromo Pad™bacterial assay (Kwan 1995)	Acute Toxicity(EC_{50})	1.Davoren et al. 2005b, 2.Cheung et al. 1996	Intestinal Bacterium *Escherichia coli (E.coli)*	1. Whole sediment (from estuarine sediments from the coast of Ireland), 2. Whole sediments from coastal waters of Hong Kong.	Wide spectrum of pollutants		90 min.	(a) High toxicity= no blue color development, (b) moderately toxic=less than 50 % of blue color intensity, (c) low toxicity=less than 100 % but greater than 50% of color intensity, (d) non-toxic=blue color intensity equivalent to control.	2.The toxi-chromo pad test showed less sensitivity than the Microtox solid phase test in detecting differences in toxicity between sediments.
Microtox™ Solid phase Test protocol (Azur Environmental Ltd. 1995).	Acute toxicity (EC_{50})	1. Cleveland et al. 1997, 2. Klamer et al. 2005, 3. Davoren et al. 2005b, 4. Naracci et al. 2009, 5 Cheung et al. 1996	*Vibrio fischeri* (marine bacteria)	1. Whole sediment extracts and SPMD dialysates (from Antarctica marine sediments), 2. Surface sediments (fraction < 63 µm) (from the southern North Sea, The Netherlands), 4.Sediments from a marine sea (from brackish, estuarine water of the infralittoral zone)	2. PCBs and PAHs, 3.Persistent sediment-associated compounds	3.Grain size of sediments major confounding factor, Salinity of diluent in assay affects toxic response measured.	2: 5 min. 4. 20 min.	The results are expresses as Sediment Toxicity Index (S.T.I.)	5. Microtox solid phase test correlated significantly ($p<0.05$) with several physiochemical properties of the sediments and sediment elutriates. It also correlated well with results form diatom, shrimp and fish assays on the sediment elutriates.

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Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Microtox ® Solid Phase Test	Acute toxicity (EC_{50})	Brouwer et al. 1990	*Vibrio fischeri* (marine bacteria)	Whole sediment					Performed by Toxicon AB, Sweden.
Microtox® acute toxicity basic test procedures (Azur Environmental Ldt 1998); SS-EN ISO 11348-3 (2008): (Luminescent bacteria test) - Part 3: Method using freeze-dried bacteria	Acute toxicity (EC_{50}); Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri*	1. Davoren et al. 2005b, 2. Johnson and Long 1997, 3. Bicchi et al. 2009	*Vibrio fischeri* (marine bacteria)	1.Sediment pore water and sediment elutriate (from estuarine sediments from the coast of Ireland), 2. Organic extracts of sediments (from the Gulf coast of Florida , USA), 3. WWTP effluents and surface water recipients upstream and downstream (in northern Italy).	2. Insecticides, petroleum products, PCBs, PAHs	Temperature, salinity, osmotic regulation, pH, color, turbidity of sample. 1.Hormetic response seen at sub-inhibitory levels of toxicants in samples.	1.20 min., 2.5 and 15 min, 3. 5,15 and 30 min.	2. The EC_{50} values of organic extracts of sediments were expressed as mg Equivalent sediment wet weight/ ml DMSO, 3. Results were expressed as toxic units, TU = (1/EC50)x 100; (a)TU>100= extremely toxic, (b)11<TU<100 = very toxic, (c) 1<TU<10 = toxic, (d) TU<1 = weakly toxic, (e) TU =0 , not toxic.	2.The EC_{50} values tended to lie around 1.2 (0.8) mg/L for insecticides, petroleum products and PCBs
Yeast Estrogen Screen (YES)	Recombinant receptor reporter assay with a colorimetric response: Test estrogenic and antiestrogenic potency	1.Routledge and Sumpter 1996 2. Arnold et al. 1996, 3. Verslycke et al. 2005 4. Legler et al. 2002, 5.IVL 2002	*Saccharomyces cerevisiae* (Yeast cells) transfected with plasmids containing human-estrogen receptors (hERα or hERβ) and an estrogen-response element controlling the reporter gene lacZ.	3: Water and sediment extracts (from the Scheldt estuary in The Netherlands) 5.Landfill leachate water and domestic wastewater, (Siljansnäs, Sweden)	1. Alkylphenol polyethoxylates, 2. o,p´-DDT, octyl phenol (OP),17ß-estradiol, Diethylstilbestrol (DES), 3. organotins, PBDEs, hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), nonylphenol ethoxylates (NPE) and transformation products, nonylphenol (NP), nonylphenol ether carboxylates (NPEC)		1,4,5: 72 hours 2:12 hours	EEQs	Yeast-based systems are more appropriate for complex environmental samples (e.g. Sewage sludge) for they are less susceptable to non-sterile conditions than other mammalian or fish cell line-based systems.

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Yeast Androgen Screen (YAS)	Recombinant receptor reporter assay with a colorimetric response: Test androgenic and antiandrogenic potencies.	1. Sohoni and Sumpter 1998, 2. Thomas et al. 2002 3. Verslycke et al. 2005 4. IVL 2002	*Saccharomyces cerevisiae* (yeast), transfected with plasmids containing human androgen receptors (hARα) and an androgen-response element	2.Surface water, sediment particulate matter (SPM) extracts and sediment pore water (from United Kingdom est-uaries), WWTP effluents. 3.Water and sediment extracts (from the Scheldt estuary in The Netherlands)	1. Antiandrogens such as vinclozolin, p,p´-DDE, o,p´-DDT, bisphenol A, and butyl benzyl phthalate; xenoestrogen nonylphenol 2: Androgens (natural and synthetic) such as dehydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone, epi-androsterone		1. 36 hours, 2,4: 72 hours		2:High levels of androgenic activity were determined in the solvent extracts of sediments, with 10 of 39 samples exhibiting a level of androgenic activity >454 ng DHT/kg (1,020–15,300 ng DHT/kg).
Progesterone receptor transactivation assay	Recombinant receptor reporter assay with a flourescent response: Test progestative and antiprogestative potency	1. Chatterjee et al. 2008	*Saccharomyces cerevisiae* (yeast) YPH499, transfected with plasmids expressing the human progesterone receptor hPR, progesterone response element driving the expression of green fluorescent protein.	1.Water extracts from waste water from leather industries in India	1.Antiprogestagens (natural and synthetic) such as DDT (o,p´-DDT, p,p´-DDT), and its metabolites (o,p´-DDE, p,p´-DDE), nonylphenol, endosulphane.		1. 24 hours		1.DDT and its metabolites, nonylphenol, endosulphane were found to be anti-progestagenic with IC50 of 3-20 μM
DR-CALUX (dioxine responsive chemically activated luciferase expression) *assay*	Relative toxic potency or total activieties of aryl hydrocarbon receptor (AhR)-active compounds.	1.Murk et al. 1996, 2. Machala et al. 2001, 3.Stronkhorst et al. 2002, 4.Houtman et el 2004, 5.Hurst et al. 2004, 6. Klamer et al. 2005	Rat hepatoma (H4IIE) cells containig a luciferase reporter gene construct	1: Sediment and pore water extracts (from Dutch waters), 2. Surface river sediments (in the Morava river basin, Czech Republic), 4. Sediments (from the Rhine Meuse estuary area, the Netherlands), 5. Estuarine sediments in the UK 6. Surface sediments from the South North Sea	2,6: PAHs (4-6-rings) 1,4,5:PHAHs (polyhalogenated hydrocarbons): PCDDs, PCDFs, PCBs.		1: 20-24 hours, 2: 6 and 24 hours	CALUX-TEQs	1. Detection limit of <0.5 fmol of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)/well, or <1 pM TCDD. Advantages: It is insensitive to substrate inhibition which has been a problem in the EROD assay. Fish are sensitive to PHAH (polyhalogenated hydrocarbon)-induced toxicity, especially when exposed during early life stages, i.e. egg to larval stadium.

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
XDS-CALUX® Assay (EPA Method 4435)	Relative toxic potency or total activieties of aryl hydrocarbon receptor (AhR)-active compounds.				Dioxins, furanes and dioxin-like-PCBs			CALUX-TEQs	
H4IIE.luc assay	Relative toxic potency or total activieties of aryl hydrocarbon receptor (AhR)-active compounds.		Rat hepatoma (H4IIE) cells containig a luciferase reporter gene construct		Dioxins, furanes and dioxin-like-PCBs, PAHs		20-24 hours	CALUX-TEQs	
ER-CALUX (estrogen responsive chemically activated luciferase) *assay*	Recombinant receptor reporter assay with a luminescent response: Test estrogenic and anti-estrogenic activities	1.Houtman et al. 2004, 2.Legler et al. 2002, 3. Klamer et al. 2005, 4. Sonneveld et al. 2005, 5.Schriks et al. 2009, 6. Van der Linden et al. 2008	1,2,3:T47D human breast-cancer cells containing endogenous estrogen receptors and a luciferase reporter construct, 4,5,6:Human U2-OS osteosarcoma cells containing the human estrogen receptor α and a luciferase reporter construct (ERα-CALUX)	1: Sediments (form the Rhine Meuse estuary area, the Netherlands). 2: Marine sediments (on the Dutch coast) 3. Surface sediments (fraction < 63 µm) (from the southern North Sea, The Netherlands), 5. Surface water (from the Dutch part of the Rhine river basin)	2. Persistent (xeno-)estrogenic compounds: 17ß-estradiol (E2),alkylphenol ethoxylates and carboxylates; phthalates; and bisphenol A, methoxychlor.		24 hours	EEQs	2: ER-CALUX was found to be approximatlely a factor of 6- 20 times more sensitive than the YES assay in detecting estradiol (E2) and other xenoestrogens. 1: DR-CALUX and ER-CALUX can detect femtograms of TEQs or EEQs and are therefore sensitive enough to be applied in TIE analysis.
LUMI-CELL® ER bioassay	Recombinant receptor reporter assay with a luminescent response: Test estrogenic and anti-estrogenic activities	Gordon et al. 2003	BG1Luc4E2 cells (BG-1, human ovarian carcinoma cell line, transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere))		Estrogens (natural and synthetic), xenoestrogens		24 hours	EEQs	

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
AR-CALUX (androgen responsive chemically activated luciferase) assay	Recombinant receptor reporter assay with a luminescent response: Test androgenic and anti-androgenic activities	1. Sonneveld et al. 2005, 2. Van der Linden et al. 2008, 3. Schriks et al. 2009	Human U2-OS osteosarcoma cells containing the human androgen receptor and a luciferase reporter construct.	2. Raw industrial and hospital effluents, WWTP effluents from municipal area and paper mill industry, surface water, drinking water, 3. Surface water from the Dutch part of the Rhine river basin	1.Androgens (natural and synthetic) and anti-androgens such as vinclozolin, DDT, methoxychlor and its metabolite HPTE, penta-BFR		24 hours		Test can be performed in 8-10 working days
*GR-CALUX (*Glucocorticogenic responsive chemically activated luciferase gene expression) *assay*	Recombinant receptor reporter assay with a luminescent response.	1.Van der Linden et al. 2008 2. Schriks et al. 2009	Human U2-OS osteosarcoma cells	1. Raw industrial and hospital effluents, WWTP effluents from municipal area and paper mill industry, surface waters, drinking water. 2 Surface water from the Dutch part of the Rhine river basin	Glucocorticoids such as cortisol, dexamethasone, triamcinolon, prednisone and cortisone		24 hours		
TR-CALUX (Thyroid hormone responsive chemically activated luciferase gene expression) *assay*	Recombinant receptor reporter assay with a luminescent response.	1.Schriks et al. 2009	Human U2-OS osteosarcoma cells	1. Surface water from the Dutch part of the Rhine river basin	Thyroidogens		24 hours		
PR CALUX (progestagen-responsive chemically activated luciferase) assay	Recombinant receptor reporter assay with a luminescent response: Test progestative and anti-progestative activities	1. Hamers et al. 2006, 2. Van der Linden et al. 2008, 3. Shriks et al. 2009	Human U2-OS osteosarcoma cells containing the human progesterone receptor and a luciferase reporter construct.	2. Raw industrial and hospital effluents, WWTP effluents from municipal area and paper mill industry, surface waters, drinking water, 3. Surface water from the Dutch part of the Rhine river basin	1: Brominated flame retardants (BFRs), 2, 3: Progestagens (natural and synthetic)		24 hours		Progestagenic compounds are present in the environment in at least the same conc. range as other sex hormones.

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
AR-LUX (androgen receptor -mediated luciferase expression) assay	Recombinant receptor reporter assay with a luminescent response.	1. Blankvoort et al. 2001, 2.Blankvoort et al. 2005	T47D human breast carcinoma cell line containing endogenous expressed androgen receptor via an upstream rat probasin androgen response element 2 (PB-ARE2) and a luciferase reporter gene construct	2. SPM extracts and water extracts from WWTP influents and effluents and FW rivers (in the Netherlands).	Androgens (natural and synthetic)		24 hours		
MVLN bioassay	MVLN = recombinant MCF-7 cells transfected with a luciferase reporter gene under the control of estrogen response elements: Measures (anti)-estrogenic-like activity	1. Demirpence et al. (1993) 2.Khim et al. (2001), 3. Machala et al.(2001), 4. 4.Vondráček et al.(2001)	Recombinant MCF-7 cells (mammary carcinoma fibroblast) human breast cancer cells	2: Sediment, pore water and water samples (from Ulsan Bay, Korea) 3, 4: Surface river sediments (in the Morava river basin, Czech Republic)	2. PAHs (polycyclic aromatic hydrocarbons) 3.PAHs and their metabolites; oxy-PAHs, azaarenes (9-fluorenone, anthrone, anthraquinone, benzanthrone, benz[a]anthracene-7,12-dione, benz[c]acridine, and dibenz[a,h]acridine), 4. PAHs and phthalate esters		1,3 :24 hours, 2: 72 hours		3.Benzanthrone, benz[*a*]-anthracene-7,12-dione, anthraquinone, and benz[*a*]-acridine were weak inducers of *in vitro* estrogenic activity, with IEFs similar to that of benzo[*a*]pyrene. No studied compound was found to contribute significantly to estrogen receptor–mediated activity *in vitro*.
Human embryonic kidney (HEK)	Recombinant receptor reporter assay with a luminescent response.	1. Pawlowski et al. 2003	1: HEK 293 cells transfected with hERα or hERß	1.Sewage treatment plant effluents and surface water from the river (Rhine, Germany)	Estrogens (natural and synthetic): 17ß -estradiol (E2), estrone, 17α -ethinylestradiol.	Mammalian cell lines may possess endogenous hormonal receptors (but yeast cells do not) which can interfere with results	24 hours		
Reporter gene assays	Estrogenic, PXR (pregnane X receptor) and (anti-)androgenic activities	1.Kinani et al. 2009	MELN (Human breast cancer cells), HG5LN-PXR (HeLa cells) and MDA-kb2 (MDA-MB-453 human breast cancer cells) reporter cell lines.	1.Sediments (organic extracts) from small rivers in France (impacted and reference areas)	Natural estrogens, organochlorine pesticides, parabens, 16 PAH:s, bisphenol A and alkylphenols	28-96% of estrogenic activity were explained by 17ß-estradiol and estrone.	16 hours		High PXR and (anti-)androgenic activities were found but could not be explained by chemically analyzed compounds.

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
RTG-2 reporter gene assay	Recombinant receptor reporter assay with a luminescent response.	1. Ackermann et al. 2002	RTG-2, rainbow trout gonad cells transfected with rainbow trout estrogen receptor α cDNA and the reporter vector pERE-TK-luc.	1.Sewage treatmant plant effluents	1: E2 (Estradiol), 17α-ethinylestradiol, 4-nonylphenol, nonylphenoxy acetic acid, 4-tert-octylphenol, bisphenol A, o,p′-DDT, p,p′-DDT, o,p′-DDE, p,p′-DDE, o,p′-DDD, p,p′-DDD, and p,p′-DDA				Dose–response curves of nonylphenol, octylphenol, bisphenol A, and *o,p′*-DDD revealed that the RTG-2 reporter gene assay is more sensitive for these compounds when compared to transfection systems recombinant for mammalian ERs.
E-SCREEN (estrogenicity screen)	*Cell proliferation response*:Assesses the estrogenicity using the proliferative effect of estrogens on their target cells as an end point	1. Soto et al. 1995, 2.Körner et al. (1999, 2000, 2001), 3. Bicchi et al. 2009	MCF-7 breast-cancer cells	2. Sewage effluents and sludge from WWTPs, South Germany, 3. Effluents from WWTP and river water samples upstream and downstream from WWTP, Torino, Italy	1. Estrogens (natural and synthetic), alkylphenols, phthalates, some PCB congeners and hydroxylated PCBs, and the insecticides dieldrin, endosulfan, and toxaphene (all of these reacted like estrogens).		1: 6 days		
A-SCREEN (androgenicity screen)	Decreased cell proliferation response	1. Szelei et al. 1997	MCF-7-AR1 cells, transfectants of MCF7 with human androgen receptor		Androgens (natural and synthetic)		24 hours		Not yet been applied on environmental samples
T-SCREEN (thyroidogenicity screen)	*Cell proliferation response*: Assesses the thyroidogenicity, i.e. the proliferative effect of xenobiotic mimicking thyroid hormones on its target cells, and measures xenobiotics interference with T_3-receptor interaction (decreased cell proliferation)	1. Gutleb et al. 2005, 2.Hamers et al. 2006	Rat pituitary tumour cell line (GH3 cells)	1.Organic sediment extracts from The Netherlands	2. Brominated flame retardants (BFRs)	2.Cytotoxicity was observed in GH3 cells at concentrations ≥ 1 µM of test compound	1: 4 days		

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
The enzyme-linked Receptor Assay (ELRA)	Detect estrogenic and anti-estrogenic effects at the level of receptor binding. Uses the same principle as a competitive enzyme-linked immunoassay (ELISA) that analyses a ligand-protein interaction. An *In situ* model.	1. Kase et al. 2008, 2.Kase et al. 2009	A subcellular test method that uses recombinant human estrogen receptor α which is expressed in transformed yeast cells.	1.Surface water (fresh water and salt water), 2. Sediment elutriate and pore water	Estrogens (natural and synthetic)	The ELRA is usable under salinity conditions up to 20‰.The YES and E-screen methods are not applicable for liquid phase testing at higher salinities. The ELRA can be used for brackish and high salinity WWTP effluent samples as well as freshwater samples.	1.5 hours		The ELRA is very fast and reproducible, it can be used for high-throughput screening in a microplate format at low cost, it is robust to microbial contamination, and is less susceptible to cytotoxic interferences than cell culture methods
*T4-transthyretin (TTR) binding competition assay (*Lans et al. 1993)	Interference with thyroid hormone (T_4; 3, 3´,5, 5´-tetraiodothyroxine) binding to plasma transport protein	1.Houtman et al. 2004 2. Hamers et al. 2006, 3. Ishihara et al. 2009	*none*	1.Sediments (from the Rhine Meuse estuary area, the Netherlands), 3.WWTP effluents and surface water, Thailand	1.PCBs 2. Brominated flame retardants (BFRs)		O/N incubation		Can detect T_4-binding in the low nanogram range
E2 SULT inhibition assay	Inhibition of sulfation of E2: measures estrogenic activity	1. Kester et al. 2000, 2.Hamers et al. 2006	*none*		1, Hydroxylated PCB metabolites, 2.Brominated flame retardants (BFRs)		30 min.		
Cytotoxicity assay: *Neutral Red Assay*	Lysosomal function	1.Davoren et al. 2005a, 2. Ali et al. 1993	*Fish cell lines*: 1.CHSE-214 cells (from Chinook salmon embryo), EPC cells (from carp epithilium), RTG-2 cells (from rainbow trout gonads), 2. BB, Brown bullhead cells, *Ictalurus nebulosus*	1. Estuarine sediment (from Irish coast):aqueous elutriates, 2. Sediments extracts (from the Great Lakes, Canada)	Metals	1.RTG-2 cells were found to be the most suitable cell line for testing estuarine elutriates samples due to much higher salinity tolerance than the other cell lines	24 hours		

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Cytotoxicity assay*: Coomassie Blue cytotoxicity assay*	Protein content	Davoren et al. 2005a	*Fish cell lines*: CHSE-214 cells (from Chinook salmon embryo), EPC cells (from carp epithilium), RTG-2 cells (from rainbow trout gonads)	Estuarine sediment (from Irish coast): aqueous elutriates	Metals	RTG-2 cells were found to be the most suitable cell line for testing estuarine elutriates samples due to much higher salinity tolerance	24 hours		
Cytotoxicity assay: *Alamar Blue assay*	Mitochondrial function	Davoren et al. 2005a	*Fish cell lines*: CHSE-214 cells (from Chinook salmon embryo), EPC cells (from carp epithilium), RTG-2 cells (from rainbow trout gonads)	Estuarine sediment (from Irish coast):aqueous elutriates	Metals	RTG-2 cells were found to be the most suitable cell line for testing estuarine elutriates samples due to much higher salinity tolerance	24 hours		
Cytotoxicity assays : *Neutral red assay (NR), succinic acid dehydrogenase assay (MTT), Lactate dehydrogenase release assay (LDH) (photometric measurements), microscopic inspections*	Cell viability/Acute toxicity	1. Hollert et al. 2000	*Fish cell lines*:1.RTG-2, rainbow trout gonad cells	1. Settling particulatte matter (SPM), sediment extracts, Neckar river, Germany.	Cadmium, PCBs, PAHs	Permanent fish cell lines have low activity of CYP-dependant enzymes why addititon of S9 prepaparation from rat liver may be added to assay to improve accuracy of results	24 hours		
Cytotoxicity assay	Mitotic inhibition	1. Kocan et al. 1985	*Fish cell lines*: RTG-2, rainbow trout gonad cells, BF-2, bluegill fin from sunfish	1: Marine sediment (organic extracts) from Puget Sound, WA, USA	PAHs, PCBs, heavy metals, CHCs (chlorinated hydrocarbons)		96 hours		

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Cytotoxicity assay : *Bradford assay*	Protein content	1. Huuskonen et al. 2000, 2.Strmac and Braunbeck 2000	1.PLHC-1, Hepatocellular carcinoma of topminnow *(Poeciliopsis lucida) 2.*Isolated hepatocytes of Rainbow trout *(Oncohynchus mykiss)*	1,2: Sediments	2. PAHs, PCBs, Heavy metals				
Cytotoxicity assays: *ultrastructural and biochemical changes correlated*	*Time and dose-dependant morphological (ultrastructural) and biochemical changes (sublethal cytotoxic effects)*;Lactate dehydrogenase, alanine aminotransferase, catalse, glutathion S-transferase, acid phosphatase and lipid peroxidation activities analyzed (photometric measurements), electron microscopic inspections	Strmac and Braunbeck 2000	Isolated hepatocytes of Rainbow trout *(Oncohynchus mykiss)*	1: Native water and sediment extracts (from two small rivers in Germany)	PAHs, PCBs, Heavy metals, pesticides.		1-3 days		
Genotoxicity assay: *Mutatox assay*® (Johnsson, B. T. 1993)	Genotoxicity: Induction of the SOS-repair system (primary DNA damage)	1. Johnsson 1992,1993, 2.Cleveland et al. 1997, 3.Johnson and Long 1997, 4. Klamer et al. 2005	*Vibrio fischeri* (marine bacteria)	2. Whole sediment extracts and SPMD dialysates (from Antarctica marine sediments), 3.Organic sediment extracts from estuaries (along the Gulf coast of Florida, USA), 4. Surface sediment extracts (fraction < 63 µm) (from the southern North Sea, The Netherlands)	2.PCBs and PAHs 3.PAHs, PCBs, insecticides, petroleum products		16-24 hours		2. No genotoxicity could be observed in sediments although they were toxic in the Microtox and amphipod tests, 3. No genotoxins were found in any complex mixtures unless PAHs were present

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Genotoxicity assay: *SOS-chromotest* (Quillardet et al. 1982)	A reporter gene assay (encoding the enzyme ß-galactosidase) coupled to SOS repair system response: Detects primary DNA damage	1. Côté et al. 1998, 2. HydroQual Laboratories, Canada, 2000.	Intestinal Bacterium *Escherichia coli (E.coli)*	1. Freshwater sediments (from Lake Erie, Lake Ontario, and St.Lawrence river, Canada) 2. Fresh water sediments (in Viskan, Sweden)		Polar extracts of sediments or sediment pore waters may contain substances (e.g. divalent cations) that interfere with enzyme function of assay which makes test results in these matrices unreliable (Chen and White 2004)	2 hours	The genotoxic effect is expressed as the SOS induction potency (SOSIP), induction factor per nmol per assay (Quillardet el al 1982).	Test can be completed within 24 hours.
Genotoxicity assay: *Ames/microsome bacterial assay or Bacterial Reverse Mutation test* (OECD no 471 Guideline, 1997)	Mutagenicity (point mutations and frameshift mutations)	Marvin et al. (2000)	*Salmonella typhimurium* strains YG1025 with S9 and YG1024 without S9	Bottom sediment and suspended sediment extracts (from Hamilton Harbour, Western Lake Ontario, Canada)	5-7 ring PAHs (benzo[a]pyrene, indeno[cd]pyrene and dibenz[a,h]anthracene and more)		2 days	Results are usually expressed as mutagenic potency, the slope of the initial linear portion of the dose-response curve. It is measured in number of Salmonella revertants/ equivalent gram dw sediment (Chen and White 2004)	Bioassay data obtained using *S. typhimurium* strain YG1025+S9 showed PAHs were a significant source of genotoxic contamination.
ISO 13829 (2002): *Determination of the genotoxicity of water and waste water using the umu-test.*	Induction of the SOS-repair system	1. Brinkman and Eisentraeger (2008) 2. Klamer et al. (2005), 3. Vondrácek et al. 2001	*Salmonella choleraesius subsp. Chol*. (strain TA1535/pSK1002)	1.Waste water, soil extracts, water extracts 2. Surface sediments (fraction < 63 µm) (from the southern North Sea, The Netherlands), 3. Sediment extracts (from Morava river and tributaries, Czech Republic)	3. PAHs	2.The umu-C test has been shown to be less sensitive to sediment extracts than the Mutatox test.	2 hours	According to the standard (ISO 13829), a sample is supposed to be genotoxic if this induction rate exceeds 1.5. To avoid false positive interpretation of the results caused by cytotoxic effects, the growth factor must exceed 0.5.	1. A miniaturized and automated bioassay in a microplate, robotized format was developed that can analyze large amounts of environmental samples, a cost- and time-saving test.
Genotoxicity assay	Anaphase aberration	Kocan et al. (1985)	*Fish cell lines*: RTG-2, rainbow trout gonad cells, BF-2, bluegill fin from sunfish	1: Marine sediment (organic extracts)	PAHs, PCBs, heavy metals, CHCs		48 hours		

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Genotoxicity assay: *Alkaline Comet assay*	Detects DNA strand breakes, crosslinks and alkali labile sites	1.Kammann et al. (2000)	1. Leukocytes from Carp, (*Cyprinus carpio)*	1,2: Sediments (organic sediment extracts from the North Sea)	1. PAHs	Total organic carbon (TOC) and different compositions of toxicants affected the results	24 hours		Enzymatic activation by the addition of fish liver enzyme to the assay was required to reveal genotoxicity of Benzo(a)pyrene, other PAHs and genotoxic substances.
Genotoxicity assay: *Comet assay* or *Single-cell gel electrophoresis* (SCGE) *assay*	Detects DNA strand breakes, crosslinks and alkali labile sites	1. Kammann et al. (2001)	EPC, *Epithelioma papulosum cyprini,* permanent cell line form skin tumour of carp *(cyprinus carpio L.)*	1: Sediments (organic sediment extracts from the North Sea)	1. PAHs and other genotoxic substances		24 hours		Out of 10 marine sediment samples, 9 showed a dose-dependent genotoxic effect. The EC_{50} of sediment extracts ranged from 7 to 307 mg sediment dry weight/ml assay volume
Genotoxicity assay: *Unscheduled DNA synthesis (UDS)*	Level of chemically -induced DNA repair	Ali et al. (1993)	Dorsal muscle cell line from BB, Brown bullhead, *Ictalurus nebulosus*	1: Sediments (from the Great Lakes, Canada)	Organochlorinated compounds (OCCs), and PAHs	Significant synergistic interactions between the two groups of chemicals from each of the three sites were observed resulting in the induction of higher levels of UDS	18 hours		
Genotoxicity assay: *Alkaline precipitation assay*	Detects DNA strand breakes, crosslinks and alkali labile sites	1.Gagné et al. (1996), 2.Gagné and Blaise (1995)	1,2: Primary rainbow trout hepatocytes (PRTH)	1, Marine sediments (organic extracts from Pointe aux Meules Harbour, Magdalen Islands, Canada) 2. Marine sediments (organic sediment extracts)	1,2: Organochlorine pesticides, PCBs and PAHs		24 hours		
Genotoxicity assay: *In situ nick translation assay*	Level of chemically -induced DNA repair (detects single-stranded breaks or nicks in the DNA)	1. Gagné and Blaise (1995)	Primary rainbow trout hepatocytes (PRTH)	1: Marine sediments (organic sediment extracts)	Organochlorine pesticides, PCBs and PAHs		24 hours		9 of 14 samples showed a genotoxic response with one or both assays (APA or NTA)

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
EROD-activity assay	Induction of CYP1A1 (monooxygenase induction)	1. Mátlová et al. (1995) 2. Huuskonen et al. 2000, 3.La Ville et al. 2004, 4. Kinani et al. 2009.	1.Hepa-1, mouse hepatoma cell line, chicken embryo liver microsomes, 2,4: PLHC-1, Hepatocellular carcinoma of topminnow (*Poeciliopsis lucida*), 3: PLHC-1 and Primary rainbow trout hepatocytes (PRTH), 5. RTLV1 (Rainbow trout liver) cells	1: Sediment organic extracts (from river Dyje, Czech Republic), 2: Sediment extracts (from recipient rivers from oil shale industries in Estonia), 4: Sediments (organic extracts) from small rivers in France	1: PAHs and HAHs (Halogenated aromatic hydrocarbons), 4:PAH-like compounds	4:PAHs were major contributors (20-60%) to the total dioxine-like activities.	1,2: 24 hours, 4: 4 and 24 hours, 5: 72 hours	2. ED_{50} and TCDD-EQs, 3.Results were expressed as percent of EROD activity induced by the positive control (TCDD 1 nM).	4:EROD induction potency was higher after 4 hours than after 24 hours exposure indicating a major contribution by non-persistant compounds like PAHs.
P450 RGS (reporter gene system) assay (ASTM standard, 1997)	Induction of CYP1A1 (monooxygenase induction)	1. Andersson et al. 1999, 2. Koh et al. (2001)	Human hepatoma cells	1.Sediment (from County Sanitation District of Los Angeles County, CA, USA), 2. Sediment (organic extracts from Ulsan Bay, South Korea)	1. PCDDs, PCDFs, PCBs and (high molecular weight 4-6 ring)-PAHs, 2. (4-6-ring-)PAHs	P450 RGS bioassay is inhibited by TBT at concentration > 6.54 µg/g in sediment.	1: 6 and 16 hours	1.Results are expressed as B(a)PEQ (benzo(a)pyrene equivalents)/dry gr of sample, for PAHs, based on the RGS concentration–response curve of B[a]P, and as TEQ (TCDD equivalents) for CHCs.	1.The value of 60 µg B[a]PEQ/g has been indicated a threshold above which degradation of the benthic community in sediments occurr. 2. The maximum response for CHCs is after 16 hours of exposure, and that of PAHs is after 6 hours exposure (due to rapid metabolism of PAHs)
Production of vitellogenin-mRNA	Estrogenic activity	1. Pawlowski et al. 2003	Primary rainbow trout hepatocytes (PRTH)	1.Sewage treatment plant effluents and surface water (from the river Rhine, Germany)	Estrogens (natural and synthetic), xenoestrogens		96 hours	Values compared to reference estrogen E2 induction and expressed as estradiol equivalents (EEQs)	

Appendix 3: In Vitro Assays

Name of assay	Mechanism measured	Applications	Cell lines	Matrix	Chemicals (substrates) investigated	Confounding factors	Exposure time	Evaluation and validity criteria	Comments
Metallothionein induction		1.Gagné et al. (1996)	Primary rainbow trout hepatocytes (PRTH)	Marine sediments (organic extracts from Pointe aux Meules Harbour, Canada)	Heavy metals, organometallic compounds		24 hours		Induction of MT was observed in 40 % of sediment extracts.
Porphyrin content	Induction of hepatic porphyria; accumulation of porphyrins in the cells	1.Huuskkonen et al. (2000)	PLHC-1, Hepatocellular carcinoma of topminnow (*Poeciliopsis lucida*)	1: Sediments extracts(from recipient rivers from oil shale industries in Estonia)	PBCs, organochlorinated compounds (OCCs)		24 hours	ED_{50} (effective dilution), TCDD-EQs	

Appendix 4: Field Biomarkers

Name of test	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Special in /sensitivity to substances	Acute/ chronic test	Evaluation criteria	Validity criteria	Other environmental parameters	Results /comments
Missbildadae embryon av Monoporeia affinis, Swedish Environmental Protection Agency (SEPA).	Guidance document for environmental screening, version 1:3, 2005.	Amphipods	*Monoporeia affinis, Pontoporeia femorata*	Frequency of deformed eggs and embryos	Whole sediment (in Baltic Sea)	Metals and organic environmental pollutants	both (sublethal)			oxygen concentration, sulphide concentration, pH, TOC, temperature, salinity in sediment.	
Hälsotillstånd hos kustfisk-biologiska effekter på subcellulär och cellulär nivå,Swedish Environmental Protection Agency (SEPA).	Guidance document for environmental screening, version 1:1, 2006.	Fish	*Zoarces viviparus*, Viviparous Eelpout*, Perca fluviatilis*, perch	GSI (gonadsomatic index), LSI (liver somatic index), white blood cells, histology, carbohydrate metabolism, EROD-activity, Protein conc., Glutathion reductase activity, Metallothionein induction and DNA adducts in the liver, Vitellogenin level in blood, Ion conc. in blood	Swedish coastal waters		sublethal			salinity, sight depth, nutrient supply, meteriological data	
Biologisk effektövervakning av organiska tennföreningar, Swedish Environmental Protection Agency (SEPA).	Guidance document for environmental screening, version 1:0, 2008.	Gastropoda	*Hydrobia ulvae* (Swedish east coast) and *Nassarius nitidus* (Swedish west coast)	Imposex (vas deference sequence index, VDSI and relative penis length index, RPLI) and tissue analysis of organotin compounds	Marine sea (Shallow waters): Point sources, natural harbours and reference areas.	Organotin compounds					
	Magnusson, M. (2008). *Pilotstudie inför biologisk effekt övervakning av organiska tennföreningar i Östersjön, 2007*	Gastropoda	*Hydrobia ulvae*	Imposex and tissue analysis of organotin compounds	Marine sea (Shallow waters): Point sources, natural harbours and reference areas.	Organotin compounds				Salinity	

Appendix 4: Field Biomarkers

Name of test	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Special in /sensitivity to substances	Acute/ chronic test	Evaluation criteria	Validity criteria	Other environmental parameters	Results /comments
	Magnusson et al. (2005). *Eventuellt samband mellan halten tennföreningar i vävnaden hos nätsnäckan Nassarius nitidus och halten tennföreningar i sedimentet.*	Gastropoda	*Nassarius nitidus*	Imposex (vas deference sequence index, VDSI) and tissue analysis of organotin compounds	Marine sea (Shallow waters): Point sources, natural harbours and reference areas.	Organotin compounds					
	Avfall Sverige AB (2009). *Fiskhälsa-Användning av biomarkörer hos exponerad regnbågs-öring och vildfångad öring i ett vattendrag med blandat dagvatten och lakvatten från deponi*	Fish	*Oncorhynchus mykiss,* Rainbow trout , *Salmo trutta*, Brown trout	Physical condition, liver somatic index (LSI), red blood count, blood glucose, Blood hemoglobin, EROD activity, PAH metabolites in gall, white blood cells, lactate, Ion conc.(K+, Na+, Ca2+, Cl-) in blood, Metallothionein ind.	Moving waters (from urban run-off water mixed with landfill site leachate water and from damm with landfill site leachate water)	Dioxines, PCBs, PAHs, Heavy metals	both			Rainfall, water flow, oxygen supply	
	Toxicon AB (2003a). *Hälsotillsstånd och fortplantning hos Tånglake i recipienten till Stora Enso Nymölla AB hösten 2002.*	Fish	*Zoarces viviparus,* Viviparous eelpout	Fatty acids and sterols in gall, EROD activity in liver, CYP1A conc. in liver, ASAT/ALAT activity in blood, Histopathology of liver, physical condition, LSI, LTI, reproduction, fecundity, GSI (gonadsomatic index), GSI2, ESI (embryosomatic index), macroscopic evaluation.	Marine sea (Paper mill recipient: including reference areas)	Dioxiner, dioxinlike PCBs, PAHs					

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Name of test	Reference	Taxon	Test-organisms /Species	Endpoint	Matrix	Special in /sensitivity to substances	Acute/ chronic test	Evaluation criteria	Validity criteria	Other environmental parameters	Results /comments
	Toxicon AB (2003b). *Hälsotillstånd och fortplantning hos Tånglake i recipienten till Södra Cell Mörrum hösten 2002.*	Fish	*Zoarces viviparus*, Viviparous eelpout	Fatty acids and sterols in gall, EROD activity in liver, CYP1A conc. in liver, ASAT/ALAT activity in blood, Histopathology of liver, physical condition, LSI, LTI, reproduction, fecundity, GSI (gonadsomatic index), GSI2, ESI (embryo-somatic index), macroscopic evaluation.	Marine sea: (Paper mill recipient: including reference areas)	Dioxiner, dioxinlike PCBs, PAHs					

Appendix 4: Field Biomarkers

Reference	Taxon	Test-organisms/ Species	Endpoint	Matrix	Special in/sensitivity to substances	Acute/ chronic test	Evaluation criteria	Validity criteria	Other environmental parameters	Results/comments
Lehtonen et al. (2006). *The BEEP project in the Baltic Sea: Overview of the results and ouline for a regional biological effects monitoring strategy*.	Fish, Bivalves	*Platichthys flesus*, European Flounder, *Zoarces viviparus*, Viviparous eelpout and Blue mussel	Lysosomal membrane stabilbity (LMS)	Marine sea	General toxicity to many substances, can detect acute oil spills	both				
Lehtonen et al.(2006)	Fish, Bivalves	*Platichthys flesus*, European Flounder, *Zoarces viviparus*, Viviparous eelpout and Blue mussel	Acetylcholin-esterase inhibition (AChE)	Marine Sea (Baltic Sea)	Neurotoxic chemicals such as organo-phosphates and carbamate pesticides, and General stress caused by various substances.	both			Local abiotic factors (temperature, salinity etc), seasonal differences in the occurance of substances (from river inputs and agricultural runnoff sources)	
Lehtonen et al. (2006)	Fish, Bivalves	*Platichthys flesus*, European Flounder, *Zoarces viviparus*, Viviparous eelpout and Blue mussel	Metallothionein Induction (MT)	Marine Sea (Baltic Sea)	Heavy metals (Cd, Zn, Cu, Hg), general stress	both			Seasonal variation of exposure situation and migration behaviour of species. Affect of salinity on MT levels. Reproductive stage of animals may effect MT levels.	
Celander and Förlin (1991). (Spectrophotometry, ELISA)	Fish		CYP1A concentration	Marine Sea (Baltic Sea)	Planar halogenated dioxines, biphenyls and PAHs.					Performed by Toxicon AB.
Lehtonen et al. (2006)	Fish	*Perca fluviatilis*, Perch, *Zoarces viviparus*, Viviparous eelpout and *Platichthys flesus*, flounder.	7-Ethoxyresorufin-O-deethylase (EROD) activity	Marine Sea (Baltic Sea)	Planar halogenated dioxines, biphenyls and PAHs.	both		Only one sex should be studied at a time or treat sexes separately, use female fish with gonad wt >1% of BW e.g..	Seasonal variation due to temperature, other abiotic factors and sexual development.	Difficult but not impossible if carefully standardised procedures are followed concerning sampling strategy, catching and handling, controlling the conditions of individuals, and taking into account the effect of environmental factors.

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Reference	Taxon	Test-organisms/ Species	Endpoint	Matrix	Special in/sensitivity to substances	Acute/ chronic test	Evaluation criteria	Validity criteria	Other environmental parameters	Results/comments
Lehtonen et al. (2006)			PAH metabolites in bile	Marine Sea (Baltic Sea)	PAHs (recent exposure)	both		When applying the Fixed Wavelength Flourescence (FF) method the results cannot be compared between different laboratories.		Interspecies and gender differences in bile PAH metabolite levels have to be taken into consideration when applying this biomarker.Can be used to indicate differences betw. polluted and reference areas and demonstrate pollution gradients.
Lehtonen et al.(2006)	Fish	*Zoarces viviparus*, Viviparous Eelpout*, Perca fluviatilis*, perch	DNA adducts (genotoxicity)	Marine Sea (Baltic Sea)	Primarily PAHs (recent and earlier exposure)					Most apropriate to apply this biomarker only in areas with expected and evidenced PAH contamination and to combine it with PAH metabolites and EROD activity measurements.
Lehtonen et al. (2006)	Fish, Bivalves	*Platichthys flesus*, European Flounder, *Zoarces viviparus*, Viviparous eelpout and Blue mussel	Micronuclei frequency (MN) (genotoxicity)	Marine Sea (Baltic Sea)	DNA-reactive and non-DNA-reactive (e.g.aneugens) mutagenic compounds	both				Well characterized, easily recognisable endpoint that reveals a time-intergrated exposure of marine organisms
Lehtonen et al. (2006)	Fish, bivalves	*Platichthys flesus*, European Flounder, Blue mussel	Neutral lipid accumulation (NL) (disturbed lipid metabolism)	Marine Sea (Baltic Sea)						Provides information about the effects of pollutants on pathologically-induced alterations of liver cell metabolism

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Reference	Taxon	Test-organisms/ Species	Endpoint	Matrix	Special in/sensitivity to substances	Acute/ chronic test	Evaluation criteria	Validity criteria	Other environmental parameters	Results/comments
Lehtonen et al.(2006)	Fish	*Platichthys flesus*, European Flounder	Macrophage activity (MA-AP), i.e.enzyme activity of acid phospatase (AP) in liver macrophage aggregates (MA) (immunosystem response)	Marine Sea (Baltic Sea)	Detect acute oil spills	both				Should only be used together with other biomarkers (such as LMS and AChE) to detect general toxicity
Lehtonen et al.(2006)	Fish, Bivalves	*Platichthys flesus*, European Flounder, *Zoarces viviparus*, Viviparous eelpout, *Perca fluviatilis*, Perch and Blue mussel	Catalase (CAT) and glutathion-S-transferase (GST) activity (oxidative stress /biotransformation of xenobiotics)	Marine Sea (Baltic Sea)						Seasonal and beween-gender differences in activity levels of enzymes in fish. Seasonal variability in blue mussels was small. No definite trends could be observed. Results in fish must be interpreted with caution.
Lehtonen et al. (2006)	Fish	*Zoarces viviparus*, Viviparous eelpout	Endocrine disruption (Reproductive disorders) such as: intersex, ovotestis in males, and degeneration of the oocytes in developing gonad (follicular atresia), reproductive success and impaired development of fry in females.	Marine Sea (Baltic Sea)	Endocrine disrupting chemicals (EDCs)	long term				Eelpout ideal species to monitor reproductive disorders at different stages of the reproductive cycle in the Baltic Sea. It´s more sensitive than other marine fish to develop ovotestis.
Lehtonen et al.(2006)			Liver histopathology (non-specific lesions, early non-neoplasic lesions, pre-neoplastic lesions, benign liver tumour and malignant liver tumours).	Marine Sea (Baltic Sea)	Detect oil spills and repeated harbour dredgings	long term				An integrative endpoint (used together with other biomarkers) of contaminant effects on the molecular, biochemical and physiological levels in the liver.