TECHNICAL REPORT ON AQUATIC EFFECT–BASED MONITORING TOOLS

Annex

Environment

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ANNEX

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Case Study 1
Title
Laxsjön – investigating sediment contamination, using chemical and *in vitro* bioassay approach
Bibliography and link to objectives/national use
Illustrating the following use described in this report:
Primarily prioritisation of areas for further studies (to support conclusions made in the analysis of pressures and impacts). Also supportive in the selection of future parameters to monitor as well as identification of control measures
Reporting Institution :
County Administrative Board Västra Götaland, Sweden
Web-Link
http://www.lansstyrelsen.se/vastragotaland/Sv/publikationer/2012/Pages/2012-47.aspx
Main sources for further information; literature:
Gunnarsson F, Wernersson A-S. 2012. Sediment i Laxsjön. Halter av miljöfarliga ämnen. Länsstyrelsen i Västra Götaland. Report 2012:47 (In Swedish)
Background, purpose, , approach, resultsand conclusions
Background The lake Laxsjön was suspected to be exposed to emissions from primarily point sources, including both on-going and historic activities (such as a sewage treatment plant, paper mill, previous saw mill and area for timber treatment) but also diffuse emissions from an urbanised area and a channel that is frequently used by pleasure craft boats. The lake is of interest to sport fishing and other recreational activities. Some fish samples had previously been taken but exclusively analysed metals and dioxins and was performed on fish caught in the lower (presumably less exposed) part of the lake, not showing elevated concentrations. Also some previous sediment investigations close to the historic saw mill activities (LX4 below) had been performed. The results indicated that the PAHs analysed were present both at exposed and reference locations at more or less equal levels, suggesting that there is either an impact from local sources on the whole lake,or the results reflect the PAH situation in a larger geographical perspective[1].
Purpose The Laxsjön water body was identified to be at risk of not achieving good status in the analysis of pressures and impacts assessment, involving a potentially large number of substances. The main purpose was therefore to make an initial investigation of whether hazardous substances were elevated in the upper sediments, to confirm or reject the conclusions made in the analysis of pressures and impacts assessment.If confirmed, the investigation should also provide information about what parts of the lake were most impacted and from what type of substances. Such information would also act asdecision support to identify potential needs for remediation and/or other control measures. **Why effect based tools were included (why not an entirely chemical approach?)** To cover a large number of substances within a limited budget, the chemical analyses were primarily focused on relevant priority substances that can be suspected to be found in sediment.This approach was combined with a battery of *in vitro* bioassays, to also cover substances that could be considered to be specific pollutants in this water body, but perhaps not individually identified and for which assessment criteria are not yet established. Furthermore, to investigate whether dioxins and dioxin-like compounds could be screened using *in vitro* bioassays instead of chemical analyses, both were performed in parallel on the same samples. Such an approach could save costs in future investigations of this area.

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[1]However, only few limnic sediment data on PAHs to be used for comparison are available from this geographical region, and it was therefore not clear whether the PAHs found were elevated compared to the ambient background situation.

Approach
The substances primarily suspected to be present at elevated concentrations are known to accumulate in sediments and/or biota. Some are probably found in sediment or lower trophic level biotarather than fish (or water), because of their hydrophobicity and high metabolisation potential in fish. Because of limited experience in biota monitoring of other species than fish in limnic environments, and due to the potential impact from historic[2] activities (sediments could act as sources), these initial studies were performed on surface sediments. Samples from five locations in the lake were taken. The chemical analyses included DEHP and other phthalates, 16 PAH, octylphenols, organic tin compounds, metals, PCDD/Fs and dioxin-like PCBs and a battery of in vitro assays based on different types of receptor binding (DR-, PAH-, ERα-, anti-AR- and PPARγ-CALUX). In this way, dioxin-like, benzo(a)pyrene-like, estrogenic, anti-androgenic and peroxisome proliferator-activated receptor (PPAR) gamma binding compounds[3] present in the surface sediment were covered.

Results and conclusions

Primarily two of the locations were found to be contaminated, based on both chemical and *in vitro* analyses. ER CALUX primarily responded at one of the locations (LX4) (first graph below), which is not surprising due to the proximity to a sewage treatment plant. Nonylphenol and octylphenol were analysed chemically but not detected, although unfortunately the detection limits were not sufficiently low to allow any conclusions about status to be made. PPARγ-CALUX results suggest that primarily LX2 and LX4 are the most impacted locations (second graph below).

Some of the priority substances found in elevated levels, such as the very high concentrations of DEHP in LX2, could be responsible for the peak observed in the *in vitro* bioassay to detect anti-androgenic compounds (third graph below).However, it is also likely that the signals observed in vitro are due to several other compounds that were not included or detected in the chemical analysis.

The DR CALUX assay more or less confirmed results from chemical analyses, suggesting that the major dioxin-like contamination was to be found at LX2. This suggests a potential to reduce the costs in future follow-up studies of dioxin and dioxin like PCB load in the lake (fourth graph below[4]). A proposed Swedish assessment criteria for dioxins and dioxin-like PCBs in sediment based on WHO-TEQ is 0.9 ng TEQfish/kg (Naturvårdsverket 2008). This value is clearly exceeded at all sites, but sediment exceedences to some extent are anticipated to occur at many locations, suggesting dioxins and dioxin-like compounds to be considered ubiquitous in sediment. To check status biota data should rather be used. However, the exceedance at LX2 is more than 300 timesif based on *in vitro* results (and almost 200 times based on chemical analysis), suggesting that the sediment quality is not good.

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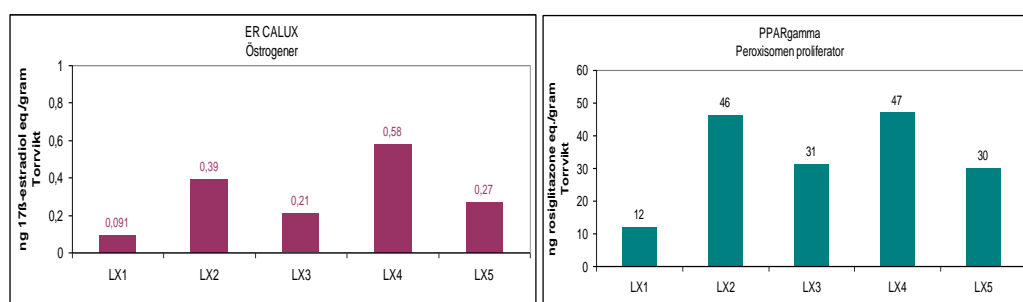
[2]It was e.g. known that phthalates had previously been used in large amounts in the paper mill processes

[3] Binding to the gamma receptor (found in adipose tissue) involved in peroxisome proliferation is analysed, thus responding to compounds that influence glucose and lipid metabolism. Examples of compounds that activate PPARy are TBT, phthalates and perfluorinated compounds

[4]Light blue bars are based on chemical analysis, whereas green bars are based on *in vitro* assay. The *in vitro* assays possibly even further indicate that LX2 is the most contaminated site from this type of compounds.

5
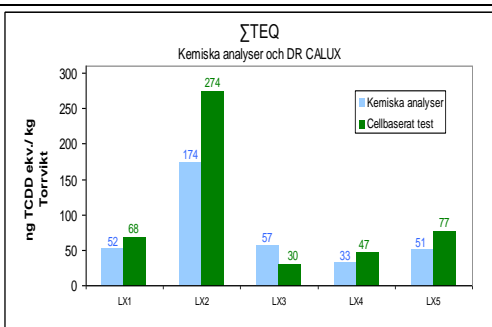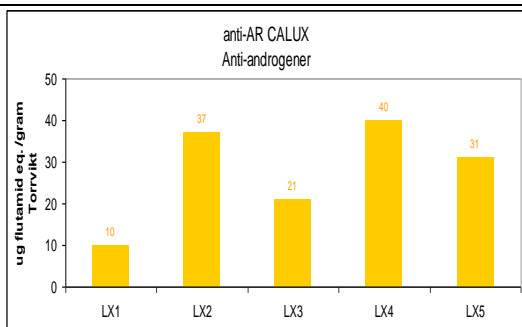
Interestingly, the PAH CALUX assay primarily responded to the two most exposed locations (LX2 and LX4). The chemical analysis of individual priority PAHson the other hand would rather confirm previous conclusions (see graphs below), suggesting that PAHs in general has an impact on the whole lake at about equal levels. If analysing each individual PAH though, there is some variability between sites[5], although at least LX2 is not suggested to be of particular concern by any individual compound.Taking *in vitro* data into account, it could instead be concluded that both LX2 and LX4 are the main areas of concern and that future studies should focus on those.
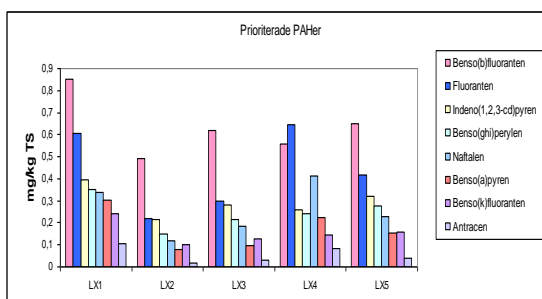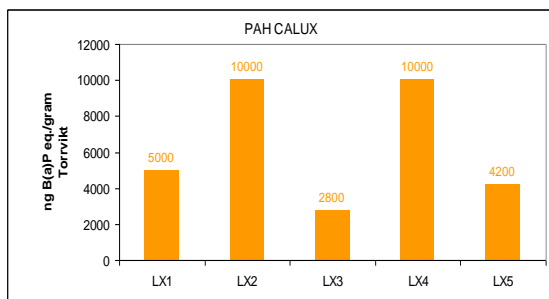
The responses observed in the *in vitro* assays were, except for DR CALUX, not interpreted in absolute terms but rather on a relative scale between the different sites of the same lake, to identify the most contaminated locations. If additional data becomes available on *in vitro* results from sediment testing using this battery, the opportunities to also compare the results with the situation in other areas would increase. Nevertheless, if comparing PAH CALUX results (expressed as BaP equivalents) with the QSbent for BaP, the results indicate a clear exceedence at LX2 and LX4, a slight exceedence at LX1 and LX5 and below at LX3. The PAH CALUX results (using BaP equivalents compared to BaP criteria) indicate that the cumulative response to PAHs other than BaP is an issue that would have been overlooked with a purely chemical approach. ER, PPAR and anti-AR CALUX are hard to evaluate at the moment because even if the results are expressed on a chemical equivalent basis, /sediment/ assessment criteria for these reference compounds are not available.

To conclude, *in vitro* assays were found to be useful as a complement to the chemical analyses, to make conclusions about which parts of the lake are the most contaminated and what type of compounds (and therefore sources), other than those analysed chemically, thatare probably of major concern. It was also concluded that the DR CALUX assay is an acceptable, cost effective alternative (about 1/5th of the price compared to the chemical approach) to track sources of dioxins and dioxin-like stable compounds in follow up studies of the same area. The PAH CALUX data indicate that PAHs other than those that are regularly monitored are of concern.

For further information about the conclusions related to chemical analyses, please refer to the full

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[5]If compared to effect based QSbent for fluoranthene, concentrations are elevated at all locations, but more so at LX1 and LX4 than at the other locations.If compared to marine sediment data, the PAH concentrations were to be considered moderate to very high, depending on the substance. Benzo(a)pyrene was primarily found at LX1, and at higher concentration than was so far reported to a national screening database, but still below a calculated QSbent using local TOC concentrations. Taken together, the chemical analysis of PAHs suggest that primarily fluoranthene is of a concern, but at all locations and primarily at LX1 and LX4 but not particularly at LX2.

report.

Costs involved: Approximately half of the budget of this project was related to the sampling of sediments, including one day spent on finding the most suitable locations by using side scan sonar mapping. Total costs for the *in vitro* battery analysed on the five samples were about 2500 Euro; total costs of project about 25 000 Euro.

Outlook - Next steps
Further studies are on-going.

Case study 2
Title
Deployment of a multi-biomarker approach to identify the origin of wild fish abnormalities reported in a French stream receiving urban and industrial effluents.
Bibliography and link to objectives/national use
Illustrating the following use described in this report: Primarily illustrating the use of a multibiomarker approach to provide robust data, useful for environmental managers to understand mechanisms of environmental degradation in a crisis context.Also illustrates how to identifysuitable measures, and should be a trigger for studies of population level effects
Reporting Institution : National Institute for Industrial Environment and Risks (INERIS), France
Web-Link http://www.ncbi.nlm.nih.gov/pubmed/21722962
Main sources for further information; literature: Sanchez, W. (2010). Rivière Dore: resultants de l'étude suite aux prélèvements realizes en 2009 et 2010. DRC-10-109441-13986A, 37p. Sanchez, W., W. Sremski, B. Piccini, O. Palluel, E. Maillot-Maréchal, S. Betoulle, A.Jaffal, S. l. Aït-Aïssa, F. Brion, E. Thybaud, N. Hinfray and J.-M. Porcher (2011). Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. Environment International**37**(8): 1342-1348.
Background, purpose, , approach, results and conclusions
Background In 2008, anglers reported the presence of gudgeons (*Gobio gobio*) exhibiting abdominal swelling associated to gonad hypertrophy in a river receiving effluents from waste water treatment plants and from a pharmaceutical industry involved in steroid production. According to the precautionary principle, local authorities have forbidden consumption of wild fish from this river.
Purpose An investigative study was initiated to identify the origin of the observed fish abnormalities. **Why effect based tools were included (why not an entirely chemical approach?)** The study was initiated by visible effects in wild fish inhabiting a good status water body. First investigations based on chemical analysis did not reveal presence of chemical contaminants. Due to the adverse effects (abdominal swelling and gonadal hypertrophy) th investigations included effect based tools (biomarker battery) with focus on endocrine disruption parameters. **Approach** A set of biomarkers including vitellogenin (VTG) as endocrine disruption end-point, biotransformation enzymes (7-ethoxyresorufin-O-deethylase (EROD), cytochrome P450 3A (CYP3A) and glutathione-S-transferase (GST) activities, together with acetylcholinesterase (AChE) and lysozyme activities respectively as neurotoxicity and immunotoxicity biomarkers was measured. To complete, histological analysis of gonads were performed for sex verification, identifying stage of development, documenting presence of intersex and other pathologies. Fish assemblage was also characterized to identify potential disturbance of fish populations complementarily to assessment of individual effects.

In this work, adult gudgeons out of reproductive period were electrofished on 3 sites located upstream and downstream from industrial effluent.

Conclusions:

Responses of biotransformation enzymes, neurotoxicity and endocrine disruption biomarkers revealed contamination of the investigated stream by a mixture of pollutants. Fish from sampled sites downstreams the industrial discharge exhibited also strong signs of endocrine disruption, including vitellogenin induction, intersex and a male-biased sex-ratio. The observed response profile appears as more complex with antagonist signals such as VTG induction and male-biased sex ratio.

No cause–effect relationship can be firmly established between fish exposure to Active Pharmaceutical Ingredients (APIs) and individual and population disturbances. However, pharmaceuticals discharged in the investigated stream could be involved in adverse effects observed on fish and their assemblage, and a set of evidence supports the hypothesis that these compounds inducedthe observed adverse effects and indicated that resident fish populations from both downstream sites could disappear.

Costs involved :

65 000 EUR including travel fees, consumables and permanent personal fees for 4 sites investigated 2-fold

Outlook - Next steps

The study was completed by an EDA approach to identify i) chemicals involved in water contamination and supporting observed adverse effects and ii) pollution source. A set of APIs discharged by a pharmaceutical manufacturer was identified. Local authorities requested specific measures to reduce environmental impact and establishment of an effect-based monitoring programme (the first in France). Now, a complementary study is performed to assess resilience of the water body.

Case study 3

Title

Endocrine Disruptors in the Irish Aquatic Environment

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

Primarily illustrating support to prioritizing areas for further monitoring and assessment of the potential risk from endocrine disrupting compounds, EDCs, to Irish freshwaters, their ecosystems and associated drinking water resources

Reporting Institution:

Irish Environmental Protection Agency;prepared by Department of Biological Sciences, Cork Institute of Technology, IRL, Aquaculture Development Centre, University of Cork, IRL and Centre for Ecology and Hydrology, Wallingford, UK.

Web-Link

http://www.epa.ie/downloads/pubs/research/water/name,12197,en.html

Main sources for further information; literature:

Endocrine Disruptors In the Irish Aquatic Environment – final report (2000-MS-2-M2) Tarrant et al, 2005.

Background, purpose, , approach, results and conclusions

Background

Before this study, there were no published studies documenting the effects of WWTP effluents on exposed fish populations in Irish rivers, or on any associated risk to drinking water supplies and, hence, the Irish population.

Purpose

The study was designed to answer the following questions

    i.   Do Irish rivers contain estrogenic compounds?

    ii.   If so, at what concentrations?

    iii.   Are these levels likely to pose a threat to aquatic ecosystems, particularly wild fish?

    iv.   Is there a risk to drinking water supplies?

Why effect based tools were included (why not an entirely chemical approach?)

By using an integrated design, including several different tools including sensitive biomarkers that directly measure endocrine disruption in exposed fish, a first assessment could be made of the risk posed by environmental estrogens to the Irish freshwater environment.

Approach

A series of WWTPs was surveyed and the level of estrogenic activity was quantified in the final effluents and, in some cases, the receiving waters. In addition, cages of male rainbow trout were placed at a series of sites in the River Lee, upstream and downstream of potential estrogenic 'hotspots' and at the intake to the Lee Road Water Treatment Works (WTW), which supplies drinking water to Cork City. After a 3-week exposure period the fish were examined for signs of endocrine disruption. Finally, a survey of wild male brown trout populations in the Rivers Liffey, Lee and Bandon was conducted and the fish were examined for signs of exposure to environmental estrogens.

The water systems studied were selected so that both rural and urban catchments were represented; putative significant EDC inputs were domestic/municipal and industrial waste from

WWTP discharges, septic tank effluents from isolated dwellings and run-off from agricultural land (in particular, animal husbandry activities). Brown trout were chosen as the indicator species in the wild fish survey, as salmonid species are of great ecological importance in the Irish context.

Results andconclusions:

Although the results obtained from this study are specific to the aquatic ecosystems investigated and to the wild brown trout populations of those systems, the study provides a scientific basis from which to assess the likely levels of environmental estrogens in other Irish freshwater systems and, to some extent, the probable effects on exposed wildlife and fish populations. Thus, with the caveat that estrogenic 'hotspots' are more likely in densely populated urban and/or industrialised areas (as demonstrated by the Liffey findings), it may cautiously be concluded that:

    i. Irish WWTP effluents are estrogenic, although levels compare favourably with other European countries and the USA.

    ii. Irish rivers and lakes do not appear to be at general risk from significant concentrations of environmental estrogens.

    iii. In general, wild fish populations do not appear to be at risk from estrogenic chemicals and

    iv. Judging from the limited number of sites examined in this study, Irish drinking water resources do not appear to be at significant risk from estrogenic chemicals.

The apparently low level of risk to Irish freshwater systems from environmental estrogens may, in part, be attributed to the coastal locations of most of the heavy centres of population and industry in Ireland. Thus, Irish rivers are not generally receiving high domestic and industrial effluent loads. This is in contrast to the situation in other countries, for example the UK, and is probably a significant factor in explaining the general absence of endocrine disruption in fish populations in Irish freshwaters.

Costs involved

€292,000

Outlook – Next steps

It is recommended that the following work should be performed in the future:

    i. Further characterisation of the Liffey is of importance, especially in the region downstream of the Osberstown WWTP and at the point of intake to Leixlip WTW. Investigations in other waterbodies, in areas of similar sensitivity, should also be considered.

    ii. A review of company IPC licences should be conducted with the aim of reducing the emissions of known EDCs to Irish fresh and marine waters. The YES (or equivalent bioassay) could be usefully employed to quantify and, subsequently, monitor estrogen levels in relevant industrial effluents.

    iii. Levels of EDCs in the marine environment, particularly estuarine waters at the sites of major agglomerations and environmentally sensitive areas of Irish coastal waters, should be Investigated.

    iv. Considering the hydrophobic nature of many EDCs, both water and sediment sampling should be performed to determine EDC levels in the estuarine/marine environment as a whole.

    v. By applying geographic information system (GIS) modelling to the whole of Ireland, potential pollution 'hotspots' for steroids, pharmaceuticals and other EDCs may be identified and, subsequently, characterised.

    vi. Agriculture as a source of estrogens (e.g. animal steroids, pharmaceuticals, pesticides and herbicides) in Irish freshwaters should be investigated.

Case Study 4

Title
Swedish national monitoring programme of fish health

Bibliography and link to objectives/national use

Illustrating the following use described in this report: Current national useto provide early warning of long term changes (surveillance monitoring on national scale including chemical, ecotoxicological and population relevant variables).Also used to fulfil OSPAR and HELCOM recommendations.

Reporting Institution :
Swedish Environmental Protection Agency and Swedish Agency for Marine and Water Management

Web-Link
Description of programme and biomarkers included: http://www.naturvardsverket.se/upload/02_tillstandet_i_miljon/Miljoovervakning/undersokn_typ/hav/fiskhalso.pdf

Main sources for further information; literature:
About the programme and results: Naturvårdsverket 2006. Undersökningstyp : Hälsotillstånd hos kustfisk - biologiska effekter på subcellulär och cellulär nivå (In Swedish) Sandström O, Larsson Å, Andersson J, Appelberg M, Bignert A, Ek H, Förlin L and Olsson M (2005).Three decades of Swedish experience demonstrates the need for integrated long-term monitoring of fish in marine coastal areas. *Water Quality Research Journal of Canada.* 40 (3), 233-250. Ronisz D, Lindesjöö E, Larsson Å, Bignert A and Förlin L. (2005). Thirteen years of monitoring selected biomarkers in Eelpout (Zoarches viviparus) at reference site in the Fjällbacka archipelago on the Swedish west coast. *Aquatic Ecosystem Health & Management.* 8 (2), 175-184. Hansson T, Lindesjöö E, Förlin L, Balk L, Bignert, A and Larsson Å. (2006) Long-term monitoring of the health status of female perch (Perca fluviatilis) in the Baltic Sea shows decreased gonad weight and increased hepatic EROD activity. Aquatic Toxicology. 79 (4), 341-355. Results are reported annually in « Havet » publications (available from the home page of the Swedish Environmental Protection Agency). Annual reports of the results observed at each area respectively: :http://www.slu.se/sv/fakulteter/akvatiska-resurser/datainsamling/provfiske-vid   -kusten/provfiske-resultatblad/

Background, purpose, , approach, results and conclusions

Background Starting in the 80s, an integrated marine national monitoring programme of fish health, population effects and tissue concentrations of stable and accumulable compounds was established along the Swedish coastline.
Purpose One purpose of this national programme was to establish an early warning system to detect long term changes in environmental quality, if occuring in coastal fish, analysed using chemical, biomarker battery and population level variables. It also provides data to establish background baseline levels, to be used for comparison in studies performed closer to local sources. **Why effect based tools were included (why not an entirely chemical approach?)** By this integrated design, the impact from a broad range of chemicals acting on a large geographical scale

and causing effects in fish can be covered.

Approach
The Swedish integrated fish monitoring programme includes a large number of biomarkers on two species of fish (eelpout and perch):

Reproduction and endocrine disruption:
- Gonadosomatic index (GSI) in perch and Embryosomatiskt index (ESI) in eelpout
- Fecundity, sex ratio and health of embryos in eelpout
- Vitellogenin in blood plasma (males of perch and eelpout)

Morphometric measures and Pathology:
- Age, length, body weigth (total and somatic), liver and gonad size
- External visible damages (e.g deformations, wounds)
- Histology (liver and spleen)

Condition and metabolism:
- Condition factor
- Liver size
- Glucose and lactate I blood

Red blood cell status and ion balance:
- Hematocrit and hemoglobin
- Number of immature red blood cells
- Plasma ion concentrations of chloride, sodium, potassium and calcium

Immune defence:
- Lymphocytes. granulocytes and thrombocytes in blood (number)
- Total white blood cells (WBC) (number)
- Macrophage centra (number) in spleen

Liver function:
- Liver morphology/histology (structural changes, e.g. vacuols, nekrosis, parasites)
- Liver somatic index (LSI)
- Ethoxyresorufin-O-deetylase (EROD) activity
- Glutathion reductase (GR) activity
- Catalase activity
- Gluthation transferase (GST) activity
- Genotoxic response (frequency of DNA adducts)
- Metal exposure (conc of Metallothionein, MT)

Four reference locations (exposed to no known local sources) are included, one on the West Coast and three on the East Coast (Baltic Sea). Metals and organic substances as well as population relevant parameters are monitored on the same locations in an integrated manner (sampled at the same time).

Results and conclusions

The results from this national programmehave so far primarily been interpreted on a trend basis. Significant time trends since the mid 90s for up to ten health variables (such as 20-30% gonad reductions, five fold increase in EROD induction, and increased number of white blood cells) in coastal perch have been observed, especially at one of the stations (Kvädöfjärden, in the southwest Baltic).Similar trends have also been observed in both perch and eelpout at the other reference stations(Larsson et al 2011)[6].

Integrated assessment criteria to facilitate evaluation of environmental quality are under development. In this suggested approach, the variables are divided into six groups according to physiological function. Each variable is given a score that reflects its relative importance for the function. If the total score within a

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[6]Larsson Å, Förlin L, Hanson N, Parkkonen J, Bignert A, Nyberg E, Danielsson S, Andersson J, Appelberg M. 2011. Kustfisk – alltmer påverkad av miljögifter. Havet report 2011.

function reaches the limit for impact on function, the function is judged to be affected. If the function scores of the affected functions reach three, or more, fish physiology is considered to be affected. The index will probably be based on a scoring system for individual biomarkers, categorized into different groups and ecological relevance(see below):

Table. Scoring system for the evaluation of different fish biomarkers, based on a first draft proposal for the biomarkers included in the Swedish marine monitoring programme (from Larsson et al 2010)[7].

Physiological function	Marker score	Limit for impact on function	Function score
Reproduction			
Reduced gonad size	1		
Sexual maturity inhibited or delayed	1		
Dead or malformed embryos (eelpout)	2	2	3
Male vitellogenin induction	2		
Female vitellogenin reduction	1		
Condition and metabolism			
Reduced condition factor	2		
Increased condition factor	1		
Liver size change	1	3	2
Glucose concentration change	1		
Lactate concentration change	1		
Liver function			
Tissue alterations (cell death e.g.)	3		
Change in liver size	2		
Structural changes (vacuoles, parasites)	1		
DNA adducts	2	3	1
EROD	1		
Glutation reductase	1		
Metallothionein (MT)	1		
Immune response			
White blood cells	2		
Macrofague centers (increase)	2		
Lymphocytes	1	3	1
Trombocytes	1		
Granulocytes	1		
Red blood cells			
Hematocrit	2		
Hemoglobin	2	3	1
Red blood cells	2		
Immature red blood cells	1		
Ion regulation			
Potassium (K)	2	3	1
Calcium (Ca)	2		

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[7]Larsson Å, Hanson N, Förlin L, Parkkonen J. 2010.Fiskens hälsotilltstånd ger en tidig signal om miljöpåverkan.Presentation at Havsmiljöseminariet in Borgholm, April 2010.

Chloride (Cl) AND Sodium (Na)	3	
Chloride (Cl) OR Sodium (Na)	1	

The next step in developing the criteria is to determine when a specific biomarker should be considered as affected. To do this, it is necessary to establish what is "normal" for each of the biomarkers that are included. Within the Swedish National Monitoring Programme for Fish Health, data has been collected in reference sites for these variables for up to 25 years. The information that has been retrieved within this programme serves as a good base to establish background levels of the different biomarkers.

Costs involved:
The total costs for the fish health programme, including also sampling, sample preparation and reporting of data (including statistical evaluation), is about 100 000 Euro on an annual basis for four stations.

Outlook - Next steps
Besides developing assessment criteria a challenge is also to identify the reasons for the steadily increasing signal, possibly occurring on a broad geographical scale, but not excluding the possibilities that effects can be caused by a combination of substances from diffuse local-regional sources and other large scale changes, in order to be able to consider the implementation of suitable control measures. Follow up studies have recently been initiated, primarily regarding the station where the most significant changes have been observed and will be based on several different approaches, including both chemical and effect based tools as well as modelling and a more detailedanalysis of pressures upstreams. At the moment, additional sampling in Kvädöfjärden could give misleading results, because a major remediation project was recently started. However, stored material from previous sampling is available, for additional analyses.

Case study 5

Title

Evaluation of Aquatic Environmental Estrogens with Passive Sampling - EPSA

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

This case study demonstrates the combined use of passive sampling and biological and chemical analyses.

Reporting Institution:

Oekotoxzentrum Eawag/EPFL, Überlandstrasse 133, Postfach 611, 8600 Dübendorf, Switzerland.

Web-Link

http://www.nrp50.ch/fileadmin/user_upload/Dokumente/Publications/Final_Summaries/Final_summery_Giger_01.pdf

Main sources for further information; literature:

Vermeirssen ELM, Körner O, Schönenberger R, Suter MJ-F, Burkhardt-Holm P (2005) Characterization of environmental estrogens in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environmental Science & Technology 39:8191-8198.

Vermeirssen ELM, Eggen RIL, Escher BI, Suter MJ-F (2008) Estrogens in Swiss rivers and effluents - sampling matters. Chimia 62:389-394.

Vermeirssen ELM, Hollender J, Bramaz N, van der Voet J, Escher BI (2010) Linking toxicity in algal and bacterial assays with chemical analysis in passive samplers deployed in 21 treated sewage effluents. Environmental Toxicology & Chemistry 29:2575-2582.

Background, purpose, , approach, results and conclusions

Background

Effluents discharge many compounds to the aquatic environment, including endocrine disrupting compounds. Between 2003 and 2005, we applied passive sampling (POCIS, polar organic chemical integrative sampler) in river water and successfully compared uptake of environmental estrogens in passive samplers with repeated grab sampling and bioaccumulation in brown trout. Passive sampler extracts were analysed with a bioassay for estrogens (the yeast estrogen screen, YES) as well as chemical analysis. Results from biological and chemical analyses were found to be highly correlated.

Purpose

In the follow up project the idea was to apply the technique in both effluent and river water, to investigate if results from measurements made directly in the effluent match measurements made in the river, up- and downstream from the discharge. An additional aspect was to test the samples in bioassays with different endpoints and compare toxicity profiles across a large number of independent Swiss effluents. Biological measurements were compared with chemical analyses.

Why effect based tools were included (why not an entirely chemical approach?)

Compared to a chemical approach, effect based tools offer a better grip on the total toxic load for a specific

endpoint, for example, an algal assay can capture all herbicides (and their metabolites) that affect photosystem II. By combining chemical and biological measurements, we could discern which portion of the toxicity could be explained by chemically measured compounds and thus investigate to what extent chemical analyses cover the measured biological responses.

Approach

Twenty two waste water treatment plants (WWTPs) were selected where three POCIS were placed in the effluent. With one exception, the WWTPs received mainly domestic effluent. Triplicate samplers were also placed in the river bed, up- and downstream from the effluent discharges, although many of these samplers were lost due to 10-50 year high flood events at many locations. Five weeks after their deployment, passive samplers could be recovered from 21 WWTPs. Extracts from the passive samplers were analysed with a yeast based bioassay for estrogenic compounds (YES), a combined algal assay for photosystem II inhibitors (2 h endpoint, yield of photosystem II) and general toxicity (24 h endpoint, algal growth rate), and a bacterial bioluminescence assay for general toxicity.

Results and conclusions

Passive sampling combined with biological and chemical analyses allowed for a comprehensive analysis of the toxic contents of a large number of treated sewage effluent discharges. This conclusion can be illustrated by a summary of five key observations.

i. The toxicity profiles for estrogens and herbicides varied very significantly across sites but these toxicities were not correlated with each other. For example, estrogenicity varied 100-fold across the effluents, but the location showing the second highest estrogenicity value was also the one with the lowest toxicity on photosystem II.

ii. As was observed in earlier work, estrogenicity was mainly explained by the presence of steroidal estrogens (estrone, 17β-estradiol and 17α-ethinylestradiol). As inputs of these compounds to raw waste water are fairly constant across WWTPs, variation in estrogenicity of effluents is mainly a result of differences in treatment efficiencies of these compounds. Estrogenicity was lowest in WWTPs equipped with a sand filter. Sand filters are known to reduce estrogenicity but are probably also an indicator of an otherwise optimised, up-to-date and efficient WWTP design.

iii. Inhibition of photosystem II varied 160-fold across the sampled effluents. It remains to be established if this is a result of highly variable inputs to the WWTPs and/or differences in herbicide removal rates. Six photosystem II inhibitors were chemically measured and explained – on average – 65% of the biological effect. Only at two locations did chemical analysis cover less than 50% of the effect observed in the algal assay (i.e. 32 and 42%). This is an indication that there is no very significant presence of unknown photosystem II inhibitors in Swiss effluents.

iv. Results from the bacterial assay were much less variable, they differed 11-fold across the 21 WWTPs. There were two outliers, when these were removed the minimum and maximum values across 19 sites were within 5-fold from each other. The number of compounds that affect the bioluminescence endpoint is probably very large. Although individual compounds and specifically acting toxicants (e.g. estrogens or herbicides) can vary greatly across sites, the bioluminescence inhibition assay integrates thousands of compounds and thus a fairly even response across sites may not be surprising. The fact that a large number of compounds contribute to this endpoint is supported by the fact that chemical analysis of some 20 compounds was estimated to explain less than 1% of the biological response. It is interesting to note that the second highest toxicity was associated with the location receiving a higher load of industrial effluent. Furthermore, the highest toxicity was measured at a location where a fish kill occurred in the river, downstream from the effluent discharge, during the last week of passive sampling.

v. Although the chemical spill was unfortunate and unintended, the obtained results from the location highlight two positive aspects associated with the applied methods: 1) this toxicity peak was

identified by one of the bioassays used (i.e., with the bacterial bioassay but not by the algae or yeast based assays); 2) the toxicity that occurred over a period of a few hours was visible in the passive sample collected over 5 weeks. After the fish kill, water samples were collected and analysed by local environmental authorities. No candidate toxic compounds could be identified. Although it was not attempted, effect directed analysis of the POCIS sample from the toxic effluent could have been a better means to try and identify the compound that caused the fish kill.

Costs involved

Approximately €160,000

Outlook – Next steps

It is recommended that the following work should be performed in the future:

   i.   As it could not be evaluated if sampling in effluent matches river water sampling up- and downstream from effluent discharges, it remains to be established if sampling in two different matrices gives comparable results.

  ii.   Passive samplers do not sample all toxicants at the same rate. Uptake kinetics require further understanding, particularly for groups of toxicants with similar modes of action (e.g. photosystem II inhibitors).

 iii.   Although it has to be kept in mind that one cannot establish robust POCIS sampling rates for the mixtures that make up a bioassay result, effects of flow rate on the uptake process requires further understanding in order to normalise bioassay results for varying flow rates during passive sampler deployment.

Case study 6

Title

Contaminated sediments in the River Elbe basin-EDA

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

EDA performed to identify substances having a potential toxic impact (to be considered as specific pollutants), after using a large in vitro and in vivo bioassay battery

Reporting Institution :

Helmholtz Centre for Environmental Research UFZ

Web-Link

http://www.ufz.de/index.php?en=617

Main sources for further information; literature:

Heise et al 2007; Lübcke-von Varel et al 2011; Brack et al 1999; Brack et al 2008; Rotter et al 2011; Bandow et al 2009; von der Ohe et al 2012; Brack et al 2002; Brack&Schirmer 2003; Lubcke-von Varel et al 2012 (see List of references in the end of this annex)

Background, purpose, , approach, results and conclusions

Background

Several large industrial regions are drained by the River Elbe and its tributaries. Despite significant improvement of water quality within the last 20 years particularly sediments are known to be highly contaminated by historical burdens and present industrial production. Elbe sediments have been subjected to extensive monitoring and risk assessment [Heise et al 2007]. These activities focused on a set of contaminants well known to be found in the Elbe sediments at high concentrations including metals and the non-polar organic pollutants PCBs, HCB, HCH, DDX, PAHs and PCDD/Fs.

Purpose

To identify additional, so far less studied substances that could be of concern, based on information about toxic pressure

Why effect based tools were included (why not an entirely chemical approach?)

The toxic pressure had already been investigated by a large battery of bioassays, the same assays were used on fractions of the samples to reduce the number of potential substances to study further

Approach

Several EDA studies on sediment extracts from River Elbe and its tributaries downstream of the major sources of industrial contamination including Bitterfeld (Germany), Most and Pardubice (both Czech Republic) applying many different toxicological endpoints including toxicity to green algae, bacteria and daphnia, mutagenicity, dioxin-like activity, tumor promotion, estrogenicity and thyroid hormone disturbing potency were performed.

Results and conclusions

For most of the samples and toxicological endpoints, Effect Directed Analysis (EDA) could demonstrate that toxic effects were predominated by semi-polar and polar fractions, which do not

contain any of the compounds included in sediment risk assessment [Lübcke-von Varel et al 2011]. In Bitterfeld samples prometryn and n-phenyl-2-naphthylamine have been identified to cause major algal toxicity [Brack et al 1999] in sediment extracts. According to the concept of hazard confirmation [Brack et al 2008] prometryn could be confirmed as key toxicant for in situ biofilms using Pollution Induced Community Tolerance (PICT) [Rotter et al 2011]. Downstream of Most triclosan and some other polar toxicants predominated toxicity to algae when bioavailability was considered [Bandow et al 2009]. Triclosan has been confirmed as a major toxicant also in other parts of the Elbe basin using monitoring data provided by Saxony [von der Ohe et al 2012]. The significance of PCDD/Fs for dioxin-like activity downstream of Bitterfeld could be confirmed [Brack et al 2002] while several dinaphthofurans as well as a naphthalenyl benzothiophene were found to significantly contribute to dioxin-like activity [Brack & Schirmer 2003]. None of these compounds stemming from naphthol production were known as environmental pollutants so far. Mutagenicity was predominated by dinitropyrenes and 3-nitrobenzanthrones [Lubcke-von Varel et al 2012]. None of these incineration by-products is monitored in the River Elbe or other European river basins.

Costs involved: Costs are highly dependent on the depth of the study, the type of tests involved and on the compound to be identified (if known and possible to purchase or if structure elucidation and synthesis of standards is necessary).

Outlook - Next steps

The results suggests new compounds to be considered in monitoring and risk assessment of the River Elbe estuary, but possibly also in a broader context (screening for emerging substances).

Case study 7

Title

Monitoring concentrated surface water with in vivo bioassays in the Netherlands

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

Use on national level, status and trend monitoring programme

Reporting Institution :

National Institute for Public Health and the Environment and Centre of Water, Traffic and Human Environment (previously Centre of Water management) (Both the Netherlands)

Web-Link

http://www.rivm.nl/dsresource?objectid=rivmp:16209&type=org&disposition=inline

Main sources for further information; literature:

Durand A M, Rotteveel S, Collombon M T, Van der Grinten E, Maas J L, Verweij W, 2009. Toxicity measurements in concentrated water samples; evaluation and validation. RIVM report 607013010/2009; Centre of Water Management report 2009.003 (in English)

Struijs, J., van der Grinten E., Aldenberg T., 2010. Toxic pressure in the Dutch delta measured with bioassays. Trends over the years 2000-2009. RIVM report 607013013/2010 (in English)

Background, purpose, , approach, results and conclusions

Background

Since the early 1990s, water quality in the Netherlands has been regularly assessed by chemical and biological effects monitoring (RWS, 1999; Maas and Van den Heuvel-Greve, 2005). As a consequence of the improvement in water quality, toxicity tests like the Microtox® assay barely show any acute effects at the present level of contamination. However, also the risk of chronic effects need to be taken into account.Monitoring of environmental toxicity using chronic bioassays is very laborious though, and therefore expensive.

Purpose

The National Institute for Public Health and the Environment, together with the Centre of Water Management[8], developed a method for measuring toxic pressure (risk of effects) in a cost-effective way.

Why effect based tools were included (why not an entirely chemical approach?)

Primarily to cover large number of substances that are not included in the chemical analysis, but can contribute to the toxic pressure. The results were used both to identify locations with elevated risks of long term effects but also to monitor trends in toxic pressure.

Approach

The biological effects method consists of a concentration procedure, toxicity testing and (statistical) interpretation of results. First, the organic toxicants in the water sample are adsorbed to a synthetic resin, eluted with an organic solvent and concentrated into a water extract. Then, the toxicity of the extract is measured using a set of standardized acute tests. The results are interpreted in a single species approach or in a risk-based manner.

Preconcentration step

Physical-chemical techniques are used to isolate and concentrate many of the organic toxicants from the water sample. This concentration step is necessary,so that acute toxicity tests can be used to identify samples with elevated toxic pressure. The concentration step also separates toxicological stress

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[8] formerly called Institute for Inland Water Management and Waste Water Treatment

from other stress factors like minerals and nutrients.

The method aims at comprehensive adsorption to adsorbents with a high affinity for the biologically available fraction of toxic substances. A combination of synthetic resins (called XAD-4 and XAD-8) are therefore used as the adsorbent. Nevertheless, only the organic fraction of the contaminants is tested in this way, since metals do not adsorb to the XAD.

After adsorption, the substances are removed from the XAD using acetone, found to be the most suitable organic solvent. Before using the eluates for toxicity tests the acetone is removed and the substances are transferred to a water extract. The recovery of substances has been found to be about 80 % for narcotic substances like 3-chloro-nitrobenzene but is very low for e.g. organotin substances.

Bioassays

Because of the concentration step, the amount of material available is small in relation to the original sample. One factor in the choice of bioassay is therefore that it must be possible to measure the toxicity in very small volumes. The set of organisms was also tuned to obtain optimal ecological relevance, by including primary producers (algae), primary consumers (crustaceans) and decomposing organisms (bacteria). The tests are well described in protocols and mostly derived from international ISO-standard tests and endpoints include mortality, growth inhibition and photosynthetic activity. The toxicity tests vary in duration from a few hours to several days.

Data interpretation

The bioassay results are compared to developed criteria, either in a single species approach (criteria based on risk of long term effects) or in a risk-based manner (based on a species sensitivity distribution concept). Reproducibility of the tests is good and within acceptable limits, both in intra- and in inter-laboratory experiments. The reproducibility of the entire method is good and comparable to the reproducibility of the individual tests.Validating the entire method in the same way as is done for chemical methods is complex however. Other technical specifications, such as LOD, LOQ, linearity and precision are difficult to establish because the chemical composition of surface water is not known (can only be established for a single substance or a mixture of known substances). For that reason, no detection limit, linearity and 'bias' are specified. There was, however, a pragmatic solution chosen for LOQ-determination.

Results and conclusions

The concentration procedure (sample pretreatment) was evaluated thoroughly. The method appears to be suitable for many substances, although there are differences in recovery. More specifically:

• The method is especially suitable for hydrophobic substances with a narcotic mechanism of action. Recovery was typically between 88 and 100%.

• The effects of herbicides and organochloro-pesticides (insecticides) can be well demonstrated. Recovery is usually somewhat lower (60 to 75 %).

• Hydrophilic substances (like medicines) do not adsorb very well onto the applied resins (XAD 4/8). Perhaps other types of resins would lead to better results.

• Volatile substances will disappear largely during the concentration procedure.

• Metals are not extracted from the water phase.

• The extraction procedure can be applied well to surface water. Natural organic matter does not appear to influence extraction efficiency of toxic substances.

• The procedure is well described in protocols. Disturbing factors are well known and included in the protocols.

• Reproducibility of the method is good and within acceptable limits.

For more details, please refer to the specified report.

During the years 2000-2009, the effects of toxic substances in Dutch inland waters were measured. The results offer an excellent opportunity to evaluate the trend in toxic pressure in Dutch freshwater water bodies, without analysing all the individual substances involved. Trends became apparent when the results of five bioassays in several water bodies collected over ten years were combined (Van der Grinten et al. in prep).

Costs involved:

Although not all substances and types of effects (mode of actions) are covered in this way, the approach has been found to offer a cost effective alternative to an approach based on chemical analysis only, if trying to cover an almost unlimited list of substances that could potentially be present in the studied waters.

Outlook - Next steps

The approach has been suggested to be used for different purposes, such as identification of locations where toxic substances may have a negative impact and for that reason it was also proposed as an additional method within WFD-monitoring (Maas, 2005).

Case study 8

Title
Monitoring imposex on water body level

Bibliography and link to objectives/national use

Illustrating the following use described in this report:
To assessimpact from organic tin compounds, but also to confirm whether additional control measures are necessary

Reporting Institution:
County Administrative Board Västra Götaland, Sweden

Web-Link
Not yet published on web

Main sources for further information; literature:
Magnusson & Hilvarsson. 2012. Effekter av TBT I Västra Götalands län. En studie av förekomst av imposex. Marine Monitoring AB

Background, purpose, , approach, results and conclusions

Background

Imposex biomarkers are highly specific to organic tin compounds. Within the national monitoring programme, annual monitoring of imposex is performed along the Swedish west and east coasts. The trends are generally decreasing but this is occurring at a significantly slower rate in the Göteborg area than at most other investigated sites. The monitoring stations so far included were primarily located either within harbour areas or at reference locations, although some intermediate locations are also included. In 2010 the County Administrative Board sampled sediments from more than 40 pleasure craft harbours, and the results indicated that at one third of the investigated harbours the surface sediments contained higher concentrations of TBT than at layers located 10 cm deeper (Bengtsson & Cato, 2011)[9]. The ratio between TBT and its degradation products DBT and MBT was also frequently above 1, suggesting that, in spite of the ban, TBT is still being emitted to pleasure craft harbours at a higher rate than what is being degraded. Within a regional monitoring programme, also sediment along the coast had been investigated, observing variable sediment concentrations and TBT/(DBT+MBT) ratios.

The concentrations found in sediments generally suggest that chemical status is not good at any location, and at some locations severe impacts can be anticipated to occur due to highly elevated TBT concentrations.

Purpose

Although severe impacts can be expected inside several of the harbours, it was not clear whether significant effects are expected to occur also on water body levels in exposed areas. The purpose of the study was therefore to investigate the occurrence of imposex on water body level, primarily in the Göteborg and Stenungsund region.

Why effect based tools were included (why not an entirely chemical approach?)

Chemical water TBT analysis is not sufficiently sensitive to fulfil the requirements in the 2009/90/EG and water concentrations can be highly variable during the year. Chemical analysis of sediments had already been performed, suggesting that effects could occur, primarily within the harbours, but the assessment criteria used for comparison is only tentative.In order to investigate the situation on water body level and to take bioavailability issues into account, other types of follow up studies were

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[9]Bengtsson H, Cato I. 2011. TBT i småbåtshamnar i Västra Götalands län 2010 - en studie av belastning och trender. Länsstyrelsen Västra Götalands län, rapport nr 2011:30

considered appropriate. Imposex biomarkers are very specific and effects are irreversible. By studying these effects, an indication about the average status regarding tributyltin compounds can be obtained. By the monitoring of imposex, one is actually also directly monitoring the effects that the chemical analysis is trying to predict.

Approach

In 2011, the City of Göteborg and the County Administrative Board initiated a coordinated investigation of imposex in Nucella, sampled from inside several harbours and at "water body level" respectively. In total 11 water bodies were investigated.

Results and conclusions:

Imposex at least to some degree was observed at all locations, even at reference sites, but there was a high variability in the number of impacted females (15-83% on water body level and 70-100% inside harbours). On water body level and if compared to OSPAR assessment criteria for this species, good status can be considered to be found at four monitored water bodies, whereas moderate status is suggested for the other seven water bodies.

The high TBT concentrations found in sediment do not always seem to cause effects and vice versa. This is most likely related to factors other than the concentration only, such as important factors that influence the bioavailability of TBT (organic carbon content, oxygenation levels and particle size etc). Therefore, imposex data combined with sediment TBT concentrations provided important information about water bodies that are of the major concern, due to TBT exposure.

Costs involved

Total costs for sampling, chemical (tin organic compounds of tissues), and effect based analyses was about 16 000 Euro for the twelve stations, of which the actual effect analysis was about 5000 Euro.

Outlook – Next steps

Another parallel project also showed that extremely high concentrations of TBT and other antifouling compounds can be found in storm water and soil samples from some of the investigated harbours (Bengtsson & Wernersson 2012)[10]. Taken together, the results show that immediate additional action is necessary to reduce the emissions of organic tin compounds from pleasure craft harbours in the investigated regions in order to achieve acceptable conditions within reasonable time frames. During the last years, boat washing equipment has been installed at many pleasure boat harbours along the Swedish West Coast, usually with a 50% funding from regional authorities. A national guidance was recently published in order to support local authorities also in other regions to promote similar emission control measures.

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[10] Bengtsson H, Wernersson A. 2012. TBT, koppar, zink och irgarol i dagvatten, slam och mark i småbåtshamnar. Länsstyrelsen Västra Götalands län, rapport 2012:16

Case study 9

Title

Bioassays for Monitoring the Offshore Platform Impacts and their main discharges

Illustrating the following use described in this report:

To support the assessment of water quality, but also to confirm whether additional control measures are necessary

Reporting Institution:

Institute for Environmental Protection and Research, Italy

Web-Link

http://www.intechopen.com/books/advances-in-natural-gas-technology/an-approach-integrating-chemistry-and-toxicity-for-monitoring-the-offshore-platform-impacts
http://www.mba.ac.uk/library-archive/
http://www.scirp.org/journal/ns/

Main sources for further information; literature:

Loredana Manfra & Chiara Maggi, 2012. An approach integrating chemistry and toxicity for monitoring the offshore platform impacts. Natural Gas, InTech ISBN 979-953-307-567-8

L. Manfra, E. De Nicola, C. Maggi, E. Zambianchi, D. Caramiello, A. Toscano, D. Cianelli, A.M. Cicero, 2011. Exposure of rotifers, crustaceans and sea urchins to produced formation waters and seawaters collected in a gas platform area. Journal of the Marine Biological Association of the United Kingdom 91(1), 155–161.

L. Manfra, C. Maggi, J. Bianchi, M. Mannozzi, O. Faraponova, F. Onorati, L. Mariani, A. Tornambè, C. Virno Lamberti, E. Magaletti, 2010. Toxicity evaluation of produced formation waters after filtration treatment. Natural Science vol. 2, n. 1, 33-40.

Background, purpose, , approach, results and conclusions

Background

The main pressures on the marine environment from oil and gas activities include the placement of installations on the seabed and the discharge of Produced Formation Water (PFW).
The placing a structure on the seabed can lead to contamination phenomena in water, sediment and biota, and then may cause biotic modifications, affecting the benthic community composition and the responses of organisms exposed to eventual contaminations linked to installation phase.
PFW is the main waste from gas and oil platforms and is discharged into the sea according to existing laws (in Italy this is ruled by the Legislative Decree no. 152 of 2006). Chemical characteristics of PFW are very unusual and the exposure of marine organisms to contaminants contained in PFW may cause different responses (e.g., narcosis, alterations of the permeability of cell membranes and developmental defects).

Purpose

The purpose of three studies were: a) to explain the importance of an integrated chemical and ecotoxicological approach for offshore platform monitoring; b) to investigate the PFW toxicity of some Adriatic platforms (Mediterranean Sea) by means test-organisms belonging to different trophic levels and c) to investigate the occurrence of toxic effects on seawater samples collected around these platforms

Why effect based tools were included (why not an entirely chemical approach?)

The traditional chemical analyses allow to evaluate the behaviour of some environmental parameters (e.g. dissolved oxygen, chlorophyll, nutrients) and contaminants when a platform is installed and/or in production phase. Chemistry gives information on the presence, quantity and chemical form of substances linked to platform activities. The integration of biological investigations allows an assessment of toxicity and bioavailability of contaminants, to understand the mechanisms of their toxic action and identification of the area of potential biological impact of platforms and/or their discharges.

A series of chemical and biological analysis tools may be employed to monitor environmental effects when a gas platform is installed and/or a PFW is discharged into the sea. In fact, contaminants associated to offshore activities (installation and/or production phase, with operational or accidental discharges) could be accumulated within sediments and marine organisms and result in significant negative effects on the marine environment.

Approach

In 2005 and 2006, PFW samples were collected from some gas platforms in the western Adriatic Sea; surface seawaters were also sampled around the platforms, in correspondence to the discharge port and 25 m downstream the observed ambient current. (Manfra et al., 2010, 2012).

Results and conclusions:

Some differences among the PFW were observed both for toxicity and chemical composition: the highest toxicity was recorded in the PFW containing the highest concentrations of some metals (barium, manganese and zinc) and/or volatile aromatic compounds (BTEX). The authors observed that PFW particle presence induces toxicity due to chemical effect of contaminants and/or to particle mechanical effects. Besides, the authors showed that a PFW filtration treatment, before the discharge into the sea, reduces their toxicity and consequently the ecological risk decreases. No toxicity was recorded in seawater samples collected in the vicinity of the platforms, even in coincidence of the PFW discharge operations. This is probably a consequence of a fast and efficient initial dilution of PFW into the sea.

Case study 10

Title

Evaluation of the utility of microarrays as a biomonitoring tool in field study.

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

Not relevant, refers to chapter 6 (OMICS)

Reporting Institution :

European Commission Joint Research Centre, Institute for Environment and Sustainability, Molelcular Ecology Laboraotries, Ispra-Italy

Web-Link http://pubs.acs.org/doi/abs/10.1021/es2039097

Main sources for further information; literature:

Marlo K. Sellin Jeffrieshttp://pubs.acs.org/doi/abs/10.1021/es2039097 - notes-1http://pubs.acs.org/doi/abs/10.1021/es2039097 - notes-2, Alvine C. Mehintohttp://pubs.acs.org/doi/abs/10.1021/es2039097 - notes-1, Barbara J. Carterhttp://pubs.acs.org/doi/abs/10.1021/es2039097 - notes-3, Nancy D. Denslow, and Alan S. Kolok. Taking Microarrays to the Field: Differential Hepatic Gene Expression of Caged Fathead Minnows from Nebraska Watersheds

Falciani, F.; Diab, A. M.; Sabine, V.; Williams, T. D.; Ortega, F.;George, S. G.; Chipman, J. K. Hepatic transcriptomic profiles of European flounder (Platichthysflesus) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants.Aquat. Toxicol. 2008, 90, 92−101.

Background, purpose, , approach, results and conclusions

Background

The DNA microarray has been tested and validated so far to investigate the gene expression changes upon exposure in the laboratory to specific compounds (mainly as single compounds) to identify molecular biomarkers. However the application of this tool to investigate the effects from field sites was still unexplored. Therefore there was an interest to test this tool in the field.

Purpose

To assess the utility of DNA microarray analysis as a biomonitoring tool to discriminate field sites with different levels or types of contaminant from one another, but also to link the contaminant load and water quality with the effects at molecular level. If possible, also to identify the specific contaminants present at each of the field sites based upon the gene expression changes characterized.

Why effect based tools were included (why not an entirely chemical approach?)

In this case study, the aim has been to test using the DNA microarray as biomonitoring tool in the field.

Approach

Caged fathead minnows (*Pimephalespromelas*) were used to investigate effects from exposure to a variety of pesticides and nutrients associated with local agricultural practices. The authors analysed the hepatic gene expression profile using a 15,000-oligonucleotide microarray.Four sites were investigated; two of them locatedin low-impact agricultural watersheds, and the other two in high-impact agricultural watersheds. It was hypothesized that differences in gene expression profiles would correlate with the overall impact of chemical stressors,referring to both chemicals and nutrients.

Results and conclusions:

Link of gene expression profile change to the field sites: Low impact < high-impact

The results of this study revealed that gene expression patterns among male minnows were site-specific as evidenced by clustering analysis which revealed that the gene expression profiles in minnows segregated based upon deployment location. Site-specific gene expression patterns have also been documented for fish caged in waterways impacted by wastewater treatment plant effluent as well as in fish sampled from different field sites in Europe. In this study, the clustering of fish into four site-specific groups suggests that differences in gene expression patterns among fish are related to differences in environmental factors, such as contaminant load, between sites. Furthermore, the segregation of minnows from the low-impact and high-impact sites into two distinct groups supports the contention that microarray analysis can be
used as a biomonitoring tool to distinguish field sites with different levels/types of contaminants from one another.

Molecular Biomarkers link to class of contaminant (Correlating Gene Profiles with the Presence of Specific Contaminants).

In this study, linking the presence of even known contaminants to specific gene alterations proved to be a challenge. For instance, atrazine has been shown to induce the hepatic expression of antioxidative enzymes in zebrafish (Danio rerio).These genes were on the arrays, but their expression was not significantly increased.Atrazine has also been shown to induce the expression of CYP1A1 in common carp (Cyprinus carpio), but this was not reflected in the transcriptomes of the fish examined. In fact, CYP1A1 expression was significantly reduced at the Platte site relative to the Dismal. The conflicting results between the current study and previous studies could be due to the relatively low concentrations of agrochemicals present in the high-impact sites (e.g., average atrazine concentration <3 µg/L) compared to those used in the laboratory tests (atrazine concentration >7 µg/L).

The complex nature of surface water contaminants may explain the difficulty in linking specific gene responses to single contaminants, as surface waters are likely to contain a combination of agonistic and antagonistic compounds thatinteract differently with different receptors. In addition, biological responses identified in laboratory studies are likely to differ from those observed in natural environments due to the presence of trace amounts of natural and man-made chemicals in natural waters.

Conclusion:

These findings provide evidence that microarrays can be an effective biomonitoring tool to assess differences in overall pressure, on molecular level, at different sites. However, alterations in gene expression patterns could not be linked definitely to the presence of analysed contaminants in any of the watersheds. It is likely that the complex mixture of chemicals present at these sites contributed to the inability to correlate gene expression changes to contaminant exposures. Biological factors (i.e., age, sexual maturity, etc.) and nonchemical environmental factors (i.e., temperature, food availability, etc.) could also affect gene expression patterns. So these changes should be taken into account. Further studies aimed at investigating the effects of non-chemical environmental factors on gene expression should be undertaken, so that a greater understanding of the usefulness and limitations of microarray technology in field settings can be elucidated.

Fish deployed in or collected from field sites will rarely be exposed to just onecontaminant, but rather to a complex mixture of contaminants. The toxicity outcome of exposures to multiple contaminants is difficult to elucidate given that the interaction of chemicals can lead to additive, synergistic, potentiating, or antagonistic effects.

Costs involved:

Total cost estimates, based on similar JRC studies (in the paper these calculations are not provided): 12 800€

Based on the following costs (including working hours):
 1 slide, containing 8 arrays, costs approx: 500€
 60 arrays (7.5 slides) : 4000€
 Hybridization and scanning: 15K X 8= 12 X (400/ slide)= 4800€
 Kit for labelling and RNA extraction approx. 4000€

Outlook - Next steps

Studies on gene expression profile on mixtures should be performed since synergistic, agonist and antagonist interaction could provide a pattern of gene signature different form the single compound. A greater understanding of how complex mixtures affect gene expression patterns will be required for microarray technologies to achieve their full potential as a biomonitoring tool in field studies.

Case study 11

Title

Use of DNA microarray to test the water quality of river East Turkey Creek (bay of watershed of Florida) potentially impacted by treated wastewater from sprayfield area.

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

Not relevant, refers to chapter 6 (OMICS)

Reporting Institution :

European Commission Joint Research Centre, Institute for Environment and Sustainability, Molelcular Ecology Laboraotries, Ispra-Italy

Web-Link

http://www.sciencedirect.com/science/article/pii/S0166445X12000203#

Main sources for further information; literature:

Weil RE, Spade DJ, Knoebl I, Hemming JM, Tongue ML, SzaboNJ, Kroll KJ, Tate WB, Denslow ND.Evaluation of water quality threats to the endangered Okaloosa darter (Etheostoma okaloosae) in East Turkey Creek on Eglin Air Force Base. Aquat Toxicol. 2012, 110:177-86.

Background, purpose, approach, results and conclusions

Background

Ongoing population monitoring of the endangered Okaloosa darter (*Etheostoma okaloosae*) range has indicated that water systems such as East Turkey Creek have consistently reported decreasing numbers of darters moving downstream.

Purpose

The current study has been designed to evaluate potential negative influences on water quality in East Turkey Creek from the City of Niceville's municipal wastewater effluent after application to a designated wastewater sprayfield leased from Eglin Air Force Base (Bay of watershed of Florida). To assess potential causes of these findings, water quality parameters of the impaired systems in comparison with productive streams have been examined.

Why effect based tools were included (why not an entirely chemical approach?)

The approach of DNA microarray as biomonitoring tool can provide information about the effects at molecular level of the total mixture of contaminants in only one experiment while it would be hard to identify the full chemical load.

Approach

Use of a 8X 15,000-oligonucleotide microarray to measure the hepatic and testis gene expression of fathead minnows (*Pimephalespromelas*)

The study links the contaminant load (chemical analysis) with the effects at molecular level.

Due to the presence of diverse human activity other than the sprayfields, the authors chose three reference sites including a site just upstream of the sprayfields on East Turkey Creek, and two sites on an adjacent reference creek, Long Creek. Preliminary reports have indicated possible impacts of water quality (indicated by elevated conductivity) (on darter abundance in East Turkey Creek.Because the Okaloosa darter is protected, they focused chemical and genomic investigations on other fish species. For contaminant analysis they chose Sailfin shiners (*Pternotropis hypselopterus*), a species which is found in the same waters and shares a similar trophic level as

the darter. For genomic analyses, they chose the fathead minnow (*Pimephales promelas*, FHM) as this species is commonly used for ecotoxicologic assessment and a well-annotated microarray available is for this species. During this evaluation (1) ambient water quality parameters were measured, (2) non-polar and polar organic pollutants in ambient waters were evaluated by means of passive sampling devices, (3) the metal burdens in a similar native fish species that inhabits a similar trophic level were determined and (4) microarray responses were examined from gonad and liver tissues of a model fish species after laboratory exposure to stream waters.

Results and conclusions:

To better compare the gene expression fingerprints from fish exposed to water from the four sites, the expression data were arranged in correspondence to their locations on East Turkey Creek, starting with site D (reference site) on Long Creek, to site B (above the sprayfields), to E (within the sprayfields), and to A (below the sprayfields). For each site, the expression data was in the same order and arranged from the most up-regulated to the most down-regulated genes. Expression values are listed as $\log_2$ of the fold-expression compared to the UF control. Of the four sites, it is clear that fish exposed to water from site D (the site on Long Creek) had some differences compared to the UF controls (526 genes). Gene expression results from site B (566 altered genes) are most similar to those from site D, but do contain an additional set of genes that are not differentially expressed in site D. Sites E and A presented a total of 492 and 808 altered genes, respectively. The figure shows the gene pattern regulation for each site.

Functional enrichment analysis based on Gene Ontology biological processes in the liver indicated that at all sites, the predominant pathways affected were related to metabolism, cell cycle control, transcription, translation, remodeling of various cellular structures, and cell signaling. This implies that the difference between UF control water and site water at all sites is sufficient to cause changes in transcription of genes related to the basic processes of gene and protein expression and signal transduction. This is consistent with a generalized stress response.

Many of the changes in gene expression seen at Sites E and A (within and downstream the sprayfield, respectively), both in the liver and in the testis were related to protein synthesis at multiple levels (initiation of translation, aminoacylation of tRNAs, ribosome biogenesis, etc.). This suggests an acute response to stress, potentially through the mTOR (metazoan target of rapamycin) pathway. The mTOR signaling pathway is involved in regulating the protein translational machinery, and has been shown to be inactivated by low nutrients, low energy reserves, and hypoxia, among other stressors. Overall, the enrichment analysis consistently indicates that effects on transcription, translation, signaling, and metabolism are present in both testis and liver, and this may indicate a generalized stress response. The testis effects are more diffuse than liver effects, but include indicators of stress response and modulation of cell cycle control. The liver data meanwhile provide several key clues that exposure to site waters may result in transcriptional response consistent with liver damage. Although the contaminant analysis did not identify any specific substances to explain the findings, it is possible that the combined effects of contaminants found in these site waters could contribute to net hepatotoxicity. This indicates a stronger impact than the effect of site waters on the testis; there is no direct transcriptional evidence of effects on spermatogenesis. Therefore, liver toxicity appears to be the major concern for fish exposed to waters from the sites included in the present study.

Water quality measurements in East Turkey Creek suggest that anthropogenic influences have negatively impacted the stream system. Treated sewage water is sprayed on the land that surrounds sites E and A(within and downstream the sprayfield, respectively), creating the possibility that some residues from this water make their way into East Turkey Creek. Gene expression changes from microarray analysis demonstrated that major biological processes were affected in fish exposed to waters from the creek influenced by the spray fields, suggesting a stress response in both liver and testis, and possibly early liver damage.

Costs involved:
Total cost estimates, based on similar JRC studies (in the paper these calculations are not provided): 7700€

    Based on the following calculations: 64 array(4 fish, each four biological replicates each site),

one slide contains 8 arrays, 1 array cost is according to our studies 500€x 8= 4000€
hybridization and scanning is 15KX 8= 8X (400/ slide)= 3200€
Kit for labelling and RNA extraction approx. 500€

The cost estimates are referring to DNA microarray studies only, whereas there are other costs as well, related to e.g. chemical analysis and sampling

Outlook - Next steps
A limited analytical examination of possible contaminants including analysis of metal burdens in fish and passive sampling for non-polar organic (SPMD) and polar organic (POCIS) analytes failed to identify the causative factors. This failure demonstrates the importance of a multifaceted approach when attempting to reveal and understand complex ecological issues, and it highlights the importance of bioassays in the assessment of contaminants in aquatic environments. Results of these analyses have provided insight into the water quality degradation in East Turkey Creek. Based on these data, recommendations have been made for additional studies to improve understanding of water quality issues and adapt management strategies to promote recovery of the Okaloosa darter..

Case study 12

Title
The risk of chronic impact of pollution on the Bílina River

Bibliography and link to objectives/national use
Illustrating the following use described in this report:
Primarily prioritisation of areas for further studies (to support conclusions made in the analysis of pressures and impacts). Also supportive in the identification of control measures
Reporting Institution :
T. G. Masaryk Water Research Institute, public research institution
Web-Link
www.vuv.cz
Main sources for further information; literature:
Soldán, P. and Badurová, J. (2012) A method for screening for the risk of chronic effects of surface water pollution, Environmental Monitoring and Assessment, DOI:10.1007/s10661-012-2529-9

Background, purpose, , approach, results and conclusions

Background
The small Bílina Riverflows much of its course through a man-made channel, which was created when lignite mining began in the North Bohemian region of the Czech Republic. There was a serious deterioration in the state of the river.A substantial amount of water was drawn from the river to be used in thechemical and oil industries and the release of highly polluted wastewater into the river killed all the fish in some parts of the river. The situation improved significantly in the 1990s. The river flow rate is currently artificially increased through the redirection of water from the catchment area of the Ohře River. However, the Bílina is still one of the most polluted rivers in the Czech Republic.

Purpose
The screening is intended to identify potential problems and more detailed studies with the use of a set of different bio-assays and other analyses should follow, as is the general strategy when surveying pollution hazards.

Why effect based tools were included (why not an entirely chemical approach?)
Toxicity and genotoxicity tools are additional tools to the chemical approach, to cover risk of chronic effects from substances not monitored chemically. Acute toxicity is also checked to facilitate interpretation of genotoxicity data.

Approach
The approach is largely based on the method developed by de Zwart & Sloof 1993 (see also the case study "Monitoring concentrated surface water with in vivo bioassays" above). The assessment of the risk of a chronic impact of pollution was carried out on nine selected sampling points as part of a study of the then current biological statesof the river (Havel et al. 2010), see map below.
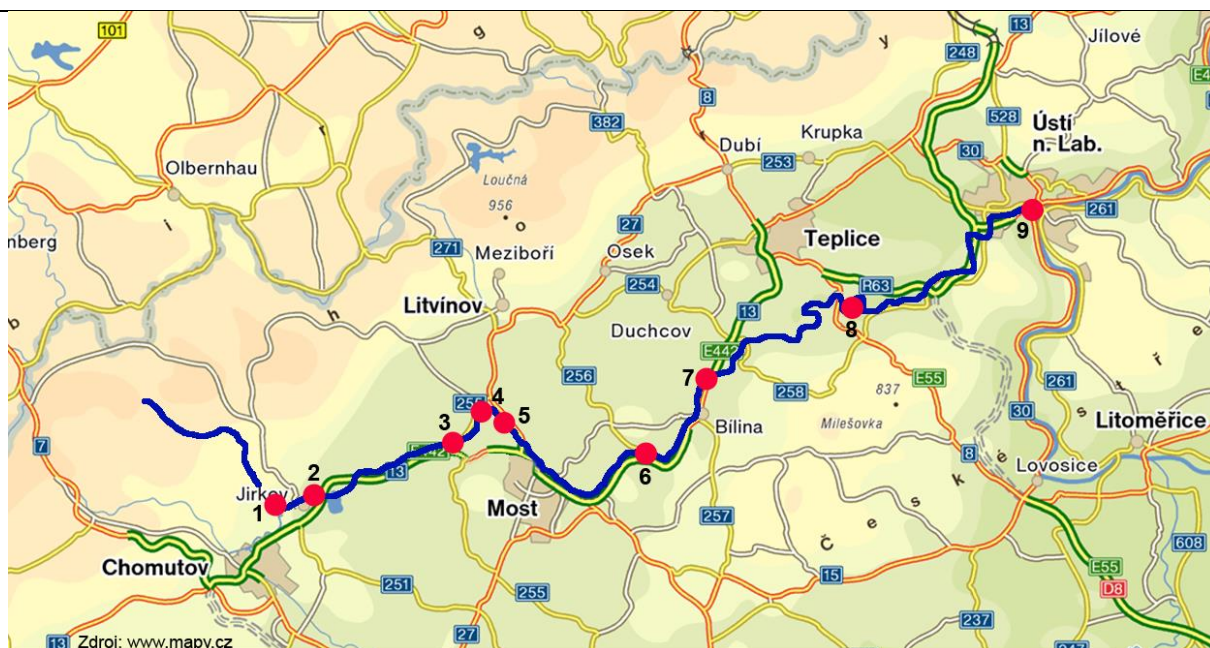
Map. Sampling stations along the Bilinia river.

These sections exhibit different types of pollution, see table below. The assessment is based on two parallel bioassays (toxicity and genotoxicity) in addition to metal analyses, to also include risks from inorganic fractions.

Table.1: Sampling points on the Bílina River

Sampling point	Map No.	Type of pollution
Březenec	1	bottom sediments, leachates from drinking water reservoir, old impairment
downstream from Jirkov WWTP[11]	2	sewage
upstream from Jiřetín weir	3	mining and machinery wastewater
downstream from Jiřetín weir	4	municipal, mining wastewater
Záluží	5	municipal, oil industry wastewater
Želenice	6	sewage, industrial, mining wastewater
downstream from Bílina WWTP	7	industrial, mining wastewater
Rtyně nad Bílinou	8	sewage
Ustí nad Labem	9	sewage, old impairment

The concentration of organic pollutants is achieved using an adsorption process (see Fig. 1). Equal measures of two types of polystyrene XAD resin (20 ml of XAD plus 20 ml of XAD 4) are added to a 20 litre sample of surface water and intensively mixed for 24 hours. After this the sieved resins, along with a very small amount of water, are transferred into a glass column. The resins in the column must be immersed in water without any air-bubbles. The adsorbed organic pollutants are washed out from the resins with 20 ml of acetone, which is injected into the column at the base. The minimum contact time of the adsorbents with the acetone is 30 minutes. Then the acetone eluant along with the rest of the water is let out from the glass column into a measuring cylinder. Subsequently the acetone is stripped out by

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[11] Waste Water Treatment Plant

intensive bubbling using nitrogen or any inert gas, and by this process the adsorbed organic pollutants are concentrated into a small volume of water. This water solution is added with additional water to make up a final volume of 20 millilitres. In this way the pollutants from the original 20 litre sample are converted into a volume of 20 millilitres and a 1000x concentrate of the organic pollutants is thus obtained.

Then the toxicity of the concentrate is evaluated. Since the amount of concentrate is very small, the evaluation of its toxicity can be done only with small volume tests. The luminescent bacteria test in accordance with ISO 11348 is one of them. In this test the level of concentration which causes a20% inhibition of bacteria luminescence during 30 minutes' exposure time (30 min EC 20) is evaluated.

Degrees of risk, their verbal descriptors and corresponding expected types of toxic impact (referring to both toxicity and genotoxicity bioassays)

Concentration interval	Degree of risk	Level of risk	Expected toxic impact
1 000 × to > 500x	I	negligible	none
500 × to > 125 x	II	low	none
125 ×> 63 x	III	maximum admissible	none
63 × to > 1x	IV	increased	chronic
1 × and less	V	serious	acute

In addition, also a chemical component is assessed, referring to the metal concentrations. The degrees of risk of toxicity of inorganic surface water pollutants are designated by the maximum values of risk of any of selected toxic metal. These risk values are obtained by the results of analyses of concentrations of metals in the collected water sample. The overall degree of risk of toxicity of surface water pollutants is obtained from the most unfavourable degree of risk of either of the two components of surface water pollution.

On the basis of the expected type of toxic impact listed in the 4th column of Tab. 1, the priority of abatement measures can be set (see Tab. 3). This step inthe interpretation of screening results provides a useful tool to water authorities in the field of surface water quality protection.

Tab. 3: Priority of abatement measures depending on assessed degree of risk

Degree of risk	Priority of measure
I	no need for abatement measures
II	strengthened care due to the risk of increase of pollution level
III	strengthened care due to the risk of increase of pollution level
IV	pollution has a chronic impact – long-term abatement strategy necessary
V	pollution has a chronic impact – need for immediate action

The concentrate of organic pollutants is tested with genotoxicity assays with and without metabolic activation (e.g. Ames fluctuation test - Rao, Lifshitz 1995). The degree of risk is determined by the levelof concentration which has a significant genotoxic impact on the test strain *Salmonella typhimurium*. The limits of concentration values are the same as those for the evaluation of toxicity risk (see Tab. 1). The resulting degree of genotoxic risk is obtained by the most unfavourable degree of risk of either of the versions of the test, i.e. with or without the addition of the S9 fraction.

In the final assessment of the risk of a chronic impact, toxicity and genotoxicity are evaluated in parallel. This is due to the fact that there is no direct linkbetween these two parameters, since they result in

different types of negative effect. Generally it may be saidthat the mechanism of impact manifestation of the volume or concentration of effective substance are completely different for toxicity and genotoxicity. Toxicity influences the physiological processes of an organism. In extreme cases it can even cause death due to the failure of basic life functions. Genotoxicity influences the mechanisms for the transfer of genetic information, which can be manifested in mutagenicity or carcinogenicity.

Results and conclusions

The conclusionsmade about risks of toxicity and genotoxicity carried out in 2009 are listed in the table below

Tab. 2: Assessment of the risk of a chronic impact of pollution on the Bílina River

Sampling point	Map No.	Risk of toxicity	Risk of genotoxicity
Březenec	1	II	V
downstream from Jirkov WWTP	2	II	IV
upstream from Jiřetín weir	3	II	IV
downstream from Jiřetín weir	4	II	IV
Záluží	5	V	I
Želenice	6	V	I
downstream from Bílina WWTP	7	II	V
Rtyně nad Bílinou	8	II	IV
Ustí nad Labem	9	II	V

Toxicity results only,without genotoxicity data, or vice versa, can lead to an incorrect evaluation of the biological quality of the water environment.There is an inverse relationship between the values of toxicity and genotoxicity. The toxic risk of the samples was evaluated using bacterial toxicity tests. In genotoxicity tests bacteria are alsoused (even if a different species). So a toxic sample can also influence, due to its toxicity, the test organisms used in genotoxicity assays and this is the most probable reason for the detection of no genotoxicity in the case of very toxic samples. This is due to the test method used in the evaluation of genotoxicity. In the Ames test, genetically modified organisms are used. These organisms are not able to synthesize histidine so they are not able to grow in the test medium, which is free from this basic amino acid. However, the genotoxic impact of the tested substances can cause reverse mutation, which restores the ability of the organism to synthesize histidine. Therefore, organisms can grow in a histidine-free media. This growth is indicated by the change in colour of chromogen (from violet to yellow), which is also present in the test medium. Due to this detection principle a negative genotoxic impact and a positive toxic one manifest themselves in the same way. This fact must be considered in the final assessment – it is extremely probable that the polluted surface water from sections 24 and 25 has high genotoxicity too. The reason why the negative impact of these pollutants is distributed over a wider area could be due to transport of pollutants with the river's flow. This can be confirmed by the results of other analyses performed in this area – high concentrations of mercury, arsenic, vanadium, polychlorinated biphenyls, hexachlorobenzene and dichloro-diphenyl-trichloroethane were detected in biofilms and sediments collected in the middle and lower part of the river (Kohušová et al. 2010).

Costs involved:

Sampling costs depend on the distance of sampling profile from analytical laboratory. Evaluation of chronic toxicity per 1 sample (with luminescent bacteria) costs approx. 480 EUR, and evaluation of genotoxicity per 1 sample (with Ames fluctuation test with and without metabolic activation) costs

approx. 630 EUR. Chemical analysis for one metal 4 EUR, usually 6 selected metals per sample:in total 25 EUR.

Outlook - Next steps

The approach is routinely used for the assessment of risk of chronic impact of surface pollution water in all three international river basins on the territory of the Czech Republic (The International Elbe /Labe/ River Basin, The International Oder /Odra/ River Basin, The International Danube /Dunaj/ River Basin**)**. To date, the assessment has been carried out for more than 80 profiles.

The method has been fully standardised and at the end of the year 2009 it was published as the Czech branch technical norm of water management (TNV).

Case study 13

Title

Mechanism-specific tools with zebrafish early life stages in EDA of surface waters

Bibliography and link to objectives/national use

Illustrating the following use described in this report:

Application of mechanism-specific methods using both *in vitro* bioassays and zebrafish early life stages tools for Effect Directed Analysis of surface waters, aiming to evaluate and improve the contribution of zebrafish bioassays for EDA studies.

Reporting Institution :

RWTH Aachen University, Germany

Helmholtz Centre for Environmental Research UFZ, Germany

Istituto Superiore di Sanità, Italy

Web-Link

http://www.bio5.rwth-aachen.de

http://www.ufz.de/index.php?en=617

http://www.iss.it

Main sources for further information; literature:

(Brack et al. 2013)**.** EDA-EMERGE: an FP7 initial training network to equip the next generation of young scientists with the skills to address the complexity of environmental contamination with emerging pollutants. *Environmental Sciences Europe.* **25**:18.

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Straehle, U., S. Scholz, R. Geisler, P. Greiner, H. Hollert, S. Rastegar, A. Schumacher, I. Selderslaghs, C. Weiss, H. Witters, and T. Braunbeck. 2012. Zebrafish embryos as an alternative to animal experiments-A commentary on the definition of the onset of protected life stages in animal welfare regulations. Reproductive Toxicology 33:128-132

Kosmehl, T., J. C. Otte, L. Yang, J. Legradi, K. Bluhm, C. Zinsmeister, S. H. Keiter, G. Reifferscheid, W. Manz, T. Braunbeck, U. Straehle, and H. Hollert. 2012. A combined DNA-microarray and mechanism-specific toxicity approach with zebrafish embryos to investi-gate the pollution of river sediments. Repro-ductive Toxicology 33:245-253.

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Wölz J, Brack W, Möhlenkamp C, Claus E, Braunbeck T, Hollert H. (2010): Identification of dioxin-like activity in suspended particulate matter of floods using effect directed analysis. Sci Tot Envrion. 408 (2010) 3327–3333

Background, purpose, approach, results and conclusions

Background

Study area: The river Holtemme has pristine waters in its source, coming from the Harz mountains. However when flowing along urbanized areas it collects effluents from municipal wastewater treatment plants, including input from health facilities as hospitals and wellness clinics. Selected sites in the Holtemme have been previously investigated, as in the Saale/Mulde monitoring study and in the EDA EMERGE project. *In vitro* bioassays results indicate mutagenicity, estrogenicity, and anti-androgenicity.

Zebrafish in EDA: The literature was reviewed for EDA studies that include zebrafish bioassays. Studies that include endpoint-specific zebrafish embryo assays have higher rate of success when compared to those that evaluate acute toxicity.

Purpose

To evaluate and improve the contribution of mechanism-specific bioassays with zebrafish early life stages in EDA studies of surface waters.

Why effect based tools were included (why not an entirely chemical approach?)

Bioassays are intrinsic elements of the EDA approach. Endpoint or mechanism-specific biotools,

rather than acute methods, are suggested to promote higher compound identification success.

Approach

Surface water samples and fractions will be evaluated by mechanism-specific *in vitro* and zebrafish assays. Endocrine potential will be assessed in the *in vitro level* by glucocorticoid-, estrogen-, and androgen-responsive cells; and in the organism level by glucocorticoid- and estrogen-responsive zebrafish. Mutagenicity will be assessed *in vitro* by p53-responsive cells and by the micronucleus assay with fish cells; while micronucleus will be evaluated in zebrafish early life stages. Results from *in vivo* tests and zebrafish assays will be compared for sensitivity and specificity.

Results and conclusions

The bioassays are expected to be completed by June-July 2014. Afterwards the results from *in vivo* tests and zebrafish assays will be compared for sensitivity and specificity.

Costs involved

Costs depend on the number of fractions detected as positive, and that therefore require further fractionation and testing.

Outlook - Next steps

Chemical analysis performed by project partners at UFZ complements the EDA procedure.

Case study 14

Title

**Multicriteria assessment of human activity effects on water ecosystems:
The case study of Tiber River basin**

Illustrating the following use described in this report:
A set of biological indicators, microbiological analysis, ecotoxicological and genotoxicity assays were used in order to describe the environmental impact of chemical contaminants coming from agricultural, industrial and urban activities.

Reporting Institution :
Italian National Istitute of Health (ISS)-L. Mancini, C. Puccinelli and S. Marcheggiani.

Bibliography and link

Web-Link

http://www.iss.it/binary/publ/cont/08-15_web.1214313128.pdf

Main sources for further information; literature:

Mancini L, Pace G. (Ed.). Strategie di protezione e indicatori delle risorse idriche: studio pilota.
Roma: *Istituto Superiore di Sanità*; 2008. (Rapporti ISTISAN 08/15).

Background, purpose, , approach, results and conclusions

Background
Environmental integrated assessment of territorial areas allows to describe the status of pollution andecosystem quality. This information can be considered as a starting point for an appropriate planning of territorial management. Integrated approach strategies will provide the possibility to obtain a sustainable balance among human social and economic needs and natural ecosystem conservation actions, extremely important for people wellness.
Despite the national and regional value of these considerations, the Tiber river basin has been chosen as a case study, due to the presence of a complete spectrum of conditions and human pressures in its area.

Purpose
The aim of this study was to assess the water quality of Tiber River Basin choosing a multicriteria approach, using biological indicators, microbiological analysis, ecotoxicological and genotoxicity assays.

Why effect based tools were included (why not an entirely chemical approach?)
The multicriteria approach is based on the study of different matrices as water, sediment and biota, providing information on the effect that chemical contaminants can have on water ecosystems. Biological indicators, microbiological analysis, ecotoxicological and genotoxicity tests are able to evaluate those effects at different trophic levels of river ecosystems.

Approach
Samplings (Biological, water and sediment) were performed seasonally on sites located on Tiber river basin, upstream, inside and downstream the city of Rome.
Biological communities (diatoms, macroinvertebrates) were investigated to assess the ecological water quality and detect impact such as eutrophication and hydromorphological alteration.
Microbiological analysis were performed on water and sediment to detect the presence of *Total Coliform* and *Faecal Coliform*, *Escherichia Coli*, *Faecal Streptococci* and sulphite –reducing bacteria (*Clostridium*).
Ecotoxicological, both acute and chronicle test (*Ceriodaphnia dubia*) and genotoxicological assays (cell roots of *Vicia faba*) were performed to evaluate the presence of chemical contaminant on

sediment.

Results and Conclusion

Overall biological communities analysis showed a poor or bad water quality for the studies sites.

Microbiological and Ecotoxicological analysis indicated, progressively worsening of environmental conditions, upstream to downstream.

Genotoxicity test detected presence of contaminants only in the sites in close proximity or inside Rome urban area.

Results revealed important and negative effects the chemical contaminants can have on water ecosystems, showing an environmental degradation conditions for all the matrices and trophic levels investigated.

Outlook - Next steps

This research can be considered as one of the first studies correlating functional and structural degradation of ecosystems with human health in Italy. The study was completed using a multicriteria approach: 1) Different environmental matrices were investigated: water, sediment and biota. 2) Effect of chemical contamination were detected at the different trophic level (producers, consumers and detritivores).

2. PROPOSALS TO ASSESS ENDOCRINE DISRUPTION

2.a.Bioassay battery to assess hormonally active substances, endocrine disruptors and reproduction toxic effects, Swiss proposal

(adapted from Kase et al. 2009 and Hecker & Hollert 2011):

In the research area of effect based tools a huge variety of biotestbattery proposals for multiple effect assessment are published. The main part includes effect based tools from different trophic levels and different *in vitro* and *in vivo* assays. The currentexample concerns a biotestbattery approach proposed to assess effects from hormonally active substances, endocrine disruptors and reproduction toxic effects, related to the specific focus of the EU on endocrine disruptors. The intention is to illustrate in which fields sufficient biotest validation is available to address relevant mode of actions in environmental samples with a combination of *in vitro* and *in vivo* assays.

As a medium-term measure, the EU strategy on endocrine disruptors (SEC, 2007) uses the Endocrine Disruptor Testing and Assessment (EDTA) Task Force of the Organisation for Economic Co-operation and Development (OECD) along with other research activities. In particular, the test methods of the OECD that are currently being validated or which have already been validated may contribute to a better understanding of the extent of endocrine disruption, especially if they are applied on environmental samples and in the context of risk-assessment strategies, for instance in waste water treatment. Further standardisation of such methods for regulatory applications, e.g. like an effect based monitoring is recommended (Kase et al. 2009).

For the integrative assessment of endocrine disruptors, hormonally active substances and reproduction toxic effects in environmental samples the Swiss Centre for Applied Ecotoxicology provided an overview about available and suitable detection methods for the identification of hormonally active and reproduction toxic effects in environmental samples.

On the basis of an extensive literature search and ongoing international validation efforts by the OECD for methods to detect endocrine disruptive effects, 15 biological test methods (5 in vivo and 10 in vitro) were selected. Comprising, for example, of eight OECD methods and three out of five *in vitro* methods mentioned in the Global Water Research Coalition (GWRC) report "Tools to detect estrogenic activity in environmental waters" (Leusch 2008). Experienced users and developers were then asked to rate the test according to given relevant criteria. The resulting criteria profiles were compiled, compared, and evaluated and checked with available literature. By combining literature research with a targeted query for information about the choosen test procedures it was possible to obtain a detailed overview about the current state-of-the-art of science and technology in the detection of hormone-active effects and reproduction toxicity. Based on the review an international workshop organized by the Swiss Centre for Applied Ecotoxicology of Eawag/EPFL (Ecotox Centre, Duebendorf, Switzerland) with participation from regulation, science and industry, a recommendation for a modular ecotoxicological test platform was given afterwards (Hecker and Hollert 2011, and Fig. below).

Sensitivity comparison of biotests for single substances
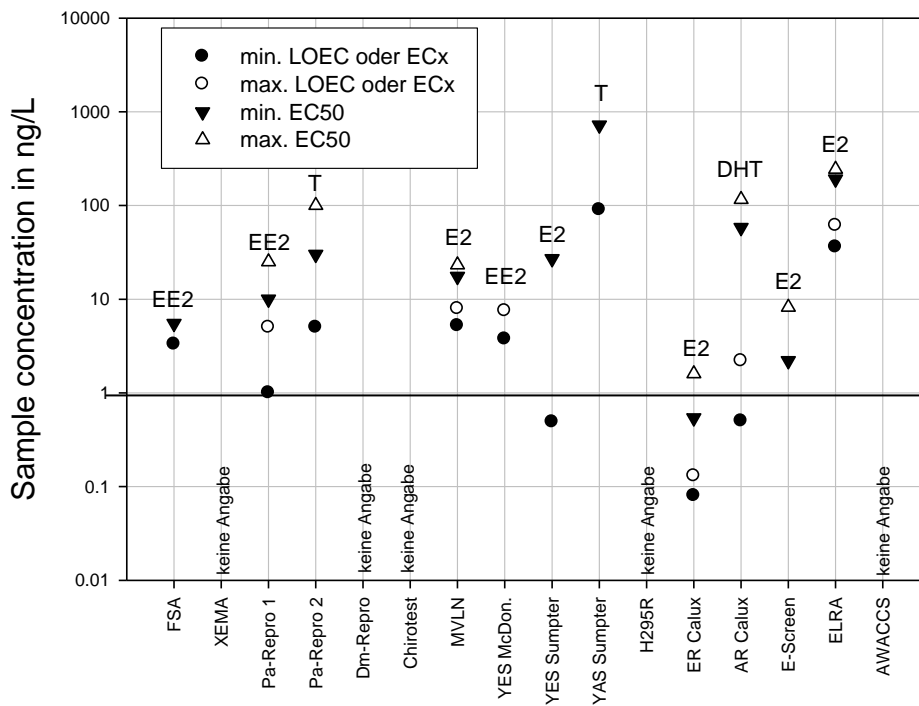
Figure: Sensitivity comparison for single substances for 5 *in vivo* and 10 *in vitro* tests,
(EE2 = 17-alpha-ethinylestradiol, E2 = 17-beta-estradiol, T= Testosterone, DHT = dihydrotestosterone)

Even bioanalytical methods reach detection limits below 1 ng/L for single substances. Moreover they can measure estrogenic effects in an integrative way, so they could be suitable as screening methods. Also important is to evaluate the different time, costs and practicability aspects of the biotests.

Comparison time expenditure, costs practicability of biotests

FigureComparison of time needs of 5 *in vivo* and 10 *in vitro* biotests

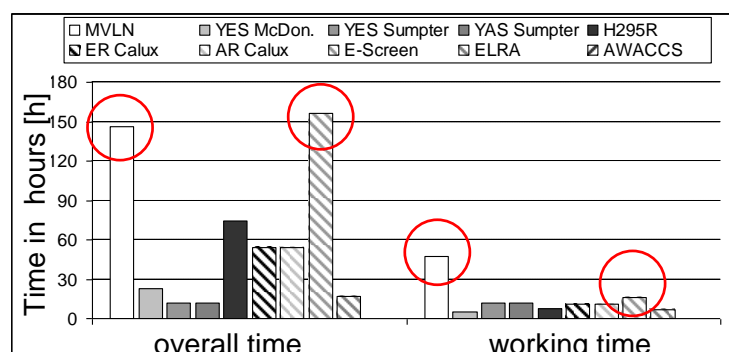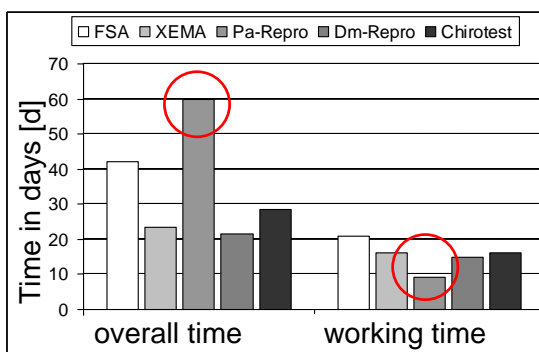In general: *in vivo*-tests consume more time than *in vitro*-tests, scaling days vs. hours

In vivo: the reproduction test with *Potamorpyrgus antipodarum* needs most overall time but minimum working time; *in vitro*: MVLN and E-Screen have the greatest overall and working time demand
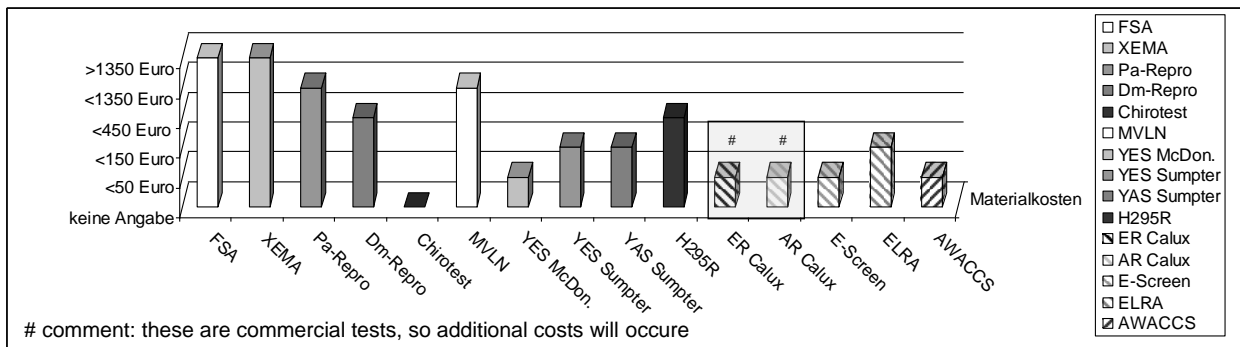

Figure: Comparison of material costs of 5 *in vivo* and 10 *in vitro* biotests. As an equivalent for the ER-CALUX also the non-commercial test system T47Kbluc is available and officially recommended by US-EPA, Additionally, for the AR-Calux the MDA-KB2 Cell Line is an adequate non-commercial alternative (Blake et al. 2010 and Hecker& Hollert 2011), which is also recommended by US-EPA.

In general *in vivo* tests are more expensive than *in vitro* tests regarding materials needed and in addition more time consuming.

In environmental samples combination effects from different chemical stressors occur. Consequently, endocrine and general-toxic effects should be detected in an integrative manner by ecotoxicological test platforms. Ecotoxicological and regulative aspects should be considered equally, and the applicability of the test procedures should be evaluated. Because of the diversity of endocrine disrupting mechanisms, a modular combination of *in-vivo* and *in-vitro* methods in a joint test platform is needed to investigate and differentiate the transitions from hormone-active effects to endocrine disruption (see Fig XX).

The detection methods were selected on the basis of validation status, distribution, their suitability for standardization, and their proven sensitivity for environmental samples. We assumed that specific YES/YAS-procedures and some ER/AR-transactivation, as the ER/AR CALUX or T47D-Kbluc/MDA-KB2 (see chapter 4.3.2), systems achieve the mentioned criteria. In the case that strong cytotoxicity of environmental samples affects the applicability of cellular reporter gene assays, alternatively a molecular receptor binding assay (e.g. ELRA) could be used. Additional molecular receptor binding assays are currently in validation by the OECD. Modulating effects on steroidogenesis and probably even on aromatase activity can be detected by the OECD validated H295R Steroidgenesis Assay.However, the results in the H295R assay can be masked by cytotoxicity in waste water assessment.

In conclusion the different mode of actions of endocrine disruptive effects and reproduction relevant effects have to be combined with their ecotoxicological relevance and their availability of response (see Fig.X in chapter 4.1).

Several test methods were proposed in an application-oriented modular test platform. These tests should be suitable to efficiently indicate different mechanisms of endocrine disruption and

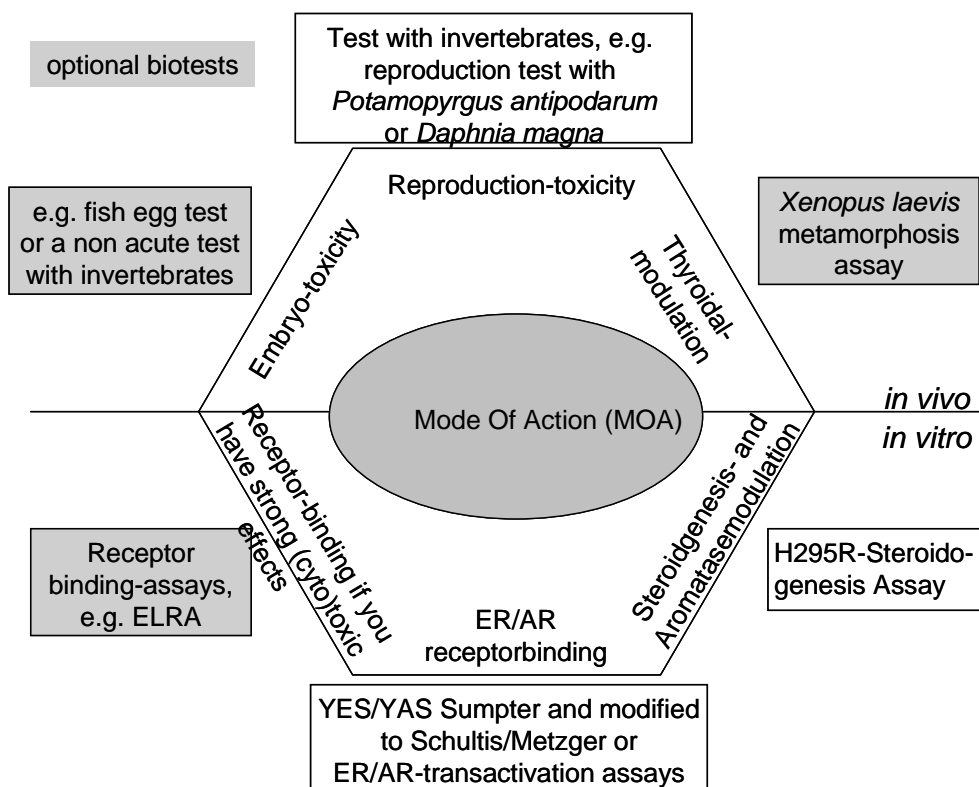reproduction toxicity, and may be employed in different situations according to their respective advantages.

Figure: Swiss proposal of amodular test battery for the detection of endocrine disruptive and reproduction toxic effects. The recommended ER/AR transactivation assays, are ER/AR-Calux or the non commercial T47D-Kbluc MDA-kb2 (see Hecker & Hollert 2011).

The modular test platform is able to detect impacts on reproduction relevant effects in invertebrates, amphibians, and fish.. Furthermore the different mode of actions of estrogenic and androgenic receptor binding, alterations of steroidogenesis and the modulation of aromatase are detectable.

We assume that the relevant mode of actions of receptor binding and steroidogenesis modulation are also able to cover some risks of endocrine disruption in other sensitive organisms like fish species. This becomes evident, because for some species most recently a link between receptor activation, vitellogenin induction (biomarker) and apical reproduction relevant endpoints (see Fig. 1), e.g. fertilization success have been identified. But it becomes evident that not all risks could be covered with biotests on *in vitro* level because indirect or delayed effects might be missed.

The modular system presented here allows the switching between test modules according to the continuously developing state-of-the-art of science and technology as well as the incorporation of novel developments. For example the US-EPA is currently working on developing an integrated screening assay for detection of thyroid hormone disruptors for environmental samples (Gilbert et al. 2011).This project proposes the application of an

innovative transgenic amphibian 'biosensor' for the screening of chemicals and environmental samples for the presence of thyroid disrupting activity, e.g., the *Xenopus laevis* metamorphosis assay (XEMA) could be replaced by this tool, when it becomes available.

The specific role of addressing effects for and with invertebrates is also addressed in Hecker and Hollert (2011): "Recent reports have shown that a number of xenobiotics in the environment are capable of interfering with the normal endocrine function in a variety of animals. The overwhelming majority of the studies on the effects of hormone-mimetic industrial chemicals were focused on findings in vertebrates (Oehlmann and Schulte-Oehlmann 2003).Although approximately 95% of known species in the animal kingdom are invertebrates, studies on the effects of EDCs on invertebrates are still scarce."

In general further standardisation of methods for regulative applications is recommended and surely most of the *in vivo* tests and some *in vitro* bioassays are too expensive for routine monitoring and are only applicable for specific case studies.

On the other hand some cost efficient biotests are already quite advanced in the validation process of the OECD and are also suitable for environmental sample testing; others are also in the preparation phase for the ISO-level standardisation which is necessary for environmental sample testing. So that probably within the next three to four years certified, standardised procedures for environmental sample testing can be expected.

2.b. **Expert opinion on the suitability of bioassays for the monitoring of EE2 and E2 in surface waters,**

Main conclusions drawn at the Koblenz workshop on bioanalytical options for the monitoring of steroidal effects in surface waters (27.2.2013 at the BfG in Koblenz):

1. The specific mode of action of EE2 an E2 allows the design of a tailor-made monitoring strategy.
2. Established bioanalytical instruments (in vitro tests) can be used to detect estrogen receptor activation in surface water with sufficient sensitivity. They are currently the only methods with sufficient sensitivity to detect effects at the very low concentrations referred to above for whichchemical analysis is not yet reliable; results represent the combined effects of all substances with endocrine disrupting properties (unknown mixtures) present in the sample.
3. Continental or coastal waters exposed to waste water or diffuse sources (e.g. agriculture) contain a mixture of unknown substances with endocrine disrupting properties. The environmental quality standards provide protection based on single-substance no effect concentrations (EQS –Directive).
4. A positive bioassay is indicative of the presence of potent estrogens (e.g. E1, E2 or EE2) in the sample, but is not equivalent to the concentration of a single substance. If this screening shows an effect above a certain threshold value, e.g. the EQS under discussion, further investigation is necessary. Thisshould include chemical analysis and information on the potential regional sources of estrogenic inputs.
5. Negative bioassay responses clearly identify water bodies without a risk-potential caused by EE2 andE2 (screening function)
6. For application to surface water samples bioassays still need validation according to commonly agreed principles (e.g. ISO 17025), guidance for sample handling and specific preparation protocols.
7. To avoid redundant work the validation should be undertaken in a coordinated European exercise and should be conducted under the CIS (Common Implementation Strategy). A collaborative mechanism like the JRC EU-wide monitoring concept offers the opportunity to organize a representative exercise in Europe. Within this exercise further crucial questions of monitoring like the financial implications should be evaluated.
8. The workshop participants recommend using freely available human in vitro cell cultures (e.g. T47DKbluc, MELN or cell lines validated/under validation by international standardization bodies; OECD, ISO) for a pre- validation study using surface water and to compare it with the ER-Calux running in one laboratory.
9. The combination of bioanalytical measurement and validated models of exposure to steroidal estrogens based on consumption data reduces uncertainties and costs of monitoring programmes. Validated models on exposure of pharmaceuticals are available for example from the German Laender projects on "Mikroschadstoffe" (micro-pollutants). A consumption based model of waste water driven substances like EE2 is able to focus monitoring efforts on polluted areas with continuous risks.(http://www.masterplan-wasser.nrw.de/ www.oekotoxzentrum.ch/projekte/stoffluss/index_EN).
10. A pre-validation and a following European standardization (ISO, CEN) of such a bioanalytical method would be desirable and should be streamlined with on-going work conducted at ISO. To avoid double work, expertise acquired in the DIN working group "Hormonelle Wirkungen" (AK 119-01-03-05-09), the German mirror

committee of ISO TC 147 SC5 WG 9 should be integrated and input from other Member States should be sought.

3.NATIVE VS PRETREATED WATER SAMPLES AND PASSIVE SAMPLING

Native vs pretreated water samples in a bioassay context

Bioassays are frequently performed using water samples. Traditionally these water samples are either grab samples or collected using an automated sampler, but in recent years also passive samplers have been used in combination with certain bioassays. Many aspects to consider are the same for both chemical and effect based analyses of water samples, but there are also differences.

Using native water samplesis the most holistic way of effect-based testing in the laboratory, although it has to be considered that the chemical mixture contained in the sample may change during storage. This situation is similar for chemical analysis, where sample storage can have an impact on what is detected. Filtration followed by solid phase extraction (SPE) is often performed to clean-up and enrich a sample.Filtration of a water sample to remove particulate matter is also the simplest clean-up step that may be required for a bioassay to work properly; for example, to remove particles that interfere with certain measurements (e.g. optical density). However, this simple step may already affect the availability of toxicants in a bioassay. The store of chemicals that was originally available in the particles is now no longer present. Consequently, when organisms in the bioassay take up chemicals from the water, or the aqueous concentration in the bioassay falls because of other reasons, there is no opportunity for the particles to supply sorbed chemicals to the water. In this way, the toxic potential of the original sample may be underestimated.

Acute toxic effects are nowadays normally not expected to occur in European surface waters. However, good status in the context of the WFD refers to conditions where also chronic effects do not occur[12]. While concentrations of pollutants in European surface waters are frequently high enough to affect communities, they are frequently too low to be directly detected by bioassaysin the water phase (Jobling et al 1998, Schäfer et al 2007, von der Ohe et al 2009). Long term bioassays would therefore be more suitable to investigate the chronic effects from toxic substances in surface waters, although the less sensitive bioassays could be useful for wastewater assessments. Long term bioassays however, are generally far more expensive and cannot easily be used within high throughput applications, limiting their applicability in a surface water monitoring context. Also in vitro tools frequently require preconcentration of samples before analysis in order to obtain sufficient sensitivity (see below).

When an extraction step is used, the distribution of the chemicals in a reconstituted extract will differ from the distribution in the native sample. Compounds with a similar mode of action can span a large range of physicochemical properties, whereas most extraction techniques target a certain group of chemicals, e.g. polar or charged compounds. Thus, whilst one part of the toxicants is enriched, another part of similarly acting toxicants may be excluded from the sample.

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[12] Refer e.g. to endpoints normally considered in the development of environmental quality standards for chemical substances

Extraction can be performed in two ways, liquid-liquid extraction and SPE (Solid Phase Extraction), but selectivity is an issue with both. Thus, it has to be realised that the chemical mixture in an extract will always differ from the mixture present in the native sample. In order to circumvent this issue it is advisable to use a combination of extraction techniques or sorbents to try to capture as much as possible of the spectrum of chemicals that is present in the sample (e.g. Kern et al 2009). For the typical endpoints involved, the vast majority can be sampled by broad scale SPE techniques. Depending on enrichment needed, assay to perform, potential subsequent analysis work/EDAs planned etc., SPE can be associated with the inconvenience of having to transport, store and handle large volumes of water. One solution to facilitate routine monitoring in combination with SPE would be to use a mobile SPE unit that merely requires having to bring a cartridge to the lab which then can be easily stored and handled.

So far, the concerns raised related to preconcentration of water samples before using bioassays were similar to both in vivo and in vitro use. However, when it comes to the evaluation of results, there are some differences.

In vivo assays and preconcentration – data interpretation

As was indicated above, the primary reason for preconcentrating water samples in the context of in vivo assays is to shorten the exposure time, in order to be able to use short term bioassays as detectors. Live organisms are expected to respond to a large variety of compounds. It is therefore crucial to be aware of what substance groups are included/excluded in extraction processes (see above), as this should also be considered in data interpretation. If elevated toxicity has been observed in a sample, it indicates that the toxic pressure from substances present in the sample (after treatment) is elevated, but absence of toxicity does not necessarily mean that the toxic pressure is of no concern at all. Certain substances (including metals, inorganic salts and ammonia) are excluded from the start, whereas others (such as TBT) are insufficiently eluted but can easily be analysed (chemically, taking bioavailability into account) in parallel. Furthermore, the prediction of chronic effects regarding certain endpoints (such as reduced growth, reproduction etc) from acute toxicity data, traditionally focusing on certain types of endpoints (lethality), is a matter of concern in this context as well as it is within chemicals testing[13]. Certain substances would not be expected to elicit a response in an acute test using regular endpoints (lethality) even if testing concentrations close to solubility limit, but can still be of high concern in longer time scales. It is sometimes simply not possible to raise the concentrations high enough to obtain acute effects, whereas effects would be detected in a long term test using other endpoints. Several PAHs could illustrate such a problem. No acute effects at all are usually detected in an immobilization assay using Daphnia magna in the concentration levels that are possible to test, whereas PAHs can induce serious and other types of effects at low levels after sufficient long exposures. This is not only due to differences in endpoints and the possibilities to discover certain mode of actions only after longer exposures, but also time for absorption of the substance.

Nevertheless, when being aware of these aspects in the interpretation of data, also acute bioassays have been proven useful in several monitoring programmes to identify locations with higher toxic pressures (see case studies "Monitoring concentrated surface water with

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[13]When EQS values are developed, acute toxicity data should not be used alone to develop overall EQS. If this is the case, large assessment factors (1000 for limnic and 10000 for marine environment) need to be applied.

in vivo bioassays" and "The risk of chronic impact of pollution on the Bílina River"). Trend monitoring based on such methods was applied in the Netherlands for more than ten years (see also Durand et al. 2009 for a full description of the method, including technical specifications and validations of the individual steps).

In vitro bioassays – data interpretation

The application possibilities of in vitro assays are to a large extent similar to those of chemical screening analyses. The sensitivity of different *in vitro* assays can differ several orders of magnitudes, and to obtain a valid screening function at current EQS levels a preconcentration of samples is sometimes necessary, which is in accordance with current analytical methods. In vitro assays are also frequently possible to apply on more or less any type of sample, even extracts of materials.

Unlike chemical screening analysis though, the results are not expressed for each individual substance. The results are instead frequently expressed on a "substance equivalent level". The response recorded is then recalculated into the concentration found in the original sample. In that sense, the concentration step can be taken into account in data evaluation. However, it needs to be clearly described, which types of substances will be included and excluded respectively when using a particular concentration procedure. This aspect is also of concern when performing "broad chemical screening analysis". Some substance groups will simply not be detected because they were excluded during the pretreatment. However, as long as this is clearly reported alongside with the results, data interpretation is facilitated.

When it comes to certain in vitro assays, the type of compounds (polar-non polar etc) expected to elicit a response in the test is largely known, whereas other types of in vitro assays may respond to a larger range of compounds. In these cases, it is again advisable to use a combination of techniques to try to capture as much as possible of the spectrum of chemicals that is present (see above).

Passive sampling and bioassays

One issue with taking water samples is that concentrations of chemicals and the make-up of the chemical mixtures change over time; this is particularly true for smaller river catchments. Whereas organisms in the field are exposed to this situation and will integrate these fluctuations, varying concentrations make it difficult to obtain representative samples from the aquatic environment. This is an aspect to consider also in a chemical monitoring approach. On-line monitoring systems would be a solution but these are expensive and only practical for key locations. As an alternative approach, passive sampling was developed to obtain an integrated measure of fluctuating concentrations.

Passive sampling is based on the principle that chemicals diffuse from the bulk water to the surface of a passive sampler where the chemicals sorb to the device (Seethapathy et al 2008;Vrana et al 2010; Mills et al 2009). Two types of passive sampling can be performed: 1) time integrated sampling, and 2) equilibrium sampling. An ideal integrative

passive sampler will show a sampling rate (often denoted in L/d) that remains constant for a long time and where loss of sorbed chemicals from the sampler back to the water phase is minimal. Thus, a benefit of passive sampling is that, over its deployment time (often many weeks), it sorbs chemicals from many litres of water. This provides for a large sample that can be applied to different bioassays whilst at the same time facilitating detection as limits of quantification (LoQ) are, as for chemical analyses, more easily exceeded. An equilibrium sampler is designed to reach equilibrium with the sampled phase fairly quickly; equilibrium is achieved when the amount of chemical that is being sorbed by the device matches the amount that desorbs. Depending on the design of the sampler (i.e. the volume to area ratio) and the sampling conditions (i.e. stagnant or highly turbulent), the time to equilibrium can range from minutes to days (or longer when needed).

When sampling rates are known, the mass of a chemical sampled at the end of a deployment period can be converted into a time-weighted average (TWA) concentration. Although deriving TWA concentrations from passive samplers can work very well for chemical target analysis, it has to be realised that a TWA concentration cannot be calculated for bioassay data as unknown chemicals, with unknown physicochemical properties, will have unknown compound-specific sampling rates (Vrana et al 2010; see also Harman et al 2012).

Although historically the prime focus of passive sampling has been on chemical analysis of target compounds, by now a large number of studies have successfully combined passive sampling with effect-based tools using bioassays (e.g.Vermeirssen et al 2010). The employed bioassays cover various modes of action and cover both cell and organism-based tests. Some of these studies were recently reviewed within the NORMAN network (Vrana et al 2010). A major critical issue that was identified was that, similar to an extraction performed in the lab, a passive sampler will selectively sample a certain class of compounds from the original mixture that is present in the water (Vrana et al 2010; see also Harman submitted). These compound classes can be metals using the DGT sampler, polar organic compounds using a POCIS (polar organic chemical integrative sampler) or a Chemcatcher, or non-polar compounds using an SPMD (semi-permeable membrane device) or silicone sheets. In this sense they offer no advantage over SPE. On the other hand, it can be argued that some passive samplers offer the benefit that they are biomimetic in that, within their domain and not unlike biota, they are more likely to concentrate the more hydrophobic chemicals from the water.

There are various positive aspects to passive sampling that makes it a promising tool for monitoring purposes within the WFD, particularly for investigative monitoring and for identifying spatial and temporal trends. Main positive features are a more integrative assessment of the aqueous concentration and the potential of a larger sample that allows for better detection (Mills et al 2009). Also the combination of passive sampling and effect-based tools offers advantages. Beside the mentioned positive aspects (TWA and a large sample) a more biomimetic sample can be an added benefit. However, passive sampling also has potential weaknesses. First, as it is an extraction procedure it is inevitably selective and does not represent the toxicity of the native sample. Second, environmental conditions such as biofouling and water flow rates affect the performance of the passive sampler in the field. The last two aspects hamper an accurate calculation of TWA water concentrations. There are EU-based efforts to support the effective development of passive sampling (www.norman-network.net) and assess its possibilities for monitoring programmes related to the WFD (www.passivesampling.net/utrechtworkshop/).

4.STANDARDS/GUIDANCES AVAILABLE

In the table below is reported an overview of several available standards and guidance documents on *in vivo* and *in vitro* bioassays as well as some biomarkers. Most of the standardized bioassays were developed for the use within chemical testing and/or effluent testing and not surface water monitoring. In order to use them in a monitoring context, some adaptations to test protocols and pretreatment of samples may therefore be necessary. A second table lists some bioassays that are currently used in a monitoring context.

IN VITRO

Erod*:*

ISO/TS 23893-2, 2007. Water quality -- Biochemical and physiological measurements on fish -- Part 2: Determination of ethoxyresorufin-O-deethylase (EROD)

Ames*:*

(T98 and T100 strains): ISO 16240, 2005; Determination of the genotoxicity using the *Salmonella*/microsome test

ISO 11350, 2012Determination of the genotoxicity of water and waste water – *Salmonella*/microsome fluctuation test (Ames fluctuation test)

umuC*:*

ISO13829, 2000. Determination of the genotoxicity of water and waste water using the *umu*-test

Micronucleus test (V79)*:*

ISO 21427-2: 2006 Water quality -- Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 2: Mixed population method using the cell line V79

OECD Test No. 487: In Vitro Mammalian Cell Micronucleus Test.

Vitellogenin induction test*:*

ISO 23893-3:2013- Water quality -- Biochemical and physiological measurements on fish -- Part 3: Determination of vitellogenin

Estrogenicity[14]

(cell line:BG1Luc): OECD 457, 2012: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists

(cell line: HeLa-9903) OECD 455, 2009: Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals

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[14]ISO WDs on Yeast Estrogen Screens and human in vitro cell culture methods are also in preparation.

BACTERIA

ISO 11348-1:2007 *Water quality -- Determination of the inhibitory effect of water samples on the light emission of* Vibrio fischeri *(Luminescent bacteria test) -- Part 1: Method using freshly prepared bacteria*

ISO 11348-2:2007 *Water quality -- Determination of the inhibitory effect of water samples on the light emission of* Vibrio fischeri *(Luminescent bacteria test) -- Part 2: Method using liquid-dried bacteria*

ISO 11348-3:2007 *Water quality -- Determination of the inhibitory effect of water samples on the light emission of* Vibrio fischeri *(Luminescent bacteria test) -- Part 3: Method using freeze-dried bacteria*

ISO 13641-1:2003 *Water quality -- Determination of inhibition of gas production of anaerobic bacteria -- Part 1: General test*

ISO 13641-2:2003 *Water quality -- Determination of inhibition of gas production of anaerobic bacteria -- Part 2: Test for low biomass concentrations*

ASTM D5660-96:2009 Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium

OECD 224:2007 Determination of the Inhibition of the Activity of Anaerobic Bacteria

ALGAE

ISO 8692:2012 *Water quality -- Fresh water algal growth inhibition test with unicellular green algae*

ISO 10710:2010 *Water quality -- Growth inhibition test with the marine and brackish water macroalga* Ceramium tenuicorne

ISO 10253:2006. Water Quality - Marine Algal Growth Inhibition Test with *Skeletonema costatum* and *Phaeodactylum tricornutum*

ASTM E1218 - 04e1 Standard Guide for Conducting Static Toxicity Tests with Microalgae

OECD 201:2002 (as European Community C3 method) Freshwater algae and Cyanobacteria, growth inhibition test
OECD 221:2002 (as European Community C26 method) *Lemna sp.* Growth inhibition test

USEPA 1988. Methods for toxicity tests of single substances and liquid complex wastes with marine unicellular algae. Environmental Research laboratory, Gulf Breeze, FL, EPA/600/04
USEPA 1974. Marine algal assay procedure bottle test. Eutrophication and Lake Restoration Branch. National Environmental Research Center, Corvallis, Oregon

ROTIFERA

ISO 20666:2008 *Water quality -- Determination of the chronic toxicity to* Brachionus calyciflorus *in 48 h*

ASTM E1440 – 91:2004 Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus*

ASTM E1440 – 91:1998 Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus*

CRUSTACEA

ISO 14371:2012 *Water quality -- Determination of fresh water sediment toxicity to* Heterocypris incongruens *(Crustacea, Ostracoda)*

ISO 14380:2011 *Water quality -- Determination of the acute toxicity to* Thamnocephalus platyurus *(Crustacea, Anostraca)*

ISO 20665:2008 *Water quality -- Determination of chronic toxicity to* Ceriodaphnia dubia

ISO 16712:2005 *Water quality — Determination of acute toxicity of marine or estuarine sediment to amphipods*

ISO 10706:2000 *Water quality -- Determination of long term toxicity of substances to* Daphnia magna Straus

(Cladocera, Crustacea)

ISO 14669:1999 Water quality -- Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)

ISO 6341:1996 Water quality -- Determination of the inhibition of the mobility of Daphnia magna Straus *(Cladocera, Crustacea) - Acute toxicity test*

ASTM E1191 - 03a:2008 Standard Guide for Conducting Life Cycle Toxicity Tests with Saltwater Mysids

ASTM E1295-01:2006 Standard Guide for Conducting Three Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*

ASTM E1193-97:2004 Standard Guide for Conducting Daphnia magna Life Cycle Toxicity Tests

ASTM E1463-92:2004 Standard Guide for Conducting Static and Flow Through Acute Toxicity Tests With Mysids From the West Coast of the United States

ASTM E2317-04 Standard Guide for Conducting Renewal Microplate Based Life Cycle Toxicity Tests with a Marine Meiobenthic Copepod

ASTM E1367:99 Standard Guide for Conducting 10 day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods

OECD 202:2004 (as European Community C2 method) Short-term toxicity on invertebrates (*Daphnia sp.)*

OECD 211:1998 (as European Community C20 method) Daphnia magna Reproduction Test. Guideline for Testing of Chemicals n° 211, adopted 21/09/1998

ENVIRONMENT CANADA 1992. Biological test method: Acute test for sediment toxicity using marine or estuarine amphipods. Report SPE 1/RM/26. Ottawa, ON.

MOLLUSCA

ASTM E2122-02:2007 Standard Guide for Conducting In situ Field Bioassays With Caged Bivalves

ASTM E1022-94:2007 Standard Guide for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks

ASTM E2455-06 Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels

ASTM E724-98:2004 Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

ASTM E2122-01 Standard Guide for Conducting In situ Field Bioassays With Marine, Estuarine, and Freshwater Bivalve

USEPA, 1995. Pacific oyster, Crassostrea gigas and mussel, Mytilus spp. Shell developmental test method. In: US EPA/600/R-95/136, Short-term Methods for Estimating the Chronic Toxicity of Effluents and ReceivingWaters toWest Coast Marine and Estuarine Organisms. Cincinnati

POLYCHAETA

ISO 10872:2010 Water quality -- Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda)

ASTM E1611-00:2007 Standard Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids

ASTM E1562-00:2006 Standard Guide for Conducting Acute, Chronic, and Life Cycle Aquatic Toxicity Tests with Polychaetous Annelids

ECHINODERMATA

ASTM E1563-98:2004 e1 Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos

FISH

*ISO 15088:2007 Water quality -- Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*)*

ISO 12890:1999 Water quality -- Determination of toxicity to embryos and larvae of freshwater fish -- Semi-static method

*ISO 7346-1:1996 Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio *Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 1: Static method*

*ISO 7346-2:1996 Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio *Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 2: Semi-static method*

*ISO 7346-3:1996 Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio *Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 3: Flow-through method*

*ISO 10229:1994 Water quality -- Determination of the prolonged toxicity of substances to freshwater fish -- Method for evaluating the effects of substances on the growth rate of rainbow trout (*Oncorhynchus mykiss *Walbaum (Teleostei, Salmonidae))*

ASTM E1768-95:2008 Standard Guide for Ventilatory Behavioral Toxicology Testing of Freshwater Fish

ASTM E1711-95:2008 Standard Guide for Measurement of Behavior During Fish Toxicity Tests

ASTM E1241-05 Standard Guide for Conducting Early Life Stage Toxicity Tests with Fishes

OECD234:2011 Fish Sexual Development Test

OECD229:2009 Fish Short Term Reproduction Assay

OECD230:2009 21-day Fish Assay

OECD215:2000   (as European Community C14 method) Fish, juvenile growth test

OECD212:1998 (as European Community C15 method) Fish, short-term toxicity test on embryo and sac-fry stages

OECD305:1996 (as European Community C13 method)Bioconcentration: flow-through fish test

OECD203:1992   (as European Community C1 method) Fish, Acute Toxicity Test. In: Guideline for Testing of Chemicals n°203, adopted 17/07/1992

OECD210:1992 Fish, Early-Life Stage Toxicity Test

OECD204:1984 Fish, Prolonged Toxicity Test: 14-Day Study. In: Guideline for Testing of Chemicals n°204, adopted 04/04/1984

OTHER STANDARD GUIDANCES AND TOXICITY TEST METHODS

OECD233:2010 Sediment-water Chironimid life-cycle toxicity test using spiked water or spiked sediment

OECD218:2004 Sediment-water Chironimid toxicity using spiked sediment

OECD219:2004 Sediment-water Chironimid toxicity using spiked water

OECD225: 2007 Sediment-water *Lumbriculus* toxicity test using spiked sediment

ISO/TS 20281:2006 Water quality -- Guidance on statistical interpretation of ecotoxicity data

ASTM E1706-05:2010 Standard Test Method for Measuring the Toxicity of Sediment Associated Contaminants with Freshwater Invertebrates

ASTM E1192-97:2008 Standard Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

ASTM E1525-02:2008 Standard Guide for Designing Biological Tests with Sediments

ASTM E1367-03:2008 Standard Test Method for Measuring the Toxicity of Sediment Associated Contaminants with Estuarine and Marine Invertebrates

ASTM E1847-96:2008 Standard Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

ASTM E1391-3:2008 Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

ASTM E729-96:2007 Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

ASTM E1850-04 Standard Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests

ASTM E1525-02 Standard Guide for Designing Biological Tests with Sediments

USEPA 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition, EPA-821-R-02-012

USEPA 2002b. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Third Edition. EPA-821-R-02-014

USEPA 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. Klemm D.J., Morrison G.E., Norberg-Ring J.J., Peltier W.H., Heber M.A., U.S. Environmental Protection Agency. Report EPA-600/4-91/003, Cincinnati, OH

USEPA 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms (fourth edition). Weber C.I. Eds. EPA /600/4-90/027F, Ecological monitoring research division, Environmental monitoring system laboratory. Cincinnati, Ohio 45268

Biological effects (and metabolite) monitoring techniques in the TIMES series (ICES):

No.11. Thain, J.E. 1991. Biological effects of contaminants: oyster (Crassostrea gigas) embryo bioassay. 12 pp.

No.13. Galgani, F., and Payne, J.F. 1991. Biological effects of contaminants: microplate method for measurement of ethoxyresorufin-O-deethylase (EROD) in fish. 11 pp.

No.19. Bucke, D., Vethaak, D., Lang, T., and Mellergaard, S. 1996. Common diseases and parasites of fish in the North Atlantic: training guide for identification. 27 pp.

No.22. Bocquené, G., and Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. 12 pp.

No.23. Stagg, R., and McIntosh, A. 1998. Biological effects of contaminants: Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity. 16 pp.

No.24. Gibbs, P.E. 1999. Biological effects of contaminants: Use of imposex in the dogwhelk, (Nucella lapillus) as a bioindicator of tributyltin (TBT) pollution. 29 pp.

No.25. Reichert, W.L., French, B.L., and Stein, J.E. 1999. Biological effects of contaminants: Measurement of DNA adducts in fish by 32P-post-labelling. 45 pp.

No.26. Hylland, K. 1999. Biological effects of contaminants: Quantification of metallothionein (MT) in fish liver tissue. 18 pp.

No.28 Thain, J., and Roddie, B. 2001. Biological effects of contaminants: Corophium sp. sediment bioassay and toxicity test. 21 pp.

No.29 Thain, J., and Bifield, S. 2001. Biological effects of contaminants: Sediment bioassay using the polychaete Arenicola marina. 16 pp.

No.31 Scott, A.P., and Hylland, K. 2002. Biological effects of contaminants: Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for the measurement of marine fish vitellogenins. 21pp.

No.34 Hylland, K. 2004. Biological effects of contaminants: Quantification of d-aminolevulinic acid dehydratase (ALA-D) activity in fish blood. ICES Techniques in Marine Environmental Sciences. 9pp.

No.36 Moore, M.N. and Lowe, D. 2004. Biological effects of contaminants: Measurement of Lysosomal membrane stability. 31pp.

No.37 Oehlmann, J. 2004. Biological effects of contaminants: Use of intersex in the periwinkle (Littorina littorea) as a biomarker of tributyltin pollution. 22pp.

No.38 Feist, S.W., Lang, T., Stentiford, G.D., and Köhler,A. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (Limanda limanda L.) and flounder (Platichthys flesus L.) for monitoring. 42pp.

No.39 Ariese, F., Beyer, J., Jonsson, G., Visa, C.P., Krahn, and M.M. 2005. Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile. 41 pp.

No.40 Widdows, J. and Staff, F. 2006. Biological effects of contaminants: Measurement of scope for growth in mussels. 30 pp.

No.41 Sundelin, B., Eriksson Wiklund, A-K., and Ford, A. T. 2008. Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors. 21 pp.

No.49 Jonsson, G., Nævdal, A., and and Beyer, J. 2012. Determination of alkylphenol metabolites in fish bile using solid-phase analytical derivatization (SPAD) and gas chromatography–mass spectrometry in electron ionization mode (GC–EI–MS). ICES
Techniques in Marine Environmental Sciences No. 49. 18 pp.

No.51 Beiras, R., Durán, I., Bellas, J., and Sánchez-Marín, P. 2012. Biological effects of contaminants: Paracentrotus lividus sea urchin embryo test with marine sediment elutriates. ICES Techniques in Marine Environmental Sciences No. 51. 13 pp.

OSPAR JAMP guidelines on general and specific contaminant effects monitoring tools

JAMP Guidelines for Contaminant-Specific Biological Effects (OSPAR Agreement 2008-09):

Technical Annex 1: Metal-specific biological effects monitoring (Metallothionein, δ-amino levulinic acid dehydratase inhibition in blood (ALA-D), Oxidative stress).

Technical Annex 2: PAH-specific biological effects monitoring (Cytochrome P4501A, DNA adducts, PAH metabolites, Liver pathology, Macroscopic liver neoplasms)

Technical Annex 3: TBT-specific biological effects monitoring (imposex)

Technical Annex 4: Estrogen-specific biological monitoring (Bile estrogenicity – ER CALUX or YES recommended methods, Vtg induction, Gonadal intersex)

JAMP Guidelines for General Biological Effects Monitoring (agreement 1997-7):

Technical Annex 1: Whole sediment bioassays[15]
Technical Annex 2: Sediment pore-water bioassays
Technical Annex 3: Sediment sea water elutriates
Technical Annex 4: Water bioassays
Technical Annex 5: CYP1A
Technical Annex 6: Lysosomal stability
Technical Annex 7: Liver histopathology
Technical Annex 8: Macroscopic liver neoplasms
Technical Annex 9: Externally visible fish diseases
Technical Annex 10: Reproductive success in fish

List of in vivo bioassays applied by certain member states within marine monitoring				
Assay	**Endpoint (unit)**	**Species**	**Exposure**	**Protocol**
Bacteria (water, whole sediment, elutriate, pore water)	Bioluminescence	*Vibrio fischeri*	5-10-15minutes	ISO 2004
Algae (water, elutriate, pore water)	Growth	*Phaeodactylum tricornutum Skeletonema costatum*	72hours	ISO 2006
Rotifera (water, elutriate, pore water)	Mortality	*Brachionus plicatilis*	24-48hours	ASTM 2004
Crustacea Amphipoda (whole sediment)	Mortality	*Corophium orientale*	10days	ISO 2005
Crustacea Anostraca (water, elutriate, pore water)	Mortality	*Artemia franciscana*	24hours / 14days	ARTOXKIT 1990) / Manfra et al 2012[*]
Crustacea Copepods		*Acartia tonsa*		ISO 1999

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[15]The oyster embryo test (Thain, 1991) is recommended as in vivo bioassays in this context. However, other species e.g. the harpacticoid copepods *Tisbe* and *Nitocra*, and the polychaete *Dinophilus* may be required for specific applications

(water, elutriate, pore water)	Mortality	*Tigriopus fulvus*	96hours	ISO 1999 modified Faraponova et al. 2005
Bivalva embryo (water, elutriate, pore water)	Development	*Crassostrea gigas Mytilus galloprovincialis Tapes philippinarum*	24-72hours	USEPA 1995 ASTM 2004a His et al 1997
Sea urchins (water, elutriate, pore water)	Fertilization Development	*Paracentrotus lividus Sphaerechinus granularis*		USEPA 1995 ASTM 2004b
Policheta	Mortality	*Hediste diversicolor*	10days	ASTM 2007
Fish	Mortality and genotoxic damage	*Dicentrarchus labrax*	96hours 28days	EC 440/2008: C1 fish acute toxicity test C13fish bioconcentration test (OCSE TG 305 1996) C14 fish growth test (OCSE TG 215 2000)

* this is not a scientific article but a publication and visualization of the bioassay method

5.VALIDATION AND STANDARDISATION OF BIOASSAYS AND BIOMARKERS

As water pollution may often be a trans-frontier problem, accepted international standards allow a common understanding on water quality problems among several countries sharing the same water body. For effect-based monitoring tools within the WFD, ideally validated and standardized methods with existing Standard Operating Procedures (SOPs) should be used. Such SOPs ensure reliability, relevance and quality of the resulting data.
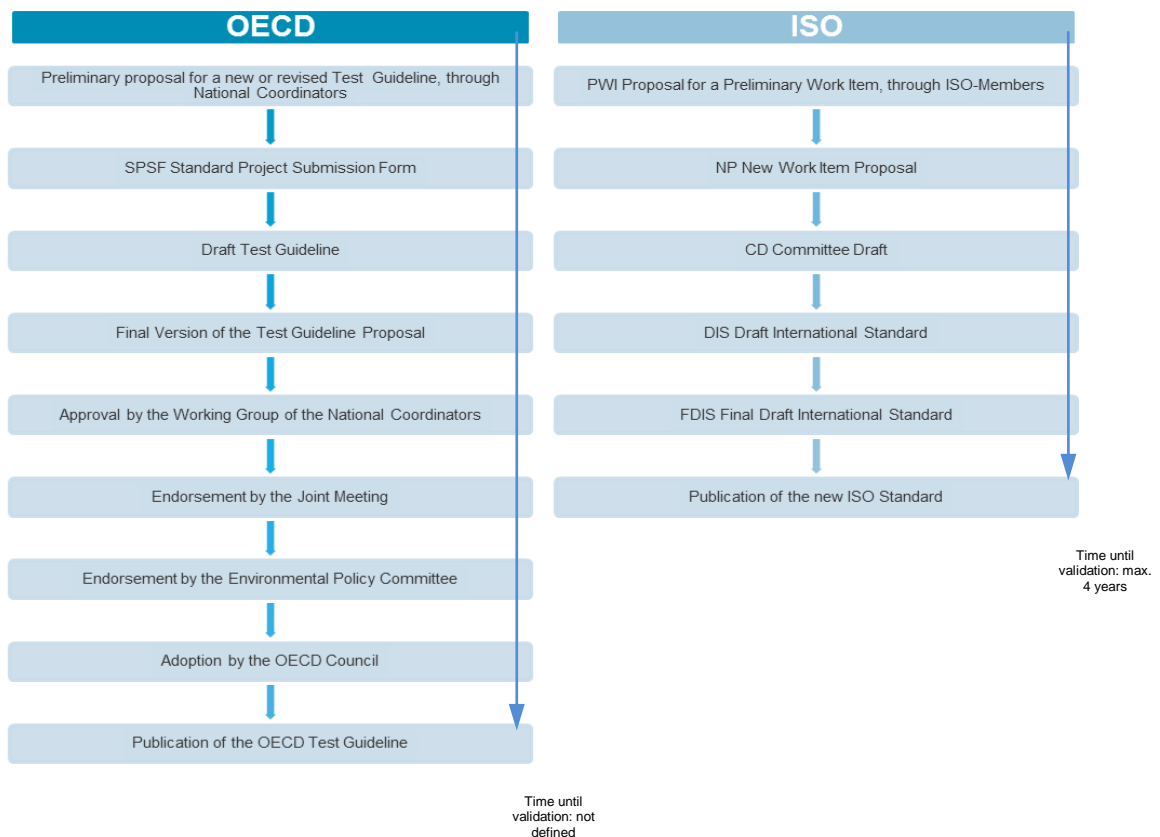
Internationally, the OECD (Organisation for Economic Co-operation and development) and ISO (International Organisation of Standardisation) are the most important bodies for development, validation and standardization of analytical as well as effect based test methods. Whereas the Test Guidelines Programme, within the Environmental Directorate of the OECD is focused on test methods for single substance testing, the Technical Committee (TC) 147 "Water Quality" of ISO is dedicated to the environmental aspects of water quality control.

Currently, several bioassay methods are under validation within ISO/TC 147. In addition, the planned standardization of several test systems to assess estrogenic and androgenic activity under the lead of Germany was officially encouraged by the ISO.

The validation of test methods is a process based on scientifically sound principles (Balls et al. 1990 a-b) in order to establish the reliability and relevance of a particular test approach or method for a specific purpose.

- **Reliability** is in this context defined as the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardised protocol (OECD, 2005).

- The **Relevance** of a test method describes the relationship between the test and the effect in the target species and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. In brief, it is the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate. Regulatory need, usefulness and limitations of the test method are aspects of its relevance (OECD, 2005).

The figure below provides an overview of the validation and standardization steps within OECD and ISO, including the time frames involved, for details see also OECD (2008).

OECD

Preliminary proposal for a new or revised Test Guideline, through National Coordinators

SPSF Standard Project Submission Form

Draft Test Guideline

Final Version of the Test Guideline Proposal

Approval by the Working Group of the National Coordinators

Endorsement by the Joint Meeting

Endorsement by the Environmental Policy Committee

Adoption by the OECD Council

Publication of the OECD Test Guideline

Time until validation: not defined

ISO

PWI Proposal for a Preliminary Work Item, through ISO-Members

NP New Work Item Proposal

CD Committee Draft

DIS Draft International Standard

FDIS Final Draft International Standard

Publication of the new ISO Standard

Time until validation: max. 4 years

At an OECD Workshop in Solna Sweden in 1996 a set of **_principles for validation_**("Solna Principles") were developed (OECD 1996). These apply to the validation of new or updated test methods, whether they are in vivo or in vitro, or tests for effects on human health or the environment. The principles are stated below; as regards point 5 in a WFD context, also environmental samples would need to be included in the evaluation of performance.

1) A rationale for the test method should be available. This should include a clear statement of scientific need and regulatory purpose.
2) The relationship of the endpoint(s) determined by the test method to the _in vivo_ biological effect and to the toxicity of interest should be addressed. The limitations of a method should be described, e.g., metabolic capability.
3) A formal detailed protocol must be provided and should be readily available in the public domain. It should be sufficiently detailed to enable the user to adhere to it, and it should include data analysis and decision criteria. Test methods and results should be available preferably in an independent peer reviewed publication. In addition, the result of the test should have been subjected to independent scientific review.
4) Intra-test variability, repeatability and reproducibility of the test method within andamongst laboratories should have been demonstrated. Data should be

provided describing the level of inter- and intra-laboratory variability and how these vary with time.

5) The test method's performance must have been demonstrated using a series of reference chemicals preferably coded to exclude bias.
6) The performance of test methods should have been evaluated in relation to existingrelevant toxicity data as well as information from the relevant target species.
7) All data supporting the assessment of the validity of the test methods including the full data set collected in the validation study must be available for review.
8) Normally, these data should have been obtained in accordance with the OECD Principles of Good Laboratory Practice (GLP) (OECD 1997).

Prior to and during the validation process, several important steps and aspects should be considered (OECD 2005). These are:

(i) The definition of the test method and related issues (e.g., purpose, decision criteria, endpoints, limitations);
(ii) the design and conduct of pre-validation studies (1st ring testing) leading to the optimization of the test method;
(iii) the design and conduct of the formal inter-laboratory validation work (2nd ring testing), based on the outcomes of the pre-validation studies and aiming at accumulation of data on the relevance and reliability of the test method, and;
(iv) the overall data evaluation and subsequent validation study conclusion, keeping in mind the requirements of regulatory authorities, for submission of information relating to new or modified test procedures.
(v) the need for and the extent of an independent evaluation, or peer review, of test methods being validated

Similar points are considered within **the ISO/TC 147 "Water Quality** (ISO/IEC 2011 a and b), and from history, emphasis of the ISO/TC 147 laid on the environmental aspect (Water quality control in respect to surface water, waste water, ground water and precipitation). Almost all standards are validated via inter-laboratory comparison tests (ring testing).

Additional points have to be considered for the validation of test systems for the assessment of environmental samples:

- Ring tests ideally should be conducted with model substances as well as environmental samples
- Depending on the test method under validation,environmental samples should be analyzed within the intra- and inter laboratory ring tests
- For environmental sample testing the test systems have also to be validated for their susceptibility towards samples with differing salinity, pH or cytotoxicity
- In order to conduct ring tests with environmental samples, the procedures used for sampling, sample handling and storage should be standardized (within the ISO/TC 147 such procedures are available).

Several biomarkers have also been widely validated by the scientific community and organizations like the International Council for the Exploration of the Sea (ICES) and the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR). Therefore, although international standards are available only for a few

analyses involved (such as EROD, vitellogenin), several additional biomarkers fulfill many of the principles that are to be considered in the context of standardization.

In particular, within ICES, the Working Group on Biological Effects on Contaminants (WGBEC) regularly categorises effect-based marine monitoring tools according to their current status. To include a method in the category of recommended methods, it needs to be established and available as a published method in the TIMES series of ICES or elsewhere. It should also have been shown to respond to contaminant exposure in the field and be able to differentiate between effects of contaminants from background variability. Although not strictly required to be categorized as a recommended tool, several such methods have also been subject to intercalibration studies, arranged by either BEQUALM(Biological Effects Quality Assurance in Monitoring Programmes), QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring) or UNEP MEDPOL (marine pollution assessment and control component of MAP, Mediterranean Action Plan for the Barcelona convention).

6.FACT SHEETS FOR CERTAIN BIOMARKERS AND BIOASSAYS

This section includes a number of fact sheets with information oncertain technical aspects that may be relevant to consider in the design of monitoring programmes within different contexts. The fact sheets are included for general information purposes only, and those included should not necessarily be considered to be recommended to include in a general sense.

The appropriateness to include a certain tool or battery of tools in a monitoring programme or campaign will often depend on the purpose of the programme and other case-specific circumstances (see e.g. case studies above). For more detailed and updated information, the reader is also recommended to check thelatest version of established protocols and technical background documents.

Because of the comparatively high availability of standards for in vivo bioassays (see section 4 above), such fact sheets are generally not included, with the exception of FET (Fish Embryo Toxicity).

Rough cost estimates for the analyses involved are included, primarily based on information from performing laboratories. Costs up to about 200 Euro are referred to as "low", between 200 and 500 as "moderate" and costs above 500 Euro (up to 1000) as "high". The costs for analysing individual endpoints are, with some exceptions, generally low or moderate, whereas costs for sampling can be high. Please note that the cost indicated in the fact sheets refer only to the analysis itself, not sampling and preparation of tissues etc.By integrated sampling (to provide samples for both chemical analyses and effect-based investigations), the total costs for sampling can be reduced. In many cases, it has been assumed that several samples are analysed at the same time. The costs for a single sample analysis can be higher.

Most of the information on biomarkers in the fact sheets is based on OSPAR/ICES documents such as WGBEC technical background documents, TIMES guidance documents and JAMP guidelines developed for tools that are currently included in the marine monitoring programmes. Certain information (such as costs and the applicability to also limnic water environments) has been obtained during a workshop on effect based monitoring tools (see Wernersson 2012), by personal communication with performing laboratoriesand experts and from scientific publications. However, some information was still not available to the authors. Fact sheets on a few other biomarkers than those that are frequently used for the marine environment, either being less established or applicable only to freshwater environments, have also been included, to better cover the WFD area of applicability.

Metallothionein (MT)

Metallothionein (MT) is a protein that is present in most vertebrate and some invertebrate cells. One third of the MT protein consists of cysteine, an amino acid that contains sulphydryl groups that can bind to metals. MT normally acts in regulation of the intracellular bioavailability of essential elements (Zn, Cu) but is also induced by and binds to non essential metals. Hepatic MT in fish is frequently used as a biomarker for certain metals (Cu, Zn, Cd). The response can be detected within a few days (Hogstrand et al 1991[16]), and disappears rather rapidly after exposure has seized, but this can depend on the species.

- **What is analysed (endpoint; unit):** Concentration of MT (common unit is ug/mg cytosolic protein).[17]
- **Tissue/cells examined:** In fish, usually liver cells
- **Method used:** OSPAR JAMP recommends any of three methods[18]. Variability between laboratories observed (OSPAR 2007). Marine Quality Assurance procedures available through BEQUALM (fish) and MEDPOL (mussels) (Davies & Vethaak 2012)
- **Amount sample needed:** Approximately 100 mg fish liver/analysis.
- **Possible to use stored samples?** Yes, if tissues are frozen in liquid nitrogen and stored at below -70°C to prevent degradation by proteases.
- **Possible to use in both limnic and marine environment?** Can be analysed in marine, brackish and limnic fish species, but also other types of organisms (such as Mytilus edulis)[19].
- **When to take samples?:** one month outside the spawning season.
- **Exposure/Effect biomarker?** Considered an exposure biomarker, it provides an early warning signal (considered sensitive, but the sensitivity varies between methods). It is recommended to compare the results to suitable reference sites to identify locations with significant responses[20], and use data in an integrated assessment[21] before making conclusions about risk of ecological effects.
- **General/Specific biomarker?** Considered a specific biomarker because it responds primarily to metals (especially Cu, Zn and Cd)[22], but also to oxidative stress (free radicals) and organics. Other influencing factors that need to be taken into account in the design of monitoring programmes and evaluation of data are: sex, season, temperature, age (OSPAR 2007), local conditions, analytical procedures (Hylland 1999), fish size, condition and GSI.
- **Costs:** Low

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[16] Hogstrand et al 1991. Significant response after 2d of injection of 2 mg of Cd, maximum response after 6d.

[17] Analysing the amount of MT protein is preferred instead of MT mRNA because physiological/toxicological response depends on MT concentration and not mRNA. Also the half life of MT is longer than that of mRNA. In addition, it is easier to preserve tissues than mRNA.

[18] ELISA, differential pulse polarography or spectrometric assay. An intercalibration study showed similar results were obtained if using metal saturation and polarographic analyses whereas ELISA and colorimetric assay results deviate from overall median. However, while the electrochemical method is also more sensitive, it involves the use of mercury (to be phased out), whereas ELISA uses antibodies and is mercury free. The spectrometric assay (also mercury free) requires larger amounts of material.

[19] Metallothioneins are generally similar but may vary in composition and number of isoforms between the species. JAMP guidelines do not recommend the wide use of MT in invertebrates in monitorings programs, because of the limited experience though.

[20] If there is background knowledge on the species, and sampling is performed during same and appropriate time period and on same sex and size etc, comparison to baseline data can also be made.

[21] Parallel analysis of Cd, Zn and Cu is strongly recommended to facilitate the evaluation of results. JAMP guidelines recommends the use of MT and ALA-D as second tier analyses at sites where sediments are contaminated by metals, to assess impact on fish. As a third tier, antioxidant enzymes and products of oxidative damage (superoxide dismutase, glutathione reductase, glutathione peroxidase and malonedialdehyde, catalase and tissue lipid peroxides; see Livingstone et al 1993) are recommended, to investigate deleterious effects.

[22] In in vitro experiments also response to inorganic mercury has been reported but the response to methylated Hg is unclear and MT is therefore currently not recommended as a biomarker for Hg contamination. MT was also shown to be induced by AgS_2O_3, AgCl and $AgNO_3$, but the latter compound is suspected to cause osmoregultory disturbance preceding MT induction (Hogstrand et al 1991).

ALA-D

The enzyme δ-amino levulinic acid dehydratase (ALA-D) is essential for haemoglobin (Hb) synthesis. It is inhibited by lead and at levels before other signs of toxicity are apparent. Responds shortly after exposure.

- **What is analysed (endpoint; unit):** ALA-D activity: porphobilinogen (PBG) formed per unit time and protein (nmol PBG/mg protein/min)
- **Tissue/cells examined:** Lysed red blood cells
- **Method used**: The amount PBG formed from added ALA. PBG is analysed spectrophotometrically after reaction with Ehrlich's reagent (Johannson-Sjobeck & Larsson, 1978). Intercalibration studies performed.
- **Amount sample needed**: approx. 20-50 ul blood
- **Possible to use stored samples**: Blood samples should be taken either prior to or within 5 minutes after killing the fish; the sample can be either centrifuged directly or frozen in liquid nitrogen.
- **Possible to use in both limnic and marine environment**: Yes[23]
- **When to take samples:** one month outside the spawning season.
- **Exposure/effect biomarker:** Considered an exposure biomarker, it provides an early warning signal of lead poisoning, but before other, more severe signs of lead poisoning occur.
- **Specific/general biomarker:** Very specific because it primarily responds to Pb levels (in the blood). Response due to other substances can however not be entirely excluded.
- **Costs:** Low

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[23] In fact, most experience from using this biomarker is on limnic fish, although also successfully applied to marine fish

Cytochrome P450 1A activity (EROD; CYP 1A activity)

EROD has been probably the most frequently analysed fish biomarker. The excretion of organic hydrophobic substances in vertebrates is facilitated by a stepwise transformation into more water soluble compounds. The first step (phase I) in this process is usually catalysed by the enzyme system called Cytochrome P450 oxygenases[24]. There are many forms of this enzyme family, also divided into subfamilies. One such subfamily is the CYPA1, considered to be particularly important and involved in transforming planar molecules. Besides being a substrate, planar molecules can also bind to the cytosolic Ah receptor, inducing the P450 1A system. This induction can be measured by adding the substrate 7-ethoxyresorufin, which will become de-ethylated into resorufin (product) by EROD (7-ethoxyresorufin-O-deethylase).

- **What is analysed (endpoint; unit):** Resorufin production (pmol/min/mg protein).
- **Tissue/cells examined:** In fish, generally liver extracts but also gill preparations
- **Method used**: Analytical procedures described by Stagg and Macintosh (1997). Intercalibration studies have been performed[25]. Marine Quality Assurance procedures available through BEQUALM, MEDPOL and between particular independent laboratories (Davies & Vethaak 2012)
- **Amount sample needed**:Approximately 100 mg fish liver/analysis.
- **Possible to use stored samples?** Yes, but requires that liver has been separated and stored in liquid nitrogen to prevent loss of activity.
- **Possible to use in both limnic and marine environment?** Can be used for both marine, brackish and limnic fish species, but is less applicable for invertebrates (not applicable for mussels).
- **When to take samples?** For trend monitoring,preferably same two week period should be sampled each year.Sampling should take place at least one month after spawning. The water temperature and the closely linked ovary development are important co-factors for enzyme activity.
- **Exposure/Effect biomarker?** Considered an exposure biomarker, it provides an early warning signal and indicates that there is a cellular response to Ah-receptor agonists. It is recommended to compare the results to suitable reference sites to identify locations with significant responses and use data only in an integrated assessment[26] before making conclusions about risk of ecological effects.
- **General/Specific biomarker?** Considered a moderately specific biomarker because it responds primarily to Ah-receptor agonists. Strong EROD inducers[27] are dioxins, planar PCBs and PAHs (such as Benzo(a)pyrene) whereas branched PAHs often do not trigger the same response. Other influencing factors that need to be taken into account in the design of monitoring programmes and evaluation of data are: developmental stage, sex[28], age, reproductive status of the organism, as well as temperature, season and dietary factors (lack of food can cause lack of EROD response), possibly also low oxygen levels. EROD induction can be inhibited by too high concentrations of the Ah-receptor agonists as well as other chemicals like metals and xenooestrogens (Brüsch et al 1996, Hanson et al 2006). In extreme situations, it is necessary to take into account, the possibility that

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[24] Previously called MFO, Mixed Function Oxidase

[25]Intercalibration studies show large variability in results, thus emphasizing the application of internal quality assurance procedures, such as the use of references for all batches.

[26]Other biomarkers of relevance to include are e.g. LSI (Liver Somatic Index), PAH metabolites in bile, DNA adducts, liver histopathology, macroscopic liver neoplasms (liver nodules), Because liver may be required also for chemical analysis, care should be taken to make sure sufficient material is available. However, 1g is considered sufficient for EROD and DNA adducts, histopathology and chemical analysis (OSPAR 2007).

[27]"Strong inducer"=substance causing >100 fold induction compared to control.

[28] It is e.g. not recommended to mix sexes or sizes.

populations that can induce EROD have disappeared. Low EROD concentrations can indicate both clean or heavily polluted areas.EROD induction also gradually decreases with time in caged studies. The reponse occurs soon after exposure, but how long it lasts after exposure has seized depends on metabolisation abilities of the compounds in question. Changes in EROD response are therefore also primarily suggested to be related to PAHs.

- **Costs:**Low

DNA adducts

DNA adducts are covalent structures that indicate exposure to genotoxic compounds, integrating several factors, including uptake, metabolism and DNA repair. Target tissues are e.g. liver. The most sensitive method is 32P postlabelling[29], being able to detect a wide range of carcinogens (prior characterisation is not necessary). DNA adducts are persistent (several months) and the response is thus cumulative, although adducts can be removed by repair processes and cell death. In chronic exposures, adducts often reach steady state concentrations.

- **What is analysed (endpoint; unit):** Number of adducted nucleotides per number of undamaged nucleotides; but also analysed as diagonal radioactive zones, DRZs (composite of multiple overlapping DNA adducts) [30].
- **Tissue/cells examined:** In fish, usually liver cells
- **Method used:** P32 labelling is the most specific and sensitive method but also time consuming. OSPAR JAMP recommends methodology described in Stein et al (1993, 1994). Marine Quality Assurance procedures not yet available (Davies & Vethaak 2012) but interlaboratory QA programmes have been conducted by BEQUALM & IARC and there is a standardised protocol available (Times technical document, IARC publications). Variability between laboratories reasonable but it is important to use external standard.
- **Amount sample needed:** Approximately 100 mg fish liver/analysis[31]
- **Possible to use stored samples?** Yes, if stored at – 70C or in liquid nitrogen
- **Possible to use in both limnic and marine environment?** The 32P post labelling method is not species specific. Can be analysed in marine, brackish and limnic fish species
- **When to take samples:** No indications that season would influence results.
- **Exposure/effect bioamarker?** Exposure biomarker in the sense that if DNA adducts are detected, it should be considered an early warning response. However, it is also a possible predictor of pathology (correlations have been observed between DNA damage and certain lesions, such as neoplastic liver disease, foci of cellular alteration and neoplasia). Therefore higher concentrations suggest that a decreased fitness could occur but threshold effect levels are not yet established.Background data usually show no detectable adducts or very faint DRZs.
- **General/specific biomarker?** DNA adducts can be caused by several genotoxic compounds but in particular, several PAHs are known to cause genotoxicity by the formation of adducts. The levels are not significantly affected by age, sex, season or dietary status. However, because detoxicification systems (e.g. CYP1A) are influenced by changes in environmental variables, these factors should always be considered (including salinity and temperature).
- **Costs:** Depends on method, the P32 labelling method: High costs (because time consuming).

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[29] A semi quantitative method; not all DNA adducts are labelled with the same efficiency and enrichment/chromatographic steps will select certain adducts over others.

[30] if exposed to complex mixture of genotoxic compounds)

[31] Because liver may be necessary for the chemical analysis care should be taken to make sure sufficient material is available. However, 1g is considered sufficient for EROD and DNA adducts, histopathology and chemical analysis (OSPAR 2007).

PAH metabolites

*Because of their rapid metabolization, the chemical quantification of PAH in fish tissues provides only limited information. An alternative but indirect indicator of PAH exposure is the concentration of PAH metabolites in the bile fluid of fish. PAH metabolites in bile/urine represent the final stage of the biotransformation process, during which more soluble compounds are formed.*The PAH metabolited in fish bile represent exposure during last hours – few days, at the most 2 weeks

- **What is analysed (endpoint; unit):** e.g. 1-hydroxypyrene or 1-hydroxyphenanthrene [ng/ml][32].
- **Tissue/cells examined:** bile fluid
- **Method used**: See e.g. review by Ariese et al 2005(ICES TIMES no 39) and Beyer et al 2010. Intercalibration studies performed.ICES Times describes different quantification methods for PAH metabolites; to be used either as screening techniques (fixed wavelength fluorescence (FWF) and synchronous fluorescence spectrometry (SFS)) or qualitative and quantitative analyses (HPLC-F and GC-MS)[33]. The latter two methods do not measure single metabolites but reflect a sum parameter. The results can be expressed as "equivalents" to one single standard such as pyrene.Some studies propose conversion factors for SFS and HPLC-F/GC-MS. So far none of the monitoring schemes recommend a single analytical method for the determination of PAH metabolites. Consequently several methods have been applied for different purposes in monitoring and research.Results from HPLC-F or GC-MS cannot be compared directly to FWF or SFS results. Marine Quality Assurance procedures available through QUASIMEME and BEAST (Davies & Vethaak 2012).
- **Amount sample needed**: 100 µl of bile
- **Possible to use stored samples**: yes, if stored frozen (-20°C)
- **Possible to use in both limnic and marine environment**: yes, without any problem
- **When to take samples:** in the same season – this marker has a seasonal trend.
- **Exposure/effect biomarker:** exposure biomarker, measuring the flux of PAHs through the fish body, and thus better related to biotic stress in the fish from PAHs than parent PAHs (in e.g. liver).
- **Specific/general biomarker:** Specific to PAH. Because of periodic release of bile into the oesophagous, being related to the digestive process, feeding status can influence volume and density of bile, which needs to be taken into account in data interpretation[34]. Parallell analysis of biliverdin/protein or bile pigments is recommended; bile pigments can be determined easily by VIS-absorption of the bile at 380 nm..
- **Costs:** Low

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[32] Using GC-MS and HPLC-F
[33] Considered to give comparable results
[34] If the individual has fed just before sampling, gall bladder may be empty.

Liver histopathology (LH)

Histological changes are pathological repsonses observed in fish livers.

- **What is analysed (endpoint; unit):**Occurrence of changes (category and preferably also type of lesion). Can be evaluated as part of FDI (Fish Disease Index).
- **Tissue/cells examined:**fish liver tissues
- **Method used**: Standard histological procedures on tissues embedded in paraffin. Categorisation into five different classes (non specific lesions, early non neoplastic lesions, foci of cellular alteration, benign neoplasms and malign neoplasms), but preferably also the type of lesions (e.g. fibrosis, granuloma and apoptosis that are examples of non specific lesions).Marine Quality Assurance procedures available through BEQUALM (Davies & Vethaak 2012).
- **Amount sample needed**: 30-50 individuals (or suitable number depending on statistical requirements)
- **Possible to use stored samples**: Yes, after fixation stored in 70% alcohol
- **Possible to use in both limnic and marine environment**: Yes
- **When to take samples:** Outside the spawning season
- **Exposure/effect biomarker:** Effects biomarker
- **Specific/general biomarker:** General but can be diagnostic depending on the type of lesion. Belongs to the five selected PAH specific biological effects monitoring tools in the OSPAR preCEMP programme
- **Costs:**

Macroscopic liver neoplasm (MLN)

Macroscopic liver neoplasms are visible tumors on the surface of fish livers.

- **What is analysed (endpoint; unit):** Occurrence of histologically confirmed cases of macroscopic liver neoplasms (malign and benign, but not including pre-neoplastic stages). Can be evaluated as part of FDI (Fish Disease Index).
- **Tissue/cells examined:**Fish liver
- **Method used**: Performed in two steps; the first involves quantification of nodules that are larger than 2 mm (Bucke et al 1996) and the second (Feist et al 2004), histolopathological examination of the nature of the tumors (classification into either malign or benign). Intercalibration studies performed andmarine Quality Assurance procedures available through BEQUALM (Davies & Vethaak 2012)
- **Amount sample needed**: 50 individuals (or suitable number depending on statistical requirements)
- **Possible to use stored samples**: Fixative used is e.g. formalin
- **Possible to use in both limnic and marine environment**: Yes
- **When to take samples:** Outside the spawning season
- **Exposure/effect biomarker:** Effects biomarker, of which malign tumors are more serious, because linked with the progression of carcinogenesis.
- **Specific/general biomarker:**Cancer inducing chemicals.Belongs to the five selected PAH specific biological effects monitoring tools in the OSPAR preCEMP programme, although neoplasms can also be the result of exposure to other types of substances.
- **Costs:**

Externally visible fish diseases

Significant changes in the prevalence of externally visible fish diseases indicate chronic stress.

- **What is analysed (endpoint; unit):** Occurrence of different categories of Externally Visible Diseases, EVD (in dab: lymphocystis, epidermial hyperplasia/papilloma, acute/healing skin ulceration, X-cell gill disease, hyperpigmentation, acute/healing fin rot/erosion, Stephanostomum baccatum, Acanthochondria comuta, Lepeophtheirus pectoralis); for most diseases expressed on three severity grades (light, medium or severe disease status). A Fish Disease Index (FDI) is calculated based on EVB, macroscopic liver neoplasms (MLN) and liver histopathology (LH). Also the impact on the host is taken into account, and adjustment factors are related to size, sex and season (for MLN and LH also age).
- **Tissue/cells examined:**Fish, external investigations
- **Method used**: Bucke et al (1996).Marine Quality Assurance procedures available through BEQUALM (Davies & Vethaak 2012); intercalibration and ring tests regularly performed, including sea-going workshops.
- **Amount sample needed**:
- **Possible to use stored samples**:
- **Possible to use in both limnic and marine environment**:
- **When to take samples:**
- **Exposure/effect biomarker:** Effect biomarker, related to ecosystem health
- **Specific/general biomarker:** General biomarker in the sense that there may be many factors behind the diseases observed. Although most wild fish diseases observed are caused by viruses/bacteria, other factors may be required before the disease develops. Contaminants can e.g. affect the immune system. Disease can also occur as a result from changes at different levels of biological organisation, without the involvement of pathogens.
- **Costs:**

Reproductive success in eelpout

The eelpout ("viviparous blenny") is a viviparous species, i. e. the females give birth to living larvae. Each female carries between 20-300 embryos/larvae. Reproductive success and malformations can therefore easily be studied. It is also known to be narrowly territorial although limited migration could occur due to change in water temperature.

- **What is analysed (endpoint; unit):** Currently agreed endpoints are Mean prevalence malformed[35] fry, late dead[36] fry, early dead fry and total abnormal fry.In addition, it is possible also to analyse the sex ratio, which, under normal conditions should be 50:50.
- **Tissue/cells examined:** Embryos (viviparous)
- **Method used**: OSPAR JAMP guidance refers to Jacobsson et al 1986; Neuman et al 1999 and Strand & Dahllöf 2005. Marine Quality Assurance procedures available through BEQUALM (Davies & Vethaak 2012)
- **Amount sample needed**: Fourty individuals (females) should be sampled as a minimum
- **Possible to use stored samples**: No
- **Possible to use in both limnic and marine environment**? For marine studies only. The eelpout inhabits coastal waters from the White Sea to southern North sea and the Baltic (both marine and brackish environments). However the species is not abundant in all areas and may be protected.
- **When to take samples:** Autumn-early winter (late October-early December), but depends on time of reproduction and can vary between geographical areas
- **Exposure/effect biomarker?**Effect biomarker. Reproductive success is directly related to expected negative effects on population level, although other factors (such as eelpouts being caught in fyke nets) can also significantly influence local populations. Risk of population effects from dead or malformed embryos will depend on other, site specific, variables as well.
- **General/specific biomarker?** General biomarker in the sense that it responds to several different types of xenobiotics, including organochlorines, pesticides, PAH, metals. Other influencing factors are increased temperature and oxygen depletion events. Year to year variability can occur.
- **Costs:**High[37]

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[35] Malformations include yolk sac or intestinal defects, bent spine, spiral shapes of spinal axis, eye defects including rudimentary or missing eye, crano-facial effects, Siamese twins, clumps of larvae.

[36] Late dead larvae are defined as larvae without malformations and having lengths >15 mm or >10 mm in Denmark.

[37]about 100 individuals can be examined by three persons in one working day; price per fish about 20-30 Euro (commercial basis); if analysing about 40 individuals (minimum recommended) total costs for the analysis (excluding sampling) would become about 1000 Euro

Vitellogenin

Vitellogenin (VTG) is normally produced in females in the liver and transported by blood stream to the ovary. As a result, the concentrations in blood plasma rise up to six orders of magnitude as compared to immature females or male fish. The protein is stored in the egg and used as nutrient by the embryo. Production is regulated in several steps, initially triggered by environmental factors such as photoperiod, temperature and food availability. The hypothalamus secretes hormones that in turn stimulate the pituitary gland to secrete other hormones that cause the ovary to release a third hormone, 17β-estradiol (E2) into the blood stream. In the liver cells, E2 binds to the oestrogen receptor (ER). The ER dimerises and binds to a promoter region of E2 inducible genes, including VTG and ER genes. Thus the E2 binding triggers both VTG and ER production. The hepatically produced VTG then is transported in blood to the ovary, causing an increase in the size of the ovaries. Normally, males do not produce VTG but they posess the VTG genes, and upon exposure to xenooestrogens, VTG can be produced in the liver and accumulate in blood plasma. Upon artificial stimulation from E2, the VTG levels can rise up to a million fold in magnitude, thus exhibiting a very high range of response and used as an extremely sensitive biomarker. The response time is slow (several days) for VTG in blood plasma. Also the half life after exposure is days-weeks. The response time of hepatic mRNA is much shorter (transcription can be measured within a few hours and decay halflife is 3-4 days).*

- **What is analysed (endpoint; unit):** VTG concentration in blood plasma (ng/ml). There are different types of VTG and some species possess several types.
- **Tissue/cells examined:** Blood plasma of male fish.
- **Method used**: It is possible to measure either the protein itself or the gene expression. OSPAR JAMP recommends the analysis of VTG (for cod lipovitellin, lv) protein in blood plasma (ICES TIMES paper available by Scott and Hylland 2002). The ELISA method can detect concentrations below 10 ng/ml[38]. It is important that the same source of Vtg antibody and antigen is used. Because the VTG is unstable, centrifugation of blood samples is required immediately (within 30 minutes), and then stored in freezer[39]. Intercalibration studies performed[40], and marine Quality Assurance procedures available through WGBEC (Davies & Vethaak 2012). Kits are available for several fish species such as salmon and cod[41].
- **Amount sample needed**:At least twelve fish of a constant size range are needed.
- **Possible to use stored samples**: Yes, if properly treated before freezing (see "method used")
- **Possible to use in both limnic and marine environment:** Can be used for both marine, brackish and limnic[42] fish species. OSPAR recommends cod or dab[43] for off shore analyses as well as flounder (estuaries).
- **When to take samples:** Sampling should be performed outside the breeding season and always at the same time of the year for trend analyses. Flounder[44] should be sampled in January/February before offshore migration.

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[38]Enzyme Linked ImmunoSorbent Assay; an immunoassay based on the fact that VTG is highly antigenic; the assays are very sensitive and have high specificity

[39]Can be stored at -20C, but not too long

[40]One ring test of VTG in cod has been performed with comparable results among participants. Variability can be large, possibly being related to varying genotypes, size, migration and prey selection, emphasizing careful field designs. Single individuals can have unexpectedly deviating VTG concentrations, an issue to consider if analysing pooled samples.

[41]although not using Lv, as recommended for this species

[42]Investigations in Swedish contaminated limnic areas used e.g. perch and caged juvenile rainbow trout.

[43]the ELISA method for VTG in dab needs to be developed because lower levels of VTG in this species

[44]Season is an important factor for male plasma VTG in flounder (being lower in the in June/July and highest in February/March according to UK data).

- **Exposure/Effect biomarker:** Exposure biomarker. Baseline levels of VTG in male blood plasma are low, and induction is considered an early warning of endocrine (oestrogenic) disruption. Elevated VTG concentrations are not problematic per see, but should trigger additional examinationsrelated to reproductive impairment.It is recommended to compare the results to suitable reference sites to identify locations with significant responses and use data only in an integrated assessment[45] before making conclusions about risk of ecological effects. VTG suppression can also be analysed in females from exposed areas.
- **General/Specific biomarker:** Specific biomarker of effects from xenooestrogens. Some pharmaceuticals, such as EE-2, are even more potent than E2. Alkylphenols, some phthalates, parabens and phytosterols posess weak activity, whereas other compounds can also be consideredAR antagonists. Other influencing factors include low oxygen levels and season can modulate VTG synthesis. VTG can also correlate with fish size if caught away from point sources, so picking fish from a narrow size range[46] is recommended. Because highly sensitive, lack of significant response probably indicates lack of effects from xenoestrogens. However, it has also been suggested that coexposure to certain compounds, such as planar compounds that interact with the AHreceptor, can inhibit VTG synthesis due to increased metabolism (observed in females). EDA/TIE fractionation can be performed on bile (to identify oestrogenic metabolites, or samples from the affected area).
- **Costs:** Low

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[45]Such as sex ratio and GSI (Gonadosomatic Index). GSI refers to gonad weight as percentage of body weight, and provides information about sexual maturity of the individual. Gonads should also be preserved, to determine intersex (presence of oocytes in testes) in case of high VTG levels and to confirm sex. Otolith samples can be used for age determination.

[46]For cod e.g. smaller individuals (30-45 cm) are recommended to assess recent xenoestrogen exposure in the JAMP guidelines.

Intersex (in male fish)

Intersex in the case of feminization of wild male fish refers to the presence of ovarian tissue in male gonads (ovotestis; oocytes are formed in testicular tissues), compromising reproductive capacity. Ovotestis found in male fish can have occurred in younger life stages[47] when sexual differentiation took place.

- **What is analysed (endpoint; unit):** Intersex prevalence (presence/absence)
- **Tissue/cells examined:** Male gonads (testis)
- **Method used**: Standard histological procedures on tissues embedded in paraffin. Marine Quality Assurance procedures will be available in the future between particular independent laboratories (Davies & Vethaak 2012)
- **Amount sample needed**: Number of individuals necessary is higher in marine off shore locations (due to lower frequencies), approximately 50 male fish required.
- **Possible to use stored samples**: To be stored in Bouins solution or buffered formal saline
- **Possible to use in both limnic and marine environment**: Yes. Well known to occur in direct vicinity of sewage treatment plant effluents, documented by numerous freshwater studies.
- **When to take samples:** Outside breeding season
- **Exposure/effect biomarker:** Early effect biomarker, in that it can have deleterious impact on reproduction. A stepwise, integrated assessment, is generally preferred[48].
- **Specific/general biomarker:** Specific to estrogenic substances, and frequently connected to the exposure to estrogenic steroids such as estrone, estradiol and ethynyl estradiol, and/or phenolic compounds (alkylphenols and their ethoxylates).If observed in marine top predator fish estrogenic disruption may also be the result of biomagnification of weak estrogens such as organochlorines and brominated flame retardants.Low prevalence of intersex can also occur in reference areas, and these levels should be taken into account in data interpretation.Severity likely increases with age.
- **Costs**: For higher cost effectiveness, gonadal histology and liver histopathology should be performed in parallel.

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[47] This is a possible explanation if finding no correlation between VTG induction and intersex, as VTG is caused by more recent exposure.

[48] JAMP recommends the three methods bile estrogenicity (using in vitro tools such as ER CALUX or YES), VTG induction and gonadal intersex to be used in an integrated approach

Lysosomal stability

The lysosomal system is involved in the intracellular recycling of macromolecules and other exogenous material, including xenobiotics. [Moore, 1985; Viarengo et al., 1985; Moore et al., 1984; Cajaraville et al., 1995; Regoli, 1992; Koehler et al., 2002]. At the subcellular level the integrity of the lysosomal membranes can be tested as a marker for non-specific toxic responses. The lysosomal membrane stability (LMS) is an integrative marker which reflects the breakdown of the adaptive capacity of cells to toxic injury. Its sensitivity to anthropogenic impact is able to show pollution induced alterations in affected areas

- **What is analysed (endpoint; unit):** Minutes destabilisation period
- **Tissue/cells examined:** Various, depending on type of organism: hemocytes (blood), liver, hepatopancreas, midgut, head kidney
- **Method used**: Moore et al.,2004, ICES TIMES. Intercalibration studies performed. Variability can be reduced by having the same person performing the analysis. Marine Quality Assurance procedures available through MEDPOL (mussels) and between particular independent laboratories (fish and mussels) (Davies & Vethaak 2012)
- **Amount sample needed**: Sufficient number of samples necessary (20 individuals recommended).
- **Possible to use stored samples**: Depends on method used. If NRR method, analysis must be performed within 24h, if histochemical method samples should be frozen immediately (in liquid nitrogen)
  Possible to use in both limnic and marine environment: Can be analysed on anyorganisms as long as they are large enough to perform serial cryostat section or where living hemocytes can be drawn[49].
- **When to take samples:** Avoid reproductive season
- **Exposure/effect biomarker:** Effect biomarker, but at cellular level (early warning).There is a linear decrease of LMS with the progress of toxically-induced health deterioration.Release of lysosomal hydrolases and accumulated chemicals might finally induce irreversible pathological alterations including cell death. Lysosomal integrity is also of high ecological relevance. It is directly correlated with physiological scope for growth (SFG) in blue mussel (*Mytilus edulis*) and is also mechanistically linked in terms of the processes of protein turnover (Allen and Moore, 2004; Moore et al., 2006a). Ringwood et al. (2004) have also shown that LMS in parent oysters is directly correlated with larval viability. It is also inversely correlated with reproductive disorders in eelpout (Broeg and Lehtonen, 2006). Finally, LMS is directly correlated with diversity of macrobenthic organisms in an investigation in Langesund Fjord in Norway (Moore et al., 2006b), and with parasite species diversity in flounder from the German Bight (Broeg et al., 1999).
- **Specific/general biomarker:** Several classes of pollutants, including polycyclic aromatic hydrocarbons, redox cycling compounds, trace metals, organochlorines and mixtures thereof.Other influencing factors are several other stress factors such as drastic changes in salinity (invertebrates only) and/or temperature.
- **Costs:**Moderate

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[49]Species so far studied include: molluscs (Mytilus galloprovincialis (Marigómez and Baybay-Villacorta, 2003, Gorbi et al. 2008), M. edulis (Schiedeck et al. 2006), M. trossolus (Kopecka et al. 2006), Pecten maximus (Hauton et al. 2001), Mercenaria mercenaria (Zaroogian and Voyer 1995), Corbicula fluminea (Champeau and Narbonne 2006), Ruditapes philippinarum (Caselli and Fabbri 2005, Da Ros and Nesto 2005, Coughlan et al. 2009), Radix peregra (Guerlet et al. 2006), Dreissena polymorpha (Guerlet et al. 2007), Physa (Zaldibar et al. 2006), fish Platichthys flesus (Koehler 1991, Broeg et al. 1999), Limanda limanda (Koehler et al. 1992), Dicentrachus labrax (Romeo et al. 2000), Zoarces viviparus (Sturve et al. 2005), Mullus barbatus (Zorita et al. 2007), Pollachius virens (Bilbao et al. 2006), Siganus rivulatus, Liza aurata, Diplodus sargus (Diamant et al. 1999, Broeg 2002)) as well as other invertebrate species, like Caddisfly larvae (Werner et al. 2000), earthworm (Lee and Kim 2009) and gastropods Helix aspersa (Regoli et al. 2005, Regoli et al. 2006).

Imposex biomarkers

Imposex is the imposition of male sex characteristics on females (whereas intersex can involve organisms with both male and female characteristics). TBT exposure displays a dose-response relationship with imposex physiological progress (Gibbs & Bryan 1994). Irreversible damages, can occur during early life stages and persist years after (depending on the length of the life cycle of the particular species investigated).

- **What is analysed (endpoint; unit):** Common endpoints areVDSI (an index for penis and vas deference development) and Relative penis size index (RPSI).
- **Tissue/cells examined:**Soft tissues of gastropods
- **Method used**:Microscope examination. References to methods included in the OSPAR JAMP guidelines on contaminants specific biological effects.Intercalibration studies performed. Marine Quality Assurance procedures available through QUASIMEME (Davies & Vethaak 2012)
- **Amount sample needed**: At least 40 indivudals from each location should be analysed. Number of individuals necessary depends also on their size, in case chemical tissue analysis is to be performed.
- **Possible to use stored samples**: Should preferably not be frozen and not stored longer than a week before analysis
- **Possible to use in both limnic and marine environment**: Primarily so far investigated on marine and brackish species on a regular basis; different species may be necessary if sampling off shore vs near coastal locations
- **When to take samples:** RPSI could be influenced by season, but VDSI is not considered season sensitive. However, the organisms could be difficult to find during cold winter conditions.
- **Exposure/effect biomarker:**Effect biomarkers with high ecological relevance. Effects that can have a direct impact on reproduction and thus population level effects.
- **Specific/general biomarker:** Specific to TBT related effects. The response can however be influenced by parasitic infections (such individuals should be excluded from the analysis).
- **Costs:**Moderate

Micronucleus assay

The Micronucleus assay is considered a good biomarker of genomic damage and can be used to obtain an indication about the occurrence of permanent and hereditary double DNA strand breaks

- **What is analysed (endpoint; unit):** frequency of MN (FMN‰) and frequency Nucleus Abnormalities (FNA) (with the need to compare results with a blank)
- **Tissue/cells examined:**Can be applied in a range of species (e.g. bivalves and fish). In fish primarily erythrocytes are examined.
- **Method used**: OECD 474 (developed for mammalian cells), Manuali e Linee Guida 32/2005 (APAT). Marine Quality Assurance procedures available through MEDPOL and between particular independent laboratories (Davies & Vethaak 2012)
- **Amount sample needed**: 1000-2000 cells for molluscs (about 20 individual mussels), higher cell numbers for fish (10-20 individual fishes).
- **Possible to use stored samples**: Should be analysed within 3d
- **Possible to use in both limnic and marine environment**: Yes
- **When to take samples:** Seasonal (at least twice a year)
- **Exposure/effect biomarker**: Effect biomarker at early stage with serious potential impact: damage to genetic material of organisms, which could affect their health and potentially also their offspring.
- **Specific/general biomarker:** Specific biomarker in the sense that it measures the effects caused by substances causing permanent and hereditary double DNA strand breaks. Can also respond to water temperature.
- **Costs:**Low

Amphipod embryo alterations

Amphipod embryo malformations have shown to correlate to sediment contaminants.

- **What is analysed (endpoint; unit):** Number (ratio) of malformed embryos
- **Tissue/cells examined:** Embryos of female amphipods
- **Method used**: Refer to: TIMES series No.41 (Sundelin et al 1998). Sundelin, B., Eriksson Wiklund, A-K., and Ford, A. T. 2008. Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors. 21 pp.; Sundelin & Eriksson 1998
- **Amount sample needed**: 50 individuals per station is recommended.
- **Possible to use stored samples**: No, live animals must be used to see malformations clearly
- **Possible to use in both limnic and marine environment**: Yes, but depends on species studied.[50]
- **When to take samples:** Preferably during later stage of embryo development[51], because high level of embryo differentiation.
- **Exposure/effect biomarker:** Effect biomarker with high ecological relevance; malformations have a direct negative impact on survival and thus population relevant effects.
- **Specific/general biomarker:** General, but primarily related to hazardous substances (strong correlation observed between effects and concentrations of metals and organic compounds in field collected sediments), rather than other variables such as oxygen depletion and temperature stress, that primarily influence number of dead and undeveloped eggs.
- **Costs:** Low[52]

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[50] Monoporeia affinis can be found in lakes below the highest coastline and in the Baltic sea.

[51] For Monoporeia affinis usually end of January is suitable (in the Baltic sea).

[52] During 1 day about 200 females can be analysed, corresponding to about 4 stations per day

Stress proteins (heat shock protein)

Depending on stressor and intensity of stress, stress proteins can be measured a short time after exposure.

- **What is analysed (endpoint; unit):** amount of protein (semi-quantitative), relative density units
- **Tissue/cells examined:**any tissue
- **Method used**: Western Blot or ELISA
- **Amount sample needed**: a few mg
- **Possible to use stored samples**: snap-frozen or frozen on dry ice and stored at -80°C or in liquid N
- **Possible to use in both limnic and marine environment**: Yes
- **When to take samples:** Any time, this may be species dependent
- **Exposure/effect biomarker:** Early stage effects, including oxidative stress[53].Very sensitive to temperature changes.
- **Specific/general biomarker:** General biomarker, responding to many types of stress factors
- **Costs:**Low

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[53] Cell ability to handle oxygen radicals and other reactive substances, that could give rise to damage to DNA and proteins

Acetylcholinesterase (AChE) assay

Neurotoxic effects in aquatic organisms can be assessed using the acetyl choline esterase assay. AChE is responsible for the rapid hydrolysis of the neurotransmitter substance acetyl choline. If AChE is inhibited an accumulation of acetyl choline will occur, causing an overstimulation at neuromuscular junctions.

- **What is analysed (endpoint; unit):**AChE inhibition (nmol/min and mg protein)
- **Tissue/cells examined:**variety of tissues possible, but highest activities observed in brain and muscle of fish and eye and muscle of prawns
- **Method used**: Marine Quality Assurance procedures available through WGBEC and between particular independent laboratories (Davies & Vethaak 2012)
- **Amount sample needed**:
- **Possible to use stored samples**:
  - **Possible to use in both limnic and marine environment**: Yes, AChE is present in most animals
- **When to take samples:**
- **Exposure/effect biomarker:**Effect biomarker. AChE inhibition causes excessive nerve stimulation (tetanus, death), but also sublethal exposures (20-50% depression in activity) can alter behaviour, which can have implications on fitness, survival and reproduction.
- **Specific/general biomarker:** Traditionally considered to be specific, and responds to organophosphate and carbamate pesticides, but has also been shown to respond to other groups of substances such as heavy metals, and detergents. It has therefore also been proposed to be used as a general stress biomarker for mussels. Natural variation of AChE activity (related to e.g. sex, size, state of gonadal maturation, seawater temperature) needs to be known to evaluate the results. . Also algal blooms can cause a significant impact.
- **Costs:**

Comet Assay

- **What is analysed (endpoint; unit):** tail moment, % DNA tail, tail length
- **Tissue/cells examined:** blood cells, most frequently circulating haemocytes or erythrocytes
- **Method used**: Single cell gel electrophoresis (SCGE), but there are no standard protocols. Marine Quality Assurance procedures available through WGBEC (Davies & Vethaak 2012)
- **Amount sample needed**: a small number of cells are needed
- **Possible to use stored samples**: protocol recently made available for conserving fish erythrocytes
o **Possible to use in both limnic and marine environment**: Yes, limnic fish has been more frequently investigated than marine so far. Can detect genotoxic effects in most eukaryotic cell types
- **When to take samples:**
- **Exposure/effect biomarker:**.effect (DNA damage), but due to its sensitivity, can also be considered early warning
- **Specific/general biomarker:** Specific to substances causing DNA strand breaks, but other factors need to be taken into account (such as reproductive status)
- **Costs:**

Mussel histopathology (gametogenesis)

- **What is analysed (endpoint; unit):** cell type composition (digestive gland epithelium), digestive tube epithelial atropy and thinning, lysosomal alterations and inflammation
- **Tissue/cells examined:**mussel tissue (digestive gland and tube)
- **Method used**: Marine Quality Assurance procedures available in the future through between particular independent laboratories (Davies & Vethaak 2012)
- **Amount sample needed**: 50 individuals
- **Possible to use stored samples**: Mussels should be processed as soon as possible after removed from water but analysis is performed on frozen tissue sections
- **Possible to use in both limnic and marine environment**:
- **When to take samples:**
- **Exposure/effect biomarker:**.Effect; provides a baseline measure of health
- **Specific/general biomarker:** General, has been related to PAHs, PCBs and heavy metals; also pathogens but it is possible to distinguish between contaminant and pathogen related pathology.
- **Costs:**

Stress on stress

Stress on stress is used to investigate general effects (sensitivity to environmental change) on mussels by exposing them to air.

- **What is analysed (endpoint; unit):** anoxic/aerial survival (LT50 and TMM, Time To Maximum mortality)
- **Tissue/cells examined:**bivalve molluscs
- **Method used**: mussels are placed on filter paper in a humidity chamber (100% humidity) and investigated daily (up to 25 days). Because of the simplicity of the method, QA procedues have not been considered necessary
- **Amount sample needed**: 40 individuals
- **Possible to use stored samples**: no, mussels need to be immediately transported to the laboratory
- **Possible to use in both limnic and marine environment**: Marine molluscs are more common (Mytilus sp)
- **When to take samples:**
- **Exposure/effect biomarker:**.Effect, whole organism response, indicating higher sensitivity to environmental change
- **Specific/general biomarker:** General, has been shown to respond to e.g. long term exposure to crude oil, and short term exposure to sub lethal concentrations of PCB, copper and DMBA respectively
- **Costs:**Low

Scope for Growth (SFG)

- **What is analysed (endpoint; unit):** Alterations in the energy available for growth and reproduction (J h–1 g–1).
- **Tissue/cells examined:**mussels
- **Method used**: See ICES TIMES series no 40. Marine Quality Assurance procedures available between particular independent laboratories (Davies & Vethaak 2012)
- **Amount sample needed**:
- **Possible to use stored samples**:
- **Possible to use in both limnic and marine environment**:
- **When to take samples:** Preferrably during the period of maximum growth potential (i.e. from early summer to early autumn) and important to avoid measurement during the spawning period.
- **Exposure/effect biomarker:**.Effect, reflects overall growth potential and it is possible to determine how near an animal is to lethally low energy levels. SFG has been correlated with concentrations of toxic contaminants in the tissues of mussels, but also with measures of biodiversity in the benthic community (Crowe et al., 2004).
- **Specific/general biomarker:**General and responds to many types of compounds, including DEHP, aromatics, PCP, copper, TBT and dichlorvos. Food absorption efficiency is kept constant during testing.
- **Costs:**

Benthic diatom malformation

Diatoms are frequently sampled to assess nutrient impacts and acidity in streams and lakes. In Sweden, France, UK and Italy there is ongoing research on the potential to add malformations to these analyses, as a way to detect changes related to pesticides and metals.

- **What is analysed (endpoint; unit):** Number (frequency) of malformed shells in combination with taxonomic studies.
- **Tissue/cells examined:**Shells of benthic diatoms (whole organisms)
- **Method used**: Sampling and storage is standardized (EN 13946 2003, EN 14407 2005). There is not yet an international standard for theclassification of malformation types but a Swedish proposal divides the deformations into different categories, see Kahlert 2012.
- **Amount sample needed**: At least 400 shells should be analysed (Kahlert 2012)
- **Possible to use stored samples**:Yes, possible to analyse stored samples (and non destructive analysis)
- **Possible to use in both limnic and marine environment**: Limnic (both streams and lakes)
- **When to take samples:** Autumn
- **Exposure/effect biomarker:** Effect biomarker on individual level (malformed shells) and of high ecological relevance. Results should be evaluated together with information on changes in taxa composition and ecological guilds[54], because certain diatom taxa can e.g. develop a toxin tolerance and then a mass growth (in turn having an impact on frequency of malformation). Motile guilds can e.g. also increase in contaminated conditions.
- **Specific/general biomarker:** Considered to respond to different types of contaminants, including metals and pesticides[55], but also Si deficit or other stress factors (Falasco et al. 2009).
- **Response time:** Elevated frequency of malformations has been found within 1-2 months after elevated concentrations were detected (Kahlert 2012).
- **Costs:**Low[56]

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[54]Total malformation frequency was e.g. found to be less than 1% in Swedish reference conditions, whereas higher values, as well as impacts on community level, were found at sites with higher concentrations of metals and/or pesticides (Kahlert M, 2012).
[55]Significant response to metals and several pesticides, but so far not found to respond to other priority substances
[56]Diatoms are frequently sampled to assess nutrient impacts and acidity in streams and partly lakes; integrated sampling to assess three types of impacts is the most cost effective approach.

BIOASSAY FACT SHEETS

DR CALUX/DR Luc assay

The Dioxin Responsive (DR) CALUX® comprises rat hepatoma cell lines (H4IIE), incorporating the firefly luciferase gene coupled to Dioxin Responsive Elements (DREs) as a reporter gene for the presence of dioxins (PCDDs) and dioxin-like compounds (e.g. furans (PCDFs) and dioxin-like PCBs (dlPCBs)). Following binding of dioxins and/or dioxin-like compounds to the cytosolic Arylhydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. Cells that are exposed to dioxins or dioxin-like compounds not only express proteins that are under normal circumstances associated to DRE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compound (2,3,7,8-TCDD). DR CALUX bioassays report total 2,3,7,8-TCDD TEQs for environmental matrices and total BEQs for food/feed matrices.

- **What is analysed (endpoint; unit):** ng 2,3,7,8-TCDD equivalents/kg sample processed
- **Test duration:** 24h
- **Method used**: Method developed originally by Wageningen University (Aarts et al., 1995; Murk et al., 1996). TIMES protocol available. QA procedures are in place and interlaboratory performance studies are organized frequently. Marine Quality Assurance procedures available in the future through between particular independent laboratories (Davies & Vethaak 2012)
- **Positive control used**: 2,3,7,8-TCDD
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample, but the substances that the assay responds to are in the aquatic environment primarily found accumulated in e.g. sediments and biota (tissues).
- **Cells examined:** Rat liver cell line
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to:** Ah receptor active compounds, e.g. Polyhalogenated dioxins/furans, dioxin like PCBs, and if using other pretreatment of samples also PAHs (see PAH CALUX).Good correlations were usually observed between bioassay results studying marine biological matrices and results from advanced chemical methods (Windal et al., 2002; Hoogenboom, 2002).
- **Sensitivity (LOD/Q):**The bioassays' LOQ is 1 pg 2,3,7,8-TCDD equivalents per amount of material processed. For example, if 5 grams of dried soil/sediment or 1 liter of water is processed, an LOQ of 0.2 ng 2,3,7,8-TCDD equivalents per gram of soil/sediment or 1 ng 2,3,7,8-TCDD equivalents per liter of water is obtained respectively.
- **Variability (e.g. CV for single substance tests) if known:**<20%
- **Influence by cytotoxicity/risk of false positives/negatives:** As the sample is cleaned up by a sulphuric acid treatment and afterwards with an additional step to separate dl-PCBs from PCDD/Fs, cytotoxicity is rarely occurring. In case of false positive/false negative guided levels has to be established to compare it with. In case of the EC project HORIZONTAL no false positive or false negative samples occurred. For such methods usually a false positive and negative ratio of 5% is reasonable.
- **Complexity/learning period:** 2 weeks of training

- **Costs:** Low[57], especially compared to chemical analysis of dioxins and dioxin-like compounds. Cost effectiveness increases even more if comparing to the price of analysing also other compounds with the same mode of action (such as brominated dioxins and furans). By applying several in vitro tools in a battery (such as PAH CALUX and ER-Luc/ER CALUX; see separate fact sheets) can further increase cost effectiveness. Generally not depending on matrix studied.
- **Commercial availability:** Commercial ISO 17025 accredited performers are available
- **WFD relevance:** Dioxins and planar PCBs included in 2008/105/EC. The bioassay analysis is significantly cheaper than chemical, and therefore valuable to include on a screening basis (see case study "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach" in section 1). Valuable to identify water bodies at risk of combined exposure to compounds that are also normally not analysed chemically but that could constitute potential RBSPs, having a relevant mode of action (Ah receptor binding).

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[57] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

PAH CALUX

The PAH Responsive (PAH) CALUX® comprises rat hepatoma cell lines (H4IIE), incorporating the firefly luciferase gene coupled to Dioxin Responsive Elements (DREs) as a reporter gene for the presence of poly aromatic hydrocarbons (PAHs). Following binding of PAHs to the cytosolic Arylhydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. Cells that are exposed to PAHs not only express proteins that are under normal circumstances associated to DRE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds benzo(a)pyrene (B(a)P). PAH CALUX bioassays report total B(a)P equivalents for environmental and food/feed matrices.

- **What is analysed (endpoint; unit):** pg B(a)P equivalents/g sample processed
- **Test duration:** 6h
- **Method used**: See DR CALUX fact sheet, but different pretreatment procedures.
- **Positive control used**: Benzo(a)pyrene
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample
- **Cells examined:** Rat liver cell line
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to**Ah receptor active compounds, e.g. Benzo(a)pyrene like compounds. Especially the higher aromatic PAHs have high activity in the PAH CALUX.
- **Sensitivity (LOD/Q):**The bioassays' LOQ is 0.45 ng B(a)P equivalents per amount of material processed. For example, 5 grams of dried soil/sediment or 1 liter of water is processed resulting in a LOQ of 0.09 ng B(a)P equivalents per gram of soil/sediment or 0.45 ng B(a)P equivalents per liter of water respectively.
- **Variability (e.g. CV for single substance tests) if known:**<20%
- **Influence by cytotoxicity/risk of false positives/negatives:** depending on the clean-up systems and separation technology (HPLC-SPE)
- **Complexity/learning period:** 1 week of training
- **Costs:** Low[58]. Costs are generally not depending on matrix studied.
- **Commercial availability:** Commercial performers available
- **WFD relevance:**The assay could be very valuable on screening level to identify water bodies at risk of exposure to the combined exposure to a large number of relevant PAHs that are normally not analysed chemically but that could constitute potential RBSPs (see case study "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach" in section 1)

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[58] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

ERα CALUX/ER-Luc (agonistic/antagonistic)

The ERα Responsive (ERα) CALUX® comprises a human bone marrow cell line (U2OS), incorporating the firefly luciferase gene coupled to Estrogen Responsive Elements (EREs) as a reporter gene for the presence of estrogens and/or estrogen-like compounds. Following binding of estrogens or estrogen-like compounds to the cytosolic estrogen receptor, the ligand-receptor complex binds the ERE. Cells that are exposed to estrogens and/or estrogen-like compounds not only express proteins that are under normal circumstances associated to ERE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds 17β-estradiol. ERαCALUX bioassays report total 17β-estradiol equivalents for environmental matrices.

- **What is analysed (endpoint; unit):** pg 17β-estradiol equivalents/g sample processed
- **Test duration:** 24h
- **Method used**: Dutch Rijkswaterstaat RIKZ-Specie-08 guideline; Australian Water Commission; Ongoing evaluations at the ISO-TC 147 standardisation group led by BFG-Germany; EPA California; China National Water Quality Monitoring in Jinan.
- **Positive control used**: 17β-estradiol (E-2)
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample.
- **Cells examined:** Human bone marrow cell line
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to** Binding to the Estrogen receptor (alpha and beta for original ER CALUX and only alpha for ERalpha CALUX)
- **Sensitivity (LOD/Q):** The bioassays' LOQ is 35 pg 17β-estradiol equivalents per amount of material processed. For example, if 5 grams of dried soil/sediment or 1 liter of water is processed an LOQ of 7 pg 17β-estradiol equivalents per gram of soil/sediment or 35 pg 17β-estradiol equivalents per liter of water is obtained respectively. Original ER CALUX: 0.1 ng EEQ/l water (see e.g. Leusch, 2008).
- **Variability (e.g. CV for single substance tests) if known:** <20%
- **Influence by cytotoxicity/risk of false positives/negatives:** Depending on the SPE extraction/clean-up as well as type of water matrix.
- **Complexity/learning period:** 1 week of training
- **Costs:** Low[59]. Costs are generally not depending on matrix studied.
- **Commercial availability:** Commercial ISO 17025 accredited performers available
- **WFD relevance:** This bioassay analysis is more sensitive than most chemical analyses (lowest LOD reported by Loos 2012 is e.g. 0.1 ng/l for a chemical analysis of EE-2 and E-2, if using USEPA method 1698; in practice the LOQ that is possible to reach by regular laboratories is generally higher). The assay could therefore be very valuable on a screening level to identify water bodies at risk due to the combined exposure to a large number of estrogenic substances that could constitute RBSPs (see case studies "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach") and to lower the frequency of analytical high end monitoring in water bodies for E2. Because EE2 is significantly (about 10-25 times) more potent *in vivo* than E2, but only 3 times more potent in ER CALUX, this should be taken into account if evaluating data in an absolute manner (comparison with EQS), when considering the need for additional studies. In vivo studies of oestrogenic response, or using precautionary EE2 equivalents can be

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[59] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

considered, if the presence of EE2 is likely, e.g. via high ratio of municipial waste water.The EU-EQS proposal for E2 is based on a SSD approach of the most sensitive aquatic organisms, and concludes that an HC5 of 800 pg/L and an AF of 2 is sufficient, resulting in an AA-EQS of 0.4 ng/L. In most cases, the commonly used TEQ approach would most likely allow a direct comparison of the EEQ value with the AA-EQS of E2 to decide where enough oestrogen-receptor binding potential is available to cause population relevant effects on the most sensitive aquatic species.

AR CALUX (agonistic/antagonistic)

The AR Responsive (AR) CALUX® comprises a human bone marrow cell line (U2OS), incorporating the firefly luciferase gene coupled to Androgenic Responsive Elements (AREs) as a reporter gene for the presence of androgens and/or androgen-like compounds (such as Bisphenol A). Following binding of androgens or androgen-like compounds to the cytosolic estrogen receptor, the ligand-receptor complex binds the ARE. Cells that are exposed to androgens or androgen-like compounds not only express proteins that are under normal circumstances associated to ARE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds di-hydro-testosterone (DHT). AR CALUX bioassays report total DHT equivalents for environmental matrices.

- **What is analysed (endpoint; unit):** pg DHT equivalents/g sample processed
- **Test duration:** 24h
- **Method used**: Dutch Rijkswaterstaat RIKZ-Specie-08 guideline; Australian Water Commission; Ongoing evaluations at the ISO-TC 147 standardisation group led by BFG-Germany; EPA California; China National Water Quality Monitoring in Jinan.
- **Positive control used**: DHT
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample.
- **Cells examined:** Human bone marrow cell line
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below). **What /type of/ substances does the assay respond to**Binding to the Androgen receptor
- **Sensitivity (LOD/Q):**The bioassays' LOQ is ca. 350 pg DHT equivalents per amount of material processed. For example, 5 grams of dried soil/sediment or 1 liter of water is processed resulting in a LOQ of ca. 70 pg DHT equivalents per gram of soil/sediment or 350 pg DHT equivalents per liter of water respectively.Original AR CALUX: ca.1 ng EEQ/l water
- **Variability (e.g. CV for single substance tests) if known:**<20%
- **Influence by cytotoxicity/risk of false positives/negatives:** Depending on the SPE extraction/clean-up as well as which kinds of water matrixes.
- **Complexity/learning period:** 1 week of training
- **Costs:** Low[60]. Costs are generally not depending on matrix studied.
- **Commercial availability:** Commercial accredited performers available
- **WFD relevance:**The assay could be valuable on screening level to identify water bodies at risk of exposure to the combined exposure to androgenic compounds that are normally not analysed chemically but that could constitute potential RBSPs (see case study "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach" in section 1).

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[60] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

YES

The Yeast Estrogen Screen is a recombinant yeast strain used for the identification of compounds/samples that can interact with the human estrogen receptor alpha (hERα)(Routledge & Sumpter, 1996). The DNA sequence of the hERα is stably integrated into the main chromosome of the yeast. In this system, the hERα is expressed in a form capable of binding to estrogen-responsive sequences (ERE). These sequenceswere situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the estrogen-occupied receptor interacts with transcription factors and other transcriptional components to modulate gene transcription. The expression plasmids also carry the reporter gene lac-Z, which after the binding of compounds/environmental samples to the hERα, encodes the enzyme b-galactosidase), which is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red-b-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm. The YES bioassay reports total Estradiol (E2) equivalents (EEQs) for environmental matrices. By coexposing a substance/sample with E2 and using Tamoxifen as the standard, the YES also allows testing for hERα-antagonistic activities (anti-estrogenicity, inhibition of E2 binding to the hERα.

- **What is analysed (endpoint; unit):** ng E2 equivalents/L (agonism), ug/L Tamoxifen equivalents/L (antagonism).
- **Test duration:** 72h
- **Method used**: SOP Sumpter, Routledge & Sumpter 1996, Ongoing evaluations at the ISO-TC 147 standardisation group led by BFG-Germany; SOP Ectotox Centre Switzerland (based on Sumpter SOP).
- **Positive control used**: E2 (agonism), Tamoxifen (antagonism).
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample.
- **Cells examined:** Recombinant yeast cells (*Saccharomyces cerevisiae*) stably transfected with hERα.
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to:**Substances/Samples that bind and transactivate the hERα.
- **Sensitivity (LOD/Q):** The bioassays' LOQ is approx. 8 ng E2/L, by concentration of the sample (i.e. SPE) the LOQ is lowered by the REF (relative enrichment factor).
- **Variability (e.g. CV for single substance tests) if known:**<20%
- **Influence by cytotoxicity/risk of false positives/negatives:** Cytotoxicity can be distinguished from effects by measuring growth of the yeast cells (620 nm) in parallel to the enzyme activity (540 nm).
- **Complexity/learning period:** 1 week of training
- **Costs:** Low
- **Commercial availability:** A test kit will be commercially available in the future.
- **WFD relevance:**See corresponding point for the ER CALUX assay, although the sensitivity of this assay is somewhat lower.

YAS

The Yeast Androgen Screen is a recombinant yeast strain used for the identification of compounds/samples that can interact with the human androgen receptor (hAR)(Sohoni and Sumpter, 1998). The DNA sequence of the hAR is stably integrated into the main chromosome of the yeast. In this system, the hAR is expressed in a form capable of binding to androgen-responsive sequences (ARE). These sequences were situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the androgen-occupied receptor interacts with transcription factors and other transcriptional components to modulate gene transcription. The expression plasmids also carry the reporter gene lac-Z, which after the binding of compounds/environmental samples to the hAR, encodes the enzyme b-galactosidase), which is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red-b-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm. The YAS bioassay reports total Testosterone (DHT) equivalents (DHT EQs) for environmental matrices. By co-exposing a substance/sample with DHT and using Flutamide as the standard, the YAS also allows testing for hAR--antagonistic activities (anti-androgenicity, inhibition of DHT binding to the hAR.

- **What is analysed (endpoint; unit):** ng DHT equivalents/L (agonism), ug/L Flutamide equivalents/L (antagonism).
- **Test duration:** 72h
- **Method used**: SOP Sumpter, Sohoni and Sumpter, 1998, Ongoing evaluations at the ISO-TC 147 standardisation group led by BFG-Germany; SOP Ectotox Centre Switzerland (based on Sumpter SOP).
- **Positive control used**: DHT (agonism), Flutamide (antagonism).
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample.
- **Cells examined:** Recombinant yeast cells (*Saccharomyces cerevisiae*) stably transfected with hAR.
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to:**Substances/Samples that bind and transactivate the hERα.
- **Sensitivity (LOD/Q):** The bioassays' LOQ is approx. 340 ng DHT/L, by concentration of the sample (i.e. SPE) the LOQ is lowered by the REF (relative enrichment factor).
- **Variability (e.g. CV for single substance tests) if known:**<20%
- **Influence by cytotoxicity/risk of false positives/negatives:** Cytotoxicity can be distinguished from effects by measuring growth of the yeast cells (620 nm) in parallel to the enzyme activity (540 nm).
- **Complexity/learning period:** 1 week of training
- **Costs:** Low
- **Commercial availability:** A test kit will be commercially available in the future.
- **WFD relevance:** This bioassay is able to measure the sum of androgenic/anti-androgenic activity present in an environmental sample, without the prerequisite to know the causing substances.

Ames Fluctuation test

The Ames Fluctuation test can be used to obtain an indication about mutagenicity due to substances present in a sample (extract).

- **What is analysed (endpoint; unit):**
- **Test duration:** 96 h
- **Method used**: ISO 16240 and 11350.
- **Positive control used**: No
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample (assay performed on extract of soils and sediments)
- **Cells examined:**
- **Sample volume or mass needed for different matrices:**
- **What /type of/ substances does the assay respond to:** Substances causing genotoxicity ; Mutations[61]
- **Sensitivity (LOD/Q):**
- **Variability (e.g. CV for single substance tests) if known:**
- **Influence by cytotoxicity/risk of false positives/negatives:**
- **Complexity/learning period:** Considered easy to perform and learn
- **Costs:** Low
- **Commercial availability:** Commercial performers available
- **WFD relevance:** Mutagenicity is a highly relevant endpoint to assess human health risks but also on wildlife (see WFD Annex VIII) and several priority substances possess the ability to cause such effects. The assay could be very valuable on screening level to identify water bodies at risk of combined exposure to a large number of relevant substances, and also those that are normally not analysed chemically but that could constitute potential RBSPs.

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[61]Responds to reactivation of bacteria (Salmonella typhimurium) that can grow without histidine. Frequently used within WEA, german standard for this purpose. Microplate tests are available. TA 98 measures frame shift mutations; TA 100 base substitutions. Further strains, some genetically modified in order to express genes of the xenobiotic metabolism are available. External metabolic activation of chemicals by S9-mix.

Micronucleus assay

The Micronucleus assay can be used to obtain an indication about the occurrence of micronuclei[62] frequency in the cytoplasm of interphase cells, due to substances present in a sample (extract).

- **What is analysed (endpoint; unit):** Micronuclei (MN) frequency
- **Test duration:** Depends on cell division cycle and treatment (1,5-2 cycles plus pretreatment)
- **Method used**: ISO 21427-2 2006; OECD 487
- **Positive control used**: Different reference substances are included in the OECD 487 Annex 3 (including Benzo(a)pyrene and Mitomycin C)
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample
- **Cells examined:**Various, including e.g. RTH-149 /CHO
- **Sample volume or mass needed for different matrices:**
- **What /type of/ substances does the assay respond to:** genotoxic (clastogenic and aneugenic) substances
- **Sensitivity (LOD/Q):**
- **Variability (e.g. CV for single substance tests) if known:**
- **Influence by cytotoxicity/risk of false positives/negatives:**Cytotoxicity tested in parallell
- **Complexity/learning period:** Considered easy to perform and learn
- **Costs:**High
- **Commercial availability:** Commercial performers available
- **WFD relevance:**Genotoxic effects are highly relevant in the assessment of risks to human health but also wildlife (see WFD Annex VIII) and several priority substances possess the ability to cause such effects. The assay could be very valuable on screening level to identify water bodies at risk of combined exposure to a large number of relevant substances that are normally not analysed chemically but that could constitute potential RBSPs.

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[62] small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis or meiosis by lagging chromosome fragments or whole chromosomes.

Fish Embryo Acute Toxicity (FET) test

In the OECD 236, fertilized eggs of zebrafish are exposed to the test chemical for a period of 96 hours, with daily observation of endpoints for lethality. In addition, sublethal endpoints and complementary methods might be included according to the specific needs of the study.

- **What is analysed (endpoint; unit):** Four apical observations are recorded daily as indicators of lethality, being coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. Acute toxicity is determined based on the occurrence of any of the lethal observations, with calculation of LC50 value.Additional sublethal endpoints can also be scored daily, with calculation of EC50 value. Plus, complementary methods can be applied to embryos by the test termination.
- **Tissue/cells examined:** wholeembryo, somites, tail-bud, and heartbeat.
- **Method used:** OECD guideline 236 FET test. It is possible to perform the test with exposure in 96-well instead of 24-well plate, which reduces the amount of required samplefor testing.
- **Amount sample needed:** Circa 180 fertilized embryos for the main test (20 embryos per test concentration, and for the control conditions: dilution-water, solvent, positive, and plate controls). When performing a range finding test, 10 embryos are recommended per concentration.
- **Possible to use stored samples:** Not for the acute test. However by the end of test the zebrafish embryos might be stored for future analysis with additional methods.
- **Possible to use in both limnic and marine environment**: Zebrafish is a freshwater fish species. However the FET method can be adapted to estuarineor marine species (e.g. stickleback). Beside the protocol for aqueous samples (Waters samples, sediment and water extracts; (OECD 236, Braunbeck et al. 2005) also a sediment contact protocol has been developed (Hollert et al. 2003) and validated (Höss et al. 2009)
- **When to take samples:** The test is initiated as soon as possible after fertilisation of the eggs.
- **Exposure/effect biomarker:** Biomarker for lethal and sublethal effects. Exposure biomarkers can be included by incorporating additional methods, as by including molecular biomarkers .
- **Specific/general biomarker:** General biomarker when lethality only is assessed (acute toxicity). Specific biomarkers can beby included by incorporating additional methods as mechanism-specific biomarkers (eg, Coment-Assay and DNA arrays; Kosmehl et al. 2006, 2012).
- **Response time:** the test is conducted for 96 hours.
- **Costs:** low for the FET acute test, however that changes accordingly with complementary methods performed by end of test.

7. Biomarkers and in vitro-assays related to certain modes of action

The following table lists a large number of available *in vitro* assays and biomarkers and the type of effects on cellular level that they respond do. The tests included and the division into type of response is based on an inquirydeveloped by the Institute for Environmental Studies (IVM), VU University Amsterdam[63].

Although it is possible to study the same type of mode of action using either in vitro bioassays or biomarkers, it should be kept in mind that *in vitro* assays are more simplified biological systems than whole organisms and therefore do not take complex interactions occurring inside an organism into account. Analyses at different levels of biological organization should therefore not be considered as alternative approaches, but the selection depends on the case specific circumstances.

Table Examples of in vitro bioassays and biomarkers responding to the same type of mode of action

Mode of action investigated	Biomarkers	In vitro bioassays
Arylhydrocarbon receptor (in)activation	CYP1A (mRNA levels, protein levels, EROD activity) UGT activity	CYP1A (mRNA levels, protein levels, EROD activity) Cell-based reporter gene assay, such as DR CALUX, PAH CALUX, H4IIE-luc
Sex hormone disruption	Estrogenicity Vitellogenin (mRNA, protein) ER (mRNA, ER protein) CYP19 mRNA levels CYP19 protein levels CYP19 (aromatase) activity Estradiolsulfotransferase activity Steroid levels Gonadal Somatic Index Oocyte/Sperm development Secondary sex characteristics	Anti/ Estrogenicity: Yeast-based reporter gene assay, such as YES Cell-based reporter gene assay, such as ER CALUX ER binding assay Proliferation assay Vitellogenin mRNA Vitellogenin protein ER mRNA levels ER protein levels CYP19 mRNA levels CYP19 protein levels CYP19 (aromatase) activity Estradiolsulfotransferase activity Anti/Andrenogenicity: AR binding assay Yeast-based reporter gene

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[63] Contact person: Dr Timo Hamers

		assay, such as YAS Cell-based reporter gene assay, such as AR CALUX CYP19 mRNA levels CYP19 protein levels CYP19 (aromatase) activity
Thyroid hormone disruption	TTR mRNA expression Thyroid hormone levels Thyroid stimulating hormone levels Amphibian tail regression rate	TTR-binding assay Proliferation assay (T-Screen)
Genotoxicity	DNA adduct measurements Comet measurements Micronucleus measurements Chromosome aberrations Sister Chromatid Exchanges Flow cytometry Sperm head abnormality	Ames assay TA98 Ames assay TA100 Ames assay TA97 Genotoxicity (continued) Ames assay TA102 SOS chromotest Mutatox assay UmuC-assay DNA adduct measurements Comet assay Micronucleus assay Chromosome aberration assay Sister Chromatid Exchange assay Flow cytometry
Oxidative stress	Lipid peroxidation Catalase activity Superoxide dismutase activity Glutathione peroxidase activity Glutathione reductase activity Reactive Oxygen Species production Glutathione levels (GSH) Oxidized glutathione levels (GSSG) Antioxidant levels	Lipid peroxidation Catalase activity Superoxide dismutase activity Glutathione peroxidase activity Reactive Oxygen Species levels
Neurotoxicity	Inhibition of acetylcholinesterase	Inhibition of acetylcholinesterase
Immunotoxicity	Phagocytic activity Lysosomal stability	
Cytotoxicity	Neutral Red Retention assay Trypan Blue Exclusion assay	MTT reductase assay Alamar Blue reductase assay CFDA-AM hydrolyse assay Neutral Red Retention assay Trypan Blue Exclusion

		assay
		LDH assay
		Microtox assay

8. ASSESSMENT CRITERIA FOR EFFECT BASED TOOLS

Assessment criteria for effect-based monitoring tools were primarily developed for the marine environment, see tables below, with BAC and EAC values developed jointly by ICES and OSPAR. Full details on how they were derived can be found in the SGIMC 2010 and SGIMC 2011 and WKIMON 2009 reports on the ICES website and in the OSPAR Background Documents for individual biological effects methods. A compilation of the Background Documents and Assessment criteria is published in Davies & Vethaak 2012. Updates and amendments to the BACs and EACs can be found in the ICES WGBEC reports from 2012 and 2013 (in press).Values are reviewed annually and can be revised as new data becomes available. Original, updated values and background documents should always be consulted before using the criteria. Tables reproduced with kind permission from ICES secretariat 16 April 2013.

Table: Current ICES assessment criteria for marine biological effects measurements for the techniques in the SGIMC integrated monitoring strategy (based on decision made at the March 2013 meeting in WGBEC). Values are given for both background assessment criteria (BAC) and environmental assessment criteria (EAC), as available.

BIOLOGICAL EFFECT	APPLICABLE TO:	BAC	EAC
VTG in plasma; µg/ml	Cod	0.23	
	Flounder	0.13	
Reproductive success in fish	Eelpout, *Zoarcesviviparus*		
	Malformed fry	1	2
Mean prevalence (%) of:	Late dead fry	2	4
	Early dead fry	2.5	5
	Total abnormal fry	5	10
EROD; pmol/mg protein pmol/min/ mg protein S9 * pmol/min/ mg microsomal protein	Dab (F)	178	
	Dab (M)	147	
	Dab (M/F)	680*	
	Flounder (M)	24	
	Plaice (M)	9.5	
	Cod (M/F)	145*	
	Plaice (M/F)	255*	
	Four spotted megrim (M/F)	13*	
	Dragonet (M/F)	202*	
	Red mullet (M)- April	208	
	Red mullet (M/F)- October 12-18 cm; GSI<1 Bottom temperature 16-20ºC	115*	
	Eelpout (F)	10	
PAHs Bile metabolites; [1] ng/ml; HPLC-F [2] pyrene-type µg/ml; synchronous scan fluorescence 341/383 nm [3] ng/g GC/MS * 1-OH pyrene ** 1-OH phenanthrene	Dab	16 [1] * 3.7 [1] ** 0.15 [2]	22 [2]
	Cod	21 [1] * 2.7 [1] ** 1.1 [2]	483 [3] * 528 [3] ** 35 [2]
	Flounder	16 [1] * 3.7 [1] **	

		1.3 [2]	29 [2]
	Haddock	13 [1] * 0.8 [1] ** 1.9 [2]	35 [2]
	Eelpout	92 [1] * 7.9 [1] **	
	Herring	143 [1] * 2.6 [1] **	
DR-Luc; ng TEQ/kg dry wt, silica clean up	Sediment (extracts)	10	40

DNA adducts; nm adducts mol DNA	Dab	1	4,0
	Flounder	1	4,0
	Long Rough Dab		4,0
	Halibut		5,8
	Herring and sprat		0,39
	Cod	1.6	6,7
	Haddock	3.0	6,7
Bioassays; % mortality	Sediment, Corophium	20	60
	Sediment, Arenicola	10	50
	Water, copepod	10	50
Bioassays; % abnormality	Water, oyster embryo	20	50
	Water, mussel embryo	30	50
	Water, sea urchin embryo	10	50
Bioassay; % growth	Water, sea urchin embryo	30	50
Lysosomal stability; minutes	Cytochemical; liver all species	20	10
	Neutral Red Retention: all species	120	50
Micronuclei; $^0/_{00}$ (frequency of micronucleated cells) [1] Gill cells [2] Haemocytes [3] Erythrocytes	*Mytilus edulis*	2.5 [1] 2.5 [2]	
	Mytilus galloprovincialis	3.9 [2]	
	Mytilus trossulus	4.5 [2]	
	Flounder	0.3 [3]	
	Dab	0.5 [3]	
	Eelpout	0.4 [3]	
	Cod	0.4 [3]	
	Red mullet (M/F) 12-18 cm; GSI<1 Bottom temperature 16-20ºC Autumn (October)	0.4 [3]	
Comet Assay; % DNA Tail	*Mytilus edulis*	10	
	Dab	5	
	Cod	5	
Stress on Stress; days	*Mytilus* sp.	10	5
AChE activity; nmol.min^{-1} mg prot^{-1} [1] gills [2] muscle tissue [3] brain tissue * French Atlantic waters ** Portuguese Atlantic waters [+] French Mediterranean Waters [++] Spanish Mediterranean Waters [+++] Baltic sea	*Mytilus edulis*	30 [1*]	21 [1*]
		26 [1**]	19 [1**]
	Mytilus galloprovincialis	29 [1+]	20 [1+]
		15 [1++]	10 [1++]
	Flounder	235 [2*]	165 [2*]
	Dab	150 [2*]	105 [2*]
	Red mullet	155 [2+]	109 [2+]
	Red mullet (M/F) 12-18 cm; GSI<1 Bottom temperature 15-16ºC Autumn (October)	118 [3++]	83 [3++]
	Eelpout	124 [2+++]	87 [2+++]

Externally visible diseases***	Dab	Fish Disease Index (FDI):	Fish Disease Index (FDI):
Ep,Ly,Ul Ep,Ly,Ul Ac,Ep,Fi,Hp,Le,Ly,St,Ul,Xc Ac,Ep,Fi,Hp,Le,Ly,St,Ul,Xc Ac,Ep,Hp,Le,Ly,St,Ul,Xc Ac,Ep,Hp,Le,Ly,St,Ul,Xc Italics: ungraded, bold: graded NA: Not applied		F: *1.32*, 0.216 M: *0.96*, 0.232 F: *1.03*, 0.349 M: *1.17*, 0.342 F: *1.09*, 0.414 M*: 1.18*, 0.398 M: males F: females	F: *NA*, 54.0 M: *NA*, 47.7 F: *50.6*, 19.2 M: *38.8*, 16.1 F: *48.3*, 21.9 M: *35.2*, 16.5
Liver histopathology-non specific	Dab	NA	Statistically significant increase in mean FDI level in the assessment period compared to a prior observation period *or* Statistically significant upward trend in mean FDI level in the assessment period
Liver histopathology-contaminant-specific	Dab	Mean FDI <2	Mean FDI ≥ 2 A value of FDI = 2 is, e. g., reached if the prevalence of liver tumours is 2 % (e. g., one specimen out of a sample of 50 specimens is affected by a liver tumour). Levels of FDI ≥ 2 can be reached if more fish are affected or if combinations of other toxicopathic lesions occur.
Macroscopic liver neoplasms	Dab	Mean FDI <2	Mean FDI ≥ 2 A value of FDI = 2 is reached if the prevalence of liver tumours (benign or malignant) is 2 % (e. g., one specimen out of a sample of 50 specimens is affected by a liver tumour). If more fish are affected, the value is FDI > 2.

Intersex in fish; % prevalence	Dab Flounder Cod Red mullet Eelpout	5	
Scope for growth Joules/hr/g dry wt.	Mussel (*Mytilus* sp.) (provisional, further validation required)	25	15
Hepatic metallothionein ìg/g (w.w.) [1] Whole animal [2] Digestive gland [3] Gills * Differential pulse polarography	*Mussel edulis*	0.6 [1*] 2.0 [2*] 0.6 [3*]	
	Mytilus galloprovincialis	2.0 [1*] 3.9 [2*] 0.6 [3*]	
Histopathology in mussels	VVbas: Cell type composition of digestive gland epithelium; $\mu m^3/\mu m^3$ *(quantitative)*	0.12	0.18
	MLR/MET: Digestive tubule epithelial atrophy and thinning; $\mu m/\mu m$ *(quantitative)*	0.7	1.6
	VVLYS & Lysosomal enlargement; $\mu m^3/\mu m^3$ *(quantitative)*	VvLYS 0.0002	V>0.0004
	S/VLYS: $\mu m^2/\mu m^3$	4	
	Digestive tubule epithelial atrophy and thinning *(semi-quantitative)*	STAGE ≤1	STAGE 4
	Inflammation *(semi-quantitative)*	STAGE ≤1	STAGE 3
Imposex/intersex in snails VDSI	*Nucella lapillus*	<0.3	<2

***: Assessment criteria for the assessment of the Fish Disease Index (FDI) for externally visible diseases in common dab (*Limandalimanda*). Abbreviations used: Ac, *Acanthochondriacornuta*; Ep, Epidermal hyperplasia/papilloma; Fi, Acute/healing fin rot/erosion; Hp, Hyperpigmentation; Le, *Lepeophtheirus sp.*; Ly, Lymphocystis; St, *Stephanostomumbaccatum*; Ul, Acute/healing skin ulcerations; Xc, X-cell gill disease.

In addition to these criteria, there are also corresponding values for the BAC and EAC assessment of imposex/intersex in other gastropod species (WGBEC 2012), see below.

Table. OSPAR Biological effect assessment (BAC and EAC) criteria for TBT related effects in Nucella lapillus, alongside with equivalent VDSI / ISI values for sympatric populations of other relevant species.

Assessment class	*Nucella*	*Nassarius*	*Buccinum*	*Neptunea*	*Littorina*	*Hydrobia*
	VDSI	VDSI	VDSI	VDSI	ISI	VDSI
A (<BAC)	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
B (>BAC<EAC)	0.3 - <2.0			0.3 - <2.0		
C (>EAC)	2.0 < 4.0	0.3 - 2.0	0.3 - 2.0	2.0 - 4.0		0.3 - <1
D (>EAC)	4.0 - 5.0	2.0 - 3.5	2.0 - 3.5	4.0 ^	0.3 - < 0.5	1 - < 2
E (>EAC)	>5.0	> 3.5	> 3.5		0.5 - 1.2	
F (>EAC)	-				> 1.2	>2

There are also values available to evaluate the reproductive success in amphipods, although this method is not included in the ICES integrated monitoring strategy, see below.

Reproductive succes in amphipods *95th percentile of % malformed embryos*	*Monoporeia affinis, Pontoporeia femorata, Gammarus* sp., *Corophium* sp., *Bathyporeia* sp.	3.4	4.6

There are also some criteria developed or suggested for other types of use as well. Within the context of contaminated sites, Swedish assessment criteria have been proposed for Microtox, UMU C and EROD (SEPA report 4918). Norwegian national guidelines on risk assessment of contaminated sediment include also assessment criteria for DR CALUX (SFT 2008). For chemical assessment of dioxins and planar PCBs, assessment criteria are available for food and feed (EU regulation), but a bioassay approach is accepted on a screening level.

9. OVERVIEW OF QUANTITATIVE REAL TIME PCR

The Quantitative Real Time PCR (qRT-PCR) has been developed to allowresearchers to studydifferences, at the quantitative level, in mRNA expression. It isbased on detecting and quantifying fluorescence signalsthatincrease in direct proportion to the amount of PCR product (or amplicon) in a reaction (Heidet al.,1996). By recording the level of fluorescence emissionateach amplification cycle, itis possible to monitor a reactionduringitsexponential phase, where the replicatesamplesamplifyexponentially and the increase in the amount of PCR product correlate to the initial amount of targettemplate.

The twopredominantmethods for the quantitative detection of the amplicon use i) generic DNA–binding agents (intercalatingdye) and ii) fluorescent oligonucleotide probes.

i) SYBR-green, is the mostcommonintercalatingdye, which shows little fluorescence in solution but has a 200-fold increase in fluorescence intensityuponbinding to the double strand (ds)DNA. Although the dyes are not expensive and requireonlya primer set for the gene of interest, there are a few caveats to their use becausetheybind to all (ds) DNA e.g. primer dimer,generatingmanyunspecificproducts.

ii) The second method for quantitative detectionisbased on fluorescence resonanceenergytransfer (FRET) probes that are specificallydesigned for the genetarget, thusovercoming the problem of the intercalatingdye. The FRET systems are the TaqMan®assay, the TaqMan®Minor Groove Binding (MGB), the MolecularBeacons, and the Scorpions. The mostpopular system isshown in Fig.1, the TaqMan®assay.

Application of Real Time PCR to Ecotoxicology

In the last few years the qRT-PCR has become an invaluabletool for scientists in many disciplines (Heid et al., 1996). In fact,itssensitivitytogetherwith the genomesequencing of manyorganisms has revolutionised the field of molecular diagnostic such as microbiology (Yang, S., et al., 2002), clinicaloncology in cancer research (BernardandWittwer, 2002), gene expression analysis and in the last years as well in ecotoxicology field.

In ecotoxicology, it has been used to estimate the expression of genes of interest as molecularindicators, diagnostic for exposure to variousstressors and for several model and non-model organisms (Islinger, et al., 2003; Carvalho, et al. 2011b;Zhanga, et al. 2008)[64]. It isenvisagedthat the dropped down price for the instruments and the standardisation of the methods (kits are availablefromseveralcompanies) makethis technique one of the promisingtools for the implementation in regulatoryagencies for ecotoxicology, microbiology and ecology fields.

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[64]There are numerous publications on the application of qRT-PCR in ecotoxicology, here it is only possible to mention a few
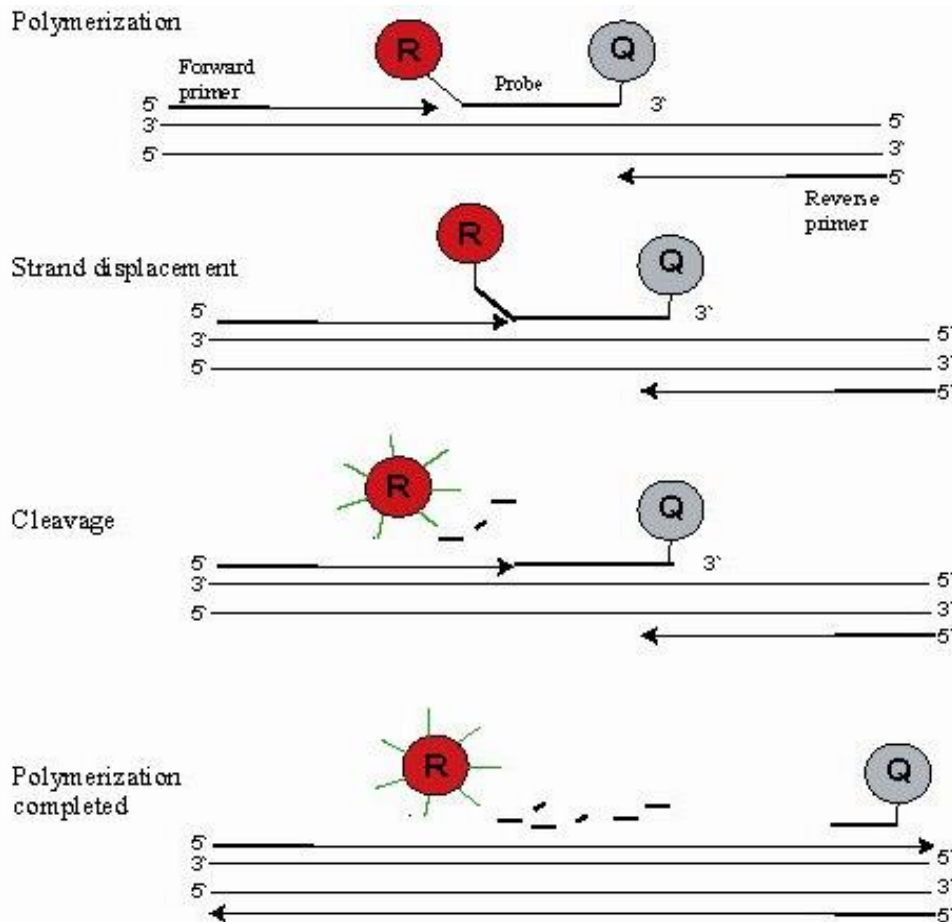
Fig.1.TaqMan assay.

The qRT-PCR reaction exploits the 5' nuclease activity of AmpliTaq Gold DNA polymerase to cleave a TaqMan probe during the PCR. The TaqMan probe contains a reporter dye at 5' end (R) and a quencher (Q) dye at 3'. During the first step, the primers and the probe anneal at the specific target, and the polymerisation starts. During the extension, the probe is displaced and cleaved by the exonuclease activity 5'-3'. The cleavage of the probe separated the reporter dye from the quencher, resulting in increased fluorescence of the reporter. Accumulation of the PCR products is detected directly by monitoring the increase in fluorescence of the reporter. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the fluorescence emission primarily by Foerster-type energy transfer(FRET).

10. OMICS – additional applications

To discover new molecular biomarkers

The biggest challenge for the utility of mode of action is the need to improve the identification of molecularbiomarkerslinked to the physiologicalphenotype as well as the patterns of gene expression associatedwithdifferentclassesof pollutants. In addition, the information on a comparative analysis of pollutanteffects in differentecologically relevant organismswouldallow the selection of genes of interest/pathwayswhich are meaningfulacrosstaxonomy.

Someexamples of molecularbiomarkers have been described*(*Ankleyet al., 2008; Miller et al., 2007), andmolecular changes in the fishhypothalamic-pituitary-gonadal axis (HPG) uponexposure to chemicals have been investigated. The HPG axis isresponsible for the steroidregulationwhich, through the vitellogenin (VTG) level in the plasma, regulates the fecundity and fertility in females. Theyshowedthat plasma VTG, in fishexposed to chemicals, decreased due to the alterationattranscriptionallevel of the HPG genes, and consequently the fecunditywasreduced, whichisexpected to have a serious impact on population status. Thus, throughunderstanding the biologicalpathwaysleading to the vitellogenesis, molecularresponseswererelated to a potential adverse outcomemeaningful to riskassessors. Similarly, Carvalho et al*.* (Carvalho et al., 2011a)exposed the marine diatom*Thalassiosirapseudonana* to benzo(a)pyrene, a polyclicaromatichydrocarbon (PAH). Theyinvestigatedwhether the gene expression profile compared to the untreatedcellscouldprovidemolecularbiomarkerslinked to a physiologicalstatus change due to the pollutanteffects. Theyshowedthat the silicificationprocesswasaffected in these conditions, particularly the down regulation of silicontransporterencodinggene, *ST1*, thuscompromisingthe silicauptakefrom the media (Fig. below). The sameresultwasconfirmedalsowhen the diatomswereexposed to marine sediment sample extracts containing PAHs.
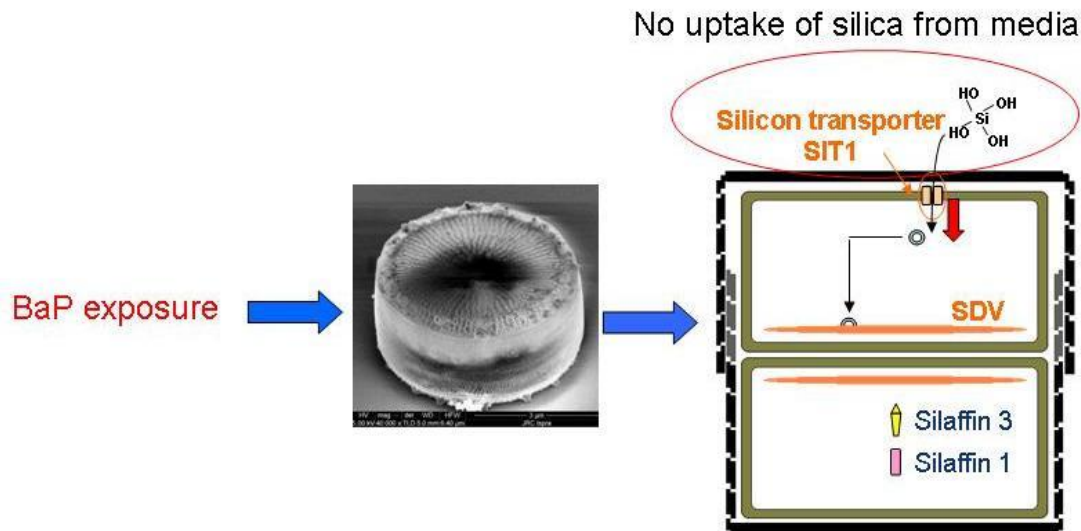
Fig.2. A molecularbiomarker in the marine diatom *Thalassiosira pseudonana* upon exposure to benzo(a)pyrene is the encoding gene for the silicon transporter *ST1.*

Other applications of omics profiles have been described by Garcia-Reyero et al (Garcia-Reyero et al., 2008) linked with the identification of the chemical(s) responsible for the effects induced by complex mixtures in wastewater effluents. Indeed they exposed male fathead minnows (*Pimephales promelas*) for 48 h to effluents at two sites, one up-stream and downstream of the wastewater sewage treatment plant, respectively. Since many pharmaceutical compounds and cosmetics are not retained by the sewage treatment plant, they investigated the gene expression profiling in the two groups. They showed that significant changes occurred in the gonad of fish exposed below the sewage plant, compared to above the treatment plant and to laboratory control fish. Among the biological processes affected were the innate immune response, response to stress, control of homeostasis, control of transcription, metabolism and cell communication. This work suggests that fish are impacted by exposures to the sewage treatment effluents, and effects can be detected rapidly by gene expression profiling.

To identify site of origin of wild organisms

In the European flounder (*Platichthys flesus*), a transcriptomics and bioinformatics approach was used to predict the site of origin of fish from environment based stress-responsive genes (Falciani et al., 2008). To determine which gene expression differences may relate to pollutant impact, they compared the transcriptomic profile with laboratory exposures of flounder to selected toxicants and determined the associated gene expression profiles. The search space for a combination of genes that effectively predicts pollutant class membership was very large. However, by combining the signatures derived from acute laboratory exposure to individual chemicals to limit the search space, a very accurate model for classification of all the different environmental sites was achieved. The final model utilised the expression profiles

of 16 genes and validation wasconfirmed by quantitative real time Polymerase Chain Reaction (PCR) arraycomprisingthesegenes. By doingthis, theycorrectlyassigned the site of origin for fishobtainedfromthree of the sites in an independentsampling. These data wouldimplythat the gene expression fingerprintsobtainedwiththesearrays are primarilyattributable to variations in chemicalpollutantresponsesat the different sites.These important observations give support to the implementation of omics as diagnostic tools in environmental monitoring.

11. OVERVIEW OF EXISTING DNA MICROARRAYS

Overview of existing DNA microarrays for several organisms and the stressors tested (4th column) and type of environmental samples (5th column).

Organism	Biological sample	Microarray platform/ Additional studies	Chemical stressors tested	Environmental samples	References
Daphnia magna (water flea)	Whole organism	DNA micoarray	Cd	-	(Connon et al., 2008)
	Whole organism	DNA micoarray	Ibuprofen	-	(Heckmann et al., 2008)
	Whole organism	DNA micoarray	pH, calcium limitation	-	-
	Whole organism	DNA micoarray	lufenuron	-	-
	Whole organism	DNA micoarray	methomyl (insecticide); propanil (herbicide)	-	(Pereira et al., 2010)
	Whole organism	DNA micoarray	Silver nanoparticles; silver nitrate	-	(Poynton et al., 2012)
	Whole organism	DNA micoarray/ Metabolomics	Cd	-	(Poynton et al., 2011b)
	Whole organism	DNA micoarray	Cd	-	(Soetaert et al., 2007)
	Whole organism	DNA micoarray	Cu, Cd, Zn (acute toxicity)	-	(Poynton et al., 2007)
	Whole organism	DNA micoarray	Cu, Cd, Zn (dose-response)	-	(Poynton et al., 2008a)
	Whole organism	DNA micoarray	Cu	copper mines	(Poynton et al., 2008b)
	Whole organism	DNA micoarray	Copper sulfate, hydrogen peroxide, pentachlorophenol, or beta-naphthoflavone	-	(Watanabe et al., 2007)
	Whole organism	DNA micoarray	ZnO NPs, ZnSO(4)	-	(Poynton et al., 2011a)
	Whole organism	DNA micoarray	Ni^{2+}, binary metal mixtures (Ni^{2+} + Pb^{2+}; Ni^{2+} + Cd^{2+})	-	(Vandenbrouck et al., 2009)
	Whole organism	DNA micoarray	Cu, Zn, Pb, and munitions constituents	-	(Garcia-Reyero et al., 2009b)
	Whole organism	DNA micoarray	Munitions constituents 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene, trinitrobenzene, dinitrobenzene, or 1,3,5-trinitro-1,3,5-triazacyclohexane well as to 8 different munitions mixtures	ground water contaminated with munitions constituents	(Garcia-Reyero et al., 2012)
	Whole organism	DNA micoarray	narcotics and polar narcotics: - 3,5-dichloroaniline; 2,3,4-trichloroaniline; 4-chloroaniline; aniline; ethanol; isopropanol; methanol	-	(Dom et al., 2012)
	Whole organism	DNA micoarray	sodium dichromate, benzo[a]pyrene	-	(David et al., 2011)
	Whole organism	DNA micoarray	Zn	-	(Vandegehuchte et al., 2010)
	Whole organism	DNA micoarray/ Metabolomics	Fluoranthene, pyrene and Flu-Pyr mixtures	-	(Vandenbrouck et al., 2010)
Daphnia pulex (water flea)	Whole organism	DNA micoarray	Cd	-	(Shaw et al., 2007)
				-	
Oryzias latipes (japanese medaka, halotolerant)	Larval medaka	DNA micoarray	17beta-estradiol (E2) (dose-response)	-	
	Whole organism	DNA micoarray	17beta-estradiol (E2)	-	(Kishi et al., 2006)
	Hypothalamic–Pituitary–Gonadal axis tissue	Real-time PCR array	17alpha-ethinylestradiol (EE2), 17beta-trenbolone (TRB)	-	(Zhang et al., 2008)
	Whole organism	DNA micoarray	CuSO4, CdCl2, tributyltin (TBT), zinc	-	(Kishi et al., 2008)
	Fish embryo	DNA microarray/Real-time PCR	Silver nanocolloids	-	(Kashiwada et al., 2012)
	Liver	DNA micoarray	4-Nonylphenol	-	(Kim et al., 2006)
	Liver	DNA micoarray	Arochlor 1260 (polychlorinated	-	(Yum et al.,

			biphenyl mixture)		2010)
	Brain	DNA micoarray	Polychlorinated biphenyls (PCBs)	-	(Nakayama et al., 2008)
	Whole organism	DNA micoarray	benzylparaben	-	(Yamamoto et al., 2007)
	Liver	DNA micoarray	1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (o,p'-DDT)	-	(Uchida et al.)
	Liver	DNA micoarray	Polychlorinated biphenyls (PCBs)	-	(Nakayama et al., 2011)
	Testis, liver and brain	DNA micoarray	17α-ethinylestradiol (EE2), estradiol benzoate (EB)	-	(Hirakawa et al., 2012)
	Liver	DNA micoarray	glyphosate agrochemical components	-	(Uchida et al., 2012)
Micropterus salmoides	neuroendocrine brain	DNA micoarray /Proteomics	dieldrin and E2		(Martyniuk et al., 2010b)
Micropterus salmoides (largemouth bass, freshwater)	Female liver	DNA micoarray /Proteomics	17alpha-ethinylestradiol (EE2)		
	Male liver	DNA micoarray	methoxychlor (MXC)		(Martyniuk et al., 2011)
	Female hypothalami	DNA micoarray	dieldrin		(Martyniuk et al., 2010a)
	Male liver and gonad	DNA micoarray	17beta-estradiol (E2)		(Garcia-Reyero et al., 2008)
Pimephales promelas (fathead minnow, freshwater)	Male liver	DNA microarray		urban effluents	not available
	Liver and gonad	DNA micoarray		comparison between low- and high-impact agricultural sites	(Sellin Jeffries et al., 2012)
	Male liver	DNA microarray		Secondary and primary treated municipal wastewater effluents	not available
	Ovary	DNA microarray	prochloraz		(Skolness et al., 2011)
	Liver, gill, brain	DNA microarray	titanium dioxide nanoparticles (TiO2)		not available
	Brain	DNA microarray	Prozac (fluoxetine), carbamazepine, venlafaxine and mixture		(Thomas et al., 2012)
	Liver and gonad	DNA microarray		effluent waste waters	(Garcia-Reyero et al., 2011)
	Liver and gonad	DNA microarray		surface waters	not available
	Brain	DNA microarray	cyclotrimethylenetrinitramine (RDX) dose-response		(Gust et al., 2011b)
	Brain, liver and Larvae	DNA microarray	RDX		(Gust et al., 2011a)
	Hypothalamus	DNA microarray		pulp and paper mill effluent	(Popesku et al., 2010)
	Female brain and liver	DNA microarray	fadrozole		(Villeneuve et al., 2007)
	Female gonad	DNA microarray	17b-trenbolone (TB)		(Dorts et al., 2009)
	Ovary	DNA microarray	trenbolone, flutamide and a mixture		(Garcia-Reyero et al., 2009c)
	Whole organism	DNA microarray	lead (Pb)		(Mager et al., 2008)
	Liver and gonads	DNA microarray		sewage treatment plant	(Garcia-Reyero et al., 2009a)

				effluents	
	Brain	DNA micoarray	propranolol		(Lorenzi et al., 2012)
	Ovary		Bisphenol A		(Villeneuve et al., 2012)
	Gonad		haloperidol (dopamine receptor antagonist)		(Villeneuve et al., 2010)
Danio rerio (zebrafish, freshwater)	Embryo	DNA microarray	methyl mercury (MeHg)		(Yang et al., 2010)
	Embryo	DNA microarray	flusilazole		(Hermsen et al., 2012)
	Embryo	DNA microarray	Silver Nanoparticles (AuNPs)		(Truong et al., 2012)
	Male liver	DNA microarray	benzo-a-pyrene (BaP)		not available
	Ovary	DNA microarray	bisphenol A (BPA)		(Villeneuve et al., 2012)
	Whole organism	DNA microarray	benzene, toluene and formaldehyde		not available
	Embryo	DNA microarray	azinphos-methyl (AMP, acetylcholinesterase)		(Kluver et al., 2011)
	Heart Ventricles	DNA microarray	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)		not available
	Embryo	DNA microarray	dimethylformamide (DMF) and dimethylsulfoxide (DMSO)		(Turner et al., 2012)
	Embryo	DNA microarray	ethanol		(Tal et al., 2012)
	Embryo	DNA microarray	cyclopamine, SANT-2 and GANT-61 (hedgehog patway inhibitors)		(Buttner et al., 2012)
	Embryo	DNA microarray	diethylmaleate (DEM)		not available
	Embryo	DNA microarray	galanthamine hydrobromide (GAL)		not available
	Brain	DNA microarray	diazepam		(Oggier et al., 2010)
	Embryo	DNA microarray	PKC412 (midostaurin, antineoplastic)		(Oggier et al., 2011)
	Embryo	DNA microarray	leflunomide		(White et al., 2011)
	Embryo	DNA microarray	trimethyltin chlorides (TMT)		not available
	Whole organism	DNA microarray	17b-estradiol (estrogen) and ICI 182,780 (anti-estrogen)		(Lam et al., 2011)
	Whole organism	DNA microarray	cyclotrimethylenetrinitramine (RDX, energetic)		not available
	Female	DNA microarray		LakeMjosa and LakeLonsa natural mixes of persistent organic pollutants	not available
	Brain	DNA microarray	methyl mercury (MeHg)		(Richter et al., 2011)
	Liver	DNA microarray	HgCl2		(Ung et al., 2010)
	Gonad	DNA microarray	haloperidol (dopamine receptor antagonist)		(Villeneuve et al., 2010)
	Embryo	DNA microarray	3,4-dichloraniline (DCA), 2,4-dichlorophenol (DCP), pentachlorophenol (PCP) and cadmium chloride		(Sawle et al., 2010)
	Embryo	DNA microarray	tert-butylhydroquinone (tBHQ), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dimethyl sulfoxide (DMSO)		not available
	Liver	DNA microarray	CuSo4, NaCl and CaCl2, and mixtures		not available
	Liver	DNA microarray		waterborne copper concentrations	(Craig et al., 2009)
	Larvae	DNA microarray	microcystin		(Rogers et al., 2011)
	Whole organism	DNA microarray	estradiol and 4-nonylphenol	screening the effect of	(Baker et al., 2009)

				environmental chemicals	
	Liver	DNA microarray	PAHs and Aps		(Holth et al., 2008)
	Brain	DNA microarray	fadrozole (aromatase inhibitor)		not available
	Ovary	DNA microarray	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)		(Heiden et al., 2008)
	Liver	DNA microarray	17-beta estradiol (E2)		(Levi et al., 2009)
	Embryo	DNA microarray	Retinoic acid (RA) and RA antagonists		not available
	Brain	DNA microarray	Domoic acid		(Lefebvre et al., 2009)
	Embryo	DNA microarray	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)		(Xiong et al., 2008)
	Brain	DNA microarray	ethanol and nicotine		(Kily et al., 2008)
	Whole organism	DNA microarray	17-beta estradiol (E2)		(Cohen et al., 2008)
	Caudal fin	DNA microarray	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)		(Andreasen et al., 2006)
	Liver	DNA microarray	17 alpha-ethynylestradiol (EE2)		(Hoffmann et al., 2006)
	Heart	DNA microarray	dioxin (TDCC)		(Carney et al., 2006)
Platichthys flesus (European flounder, marine)	Liver	DNA micoarray	pyrithione (Zn–Pt), methanol (MeOH), ethanol (EtOH), dimethyl sulfoxide	water pollution (North Europe)	(Williams et al., 2011)
	Liver	DNA micoarray	cadmium chloride, 3-methylcholanthrene, Aroclor 1254, tert-butylhydroperoxide, Lindane, perfluoro-octanoic acid		(Williams et al., 2008)
	Liver	DNA micoarray		field site prediction from stress gene responses to model toxicants	(Falciani et al., 2008)
	Liver	DNA micoarray	thiuram (TMTD), and formaldehyde (FAD).	water salinity variation	(Larsen et al., 2008)
	Liver	DNA micoarray		male transcriptomic profile according to collection site	(Williams et al., 2003)
Mytilus spp. (mussel)	digestive glands	DNA micoarray	Hg	crude North Sea oil mixture	(Dondero et al., 2006)
Mytilus galloprovincialis (Mediterranean mussel)	digestive glands	DNA micoarray	okadaic acid		(Manfrin et al., 2010)
	digestive glands	DNA micoarray	Chlorpyrifos, 17β-estradiol (E2) and mixture		(Canesi et al., 2011)
	digestive glands	DNA micoarray	nickel, Chlorpyrifos and mixture		(Dondero et al., 2011)
	digestive glands	DNA micoarray	mixtures of heavy metals or organic contaminants	Venice lagoon	(Venier et al., 2006)
	digestive glands	DNA micoarray y/ quantitative-PCR/ proteome profiling	Imidacloprid and Thiacloprid		(Dondero et al., 2010)
Phaeodactylum tricornutum (pennate marine diatom)	Whole organism	DNA micoarray	Simazine, copper, ammonia, salinity, silver, cadmium, dispersant, dispersed oil		
	Whole organism	DNA micoarray / Metabolomics	cadmium		(Brembu et al., 2011)

	Whole organism	DNA micoarray / Metabolomics	Fe limitation		(Allen et al., 2008)
	Whole organism	DNA micoarray / Metabolomics	Acclimation to High Light		(Nymark et al., 2009)
Thalassiosira pseudonana (centric marine diatom)	Whole organism	DNA micoarray/Proteomics	Benzo[a]pyrene		(Carvalho et al., 2011; Carvalho and Lettieri, 2011)
	Whole organism	DNA micoarray	Limitation of Fe, N, Si, temperature and alkaline pH/CO2		(Mock et al., 2008)

12. TERMS AND DEFINITIONS

Some of the terms that are used in the text are shortly explained below. The definitions used in this report may deviate to those used in other contexts.

Acute bioassay A short-term bioassay, using high dose exposures; effects can be detected within a relatively limited period of time in relation to the life cycle of the test organism (hours to a few weeks). They provide an initial screening and simulate a "realistic worst case". Common endpoints are death or immobility of test organism.

Agonist A substance that binds to a receptor; triggering a response

Antagonist Does not provoke a response itself upon binding to a receptor, but blocks the response from agonist

Bioaccumulation: see Cis Guidance n.27

Bioassay An experiment in which organisms (in vivo bioassays) or cell cultures (in vitro bioassays) are exposed to field collected samples of e.g. sediment, surface water or effluents. The aim is to measure possible biological effects, called endpoints, of contaminants contained in these samples. In certain applications, *in vivo* assays can also be performed *in situ*, i.e. caged and exposed in the field. Common endpoints in *in vivo* bioassays include lethality, immobility and growth, whereas common endpoints in vitro assays include induction or inhibition of enzymes.

Biological organisational level The different levels in thehierarchy of biological organization such as subcellular, cellular, tissue, organism, population and community levels

Biological quality element: Ecological elements needed for the classification of ecological status of WFD

Biomarker In this report defined as any biological response to /an/ environmental chemical/s at the individual level or below that deviate from the normal status. Thus, biochemical, physiological, histological, morphological and behavioural measurements have been considered biomarkers. By including also individual level,e.g. eelpout embryo malformation (observed inside female), egg shell thickness and imposex will be included and these tools are often referred to as biomarkers. Effects observed at higher levels are however rather refered to as ecological indicators. Effects observed on suborganism levels but on cell lines exposed to samples from the environment are called in vitro bioassays, and in a similar manner, effects observed on individual (including behavioural changes) or suborganism levels in organisms that are exposed to samples in the laboratory are referred to as in vivo assays (possibly including in vitro analyses of suborganism level endpoints). »

Chemical status: The status of the WFD based on the compliance of the substance of the list of European priority with EQS.

Chronic bioassay A long-term bioassay, using low level exposure over a period of months to years. Chronic toxicity, derived by exposure to low concentrations, can eventually lead to cumulative effects and problems much more insidious than those caused by acute exposures. Chronic bioassays are designed to simulate actual situations more closely. Endpoints include reduced reproduction or growth. They are more sensitive but also more expensive and complex in practice than acute bioassays

Community An ecological community is defined as a group of actually or potentially interacting species living in the same place. The primary focus of community ecology is on interactions between species within the ecosystem (e.g. competition, predation, parasitism, etc.). Communities are analyzed by a structural (e.g. species' abundances) and functional (species' ecological roles) point of view. Inherent in this view is the notion that whatever affects one species (direct effect) may also affect other species (indirect effects).

Ecological functioning. The ecological functioning of an ecosystem is a term used to indicate the ecological and evolutionary processes (e.g. gene flow, disturbance, nutrient cycling, etc.) which occur within the ecosystem. Natural processes are the result of complex interactions between biotic (living organisms) and abiotic (chemical and physical) components of ecosystems.The study of the ecosystem functioning is based on analysis of energy fluxes and species ecological role. It differs from the study of structure, which is based on the identification of the components of the ecosystem.

Ecological status: See WFD

Ecological indicator In this report defined as tools (such as indices or approaches) that measure variations that can be observed at higher biological organization levels, such as population and community

Effect Modification induced by a stressor (either inside or outside) the organism which can be measured at molecular, cellar, physiological or at population level.**S**ignificant differences can be expected in term of magnitude, intensity and duration. Many toxic effects are reversible and do not cause permanent damage, but complete recovery may take a long time. However, some poisons/contaminants cause irreversible (permanent) damage.

Effect biomarker Biomarker response in terms of functional changes in the biological system that is analysed. Examples are disturbed basic functions such as reproduction, respiration, changes in growth rate and feeding.

Effects Directed Analysis (EDA). EDA and TIE (Toxicity identification evaluation)- identify causative agents on complex mixtures through sequential extractions, bioassays and chemical analyses.

Endpoint The variable measured in a bioassay, such as immobility, growth, death.

Environmental Quality Standard (EQS): a concentration of a substance that should be achieved in water, sediment or biota, in order to protect environment and human health

Exposure biomarker. A biomarkerthat quantifies early stage effects from exposure to environmental agents. The effects are usually occurring on subcellular levels, such as induction of metabolism, that are not necessarily leading to negative effects on individual level (see also Biomarker).

General biomarker. Biomarker that responds to several types of substances and possibly also other types of stressors than hazardous substances.

Genomics: It is a broadly used term that encompasses numerous scientific disciplines and technologies. These disciplines include gene expression at transcription level (mRNA, transcriptomics); at protein level (proteomics) at the metabolite flux (metabolomics) and at microbial population level (metagenomics).

Hazardous substance. The term is in this report referring to substances that are hazardous to ecosystems and/or human health in or via the aquatic environment. As opposed to the terms "priority substance", "river basin specific substance" etc, the term is in this report not intended to refer to any specific regulation.

Integrated (monitoring/assessment) A combined approach to assess environmental quality, based on e.g. suites of both chemical, ecological and toxicological measurements. Integrated monitoring refers to a monitoring design by which the same tissues and individuals are sampled for analyses; the same individuals are sampled for effects and chemical analyses from the same population as is used to determine population structure parameters and at the same time. Water, sediment and biota are sampled at the same time and location, and there is a simultaneous measurement of support parameters.

In vitro **bioassay** Laboratory bioassays that use prepared cells or subcellular fractions isolated from organisms or modified bacteria. The analyses are mechanism based, of short duration and performed in small scale.

In vivo **bioassay** Whole organism bioassays, that respond in an integrated manner to all of the contaminants that are present in a test sample. These assays therefore lack specificity but are of high relevance.

Metabolomics: It describes the pattern of metabolites that are generated upon exposure to a chemical/stressor (M. R. Viant, U. Sommer, 2012). It would form a sort of genetic "signature". Techniques are NMR spectroscopy and high resolution mass spectrometry.

Metagenomics: It is the fully genome sequencing of a water sample (or any other matrix e.g. soil, gut, etc.) to identify the microorganism compositions and metabolic pathways (Kisand, V. et al. , 2012). Techniques are pyrosequencing, New Sequencing Generation (NGS) and bioinformatics tools.

Mode of action: The term mode of action is defined by Rand et al. 1995 as follows: "A mode of action is defined as a common set of physiological and behavioral signs that characterize a type of adverse biological response, while toxic mechanism(s) refer(s) to the crucial biochemical process(es) and/or xenobiotic-biological interaction(s) underlying a

given mode of action". However there are various definitions existing for both terms whereas it is sometimes difficult to separate them clearly.

PICT. Pollution-induced community tolerance (PICT) is a method used for detecting toxic effects of pollutants in biotic communities. The concept of PICT is based on the fact that organisms in a toxic environment only survive if they are tolerant to the toxicant. It relies on the assumption that sensitive components of the exposed community (species, genotypes or phenotypes) will be replaced by more tolerant ones during exposure, thus leading to an increase of community tolerance. Tolerance development can be, for example, measured as a shift in the Effect Concentration (usually EC_{50}) that is obtained with a short-term toxicity test based on a physiological endpoint. The PICT-method, which uses the theoretical basis of toxicology (the dose–response model) to quantify community effects, was proposed as a tool with strong predictive ability for causative links between toxicants and their adverse ecological effects (tolerance levels).

Priority substance: a substance of European list of priority that should be reduced from emissions, discharges and losses.

Priority Hazardous Substance a substance of European list of priority that should be eliminated from emissions, discharges and losses.

Proteomics: It describes the pattern of protein expression, changes induced upon exposure to a chemical/stressor. It would form a sort of genetic "signature". Techniques are 2D electrophoresis gels, itraq labeling (Carvalho, R. et al., 2011). Mass spectrometry for the identification of proteins .

River Basin Specific Pollutant (RBSP): See Specific pollutant

SPEAR (Species At Risk) is an index based on biological traits of freshwater invertebrate species. The traits considered are bound to species sensitivity to the presence of specific toxicants (e.g. physiological sensitivity) and to their potential exposure and recovery capacity (migration ability, presence of sensitive life stages during maximum pesticide exposure, generation time). Taking these aspects into account, the SPEAR indices measure the proportion between sensitive (SPEAR: SPEcies At Risk) and tolerant (SPEnotAR, "SPEcies not At Risk") species. The index is described in more detail in chapter 4.

Species traits: Life history characteristic. Each species is in fact characterized by biological (e.g. life cycle, respiration mode, reproduction, body size, etc.) and ecological traits (e.g. feeding habits, habitat preference, tolerance to stressors, etc.) selected by evolution as strategies to cope with environmental stress (habitat templet theory) (Townsed et al., 1994).

Specific biomarker: Biomarker that primarily responds to a specific group of substances

Specific pollutant: Chemical substances that are part of the ecological status of the WFD, they can be defined at river basin level or at national level.

Toxicity profile Toxicological "fingerprint" of a sample, ranging from a pure compound to a complex mixture, obtained by testing the sample or its extract for its activity toward a battery of biological endpoints (Hamers et al 2013)

Transcriptomics: It describes the pattern of gene expression changes induced upon exposure to a chemical / stressor . It would form a sort of genetic "signature"(T. Lettieri, 2006). Techniques used for transcritpomics are DNA microarray, quantitative Real Time PCR.

Trophic level: A group of organisms that occupy the same position in a food chain. A trophic level represents a step in the dynamics of energy flow through an ecosystem. The first trophic level is composed by the producers, which can harvest energy from an outside source, such as the sun. The second level comprises organisms who consume the producers, also known as the primary consumers. The next level would contain the secondary consumers (those who consume the primary consumers), and so on. Ecological communities with higher biodiversity form more complex trophic paths, known as food webs.

13.LIST OF ABBREVIATIONS

AA Annual Average
AChE Acetyl Cholinesterase
AhR Arylhydrocarbon Receptor
ALA-D Aminolaevulinic acid dehydratase
AR Androgen Receptor
BAC Background Assessment Concentration
BAT Best Available Technique
BCF Bioconcentration Factor
BDE Brominated diphenyl ether
BQI Benthic Quality Index
BSAP Baltic Sea Action Plan
CALUX Chemically Activated Luciferase Expression
CEMP Co-ordinated Environmental Monitoring Programme
CIRCA Communication and Information Resource Administrator
CIS Common Implementation Strategy
CMEP Chemical Monitoring and Emerging Pollutants
COHIBA Control of hazardous substances in the Baltic Sea region
COM European Commission
COMBINE Cooperative Monitoring in the Baltic Marine Environment
CR Contamination Ratio
CYP Cytochrome P450 (CYP 1A: family 1 subfamily A)
DBT dibutyltin
DDT dichlorodiphenyltrichloroethane
DEHP bis (2-ethylhexyl)phthalate
DR Dioxin Receptor
EAC Environmental Assessment Criteria
EC Effect Concentration
ECHA European Chemicals Agency
EcoO Ecological Objective
EcoQO Ecological Quality Objective
EDA Effects Directed Analysis
EDTA ethylenediaminetetraacetic acid
EE-2 17-alpha ethinyl oestradiol
EEA European Environment Agency
ELISA enzyme-linked immunosorbent assay
EN European Norm
EqP Equilibrium Partitioning
EQS Environmental Quality Standard
ER Estrogen Receptor
EROD Ethoxyresorufin-O-deethylase
FDI Fish Disease Index
GC Gas chromatography
GES Good Environmental Status
GIS Geographic Information System
GR Glucocorticoid Receptor
GSI Gonadosomatic Index
HBCDD, hexabromocyclododecane
HCB hexachlorobenzene
HCBD hexachlorobutadiene
HCH Hexachlorocyclohexane
HELCOM Helsinki Commission (The Baltic Marine Protection Commission)
HPLC High Pressure Liquid Chromatography
ICES International Council for the Exploration of the Sea
ICP Inductively Coupled Plasma

IMO International Maritime Organisation
ISO International Standardisation Organisation
JAMP Joint Assessment and Monitoring Programme
JRC Joint Research Centre of the European Commission
Koc Organic Carbon Coefficient
Kow Octanol Water Coefficient
LOD Level of Detection
LOQ Level of Quantification
LSI Liver Somatic Index
MAC Maximum Allowed Concentration
MBT monobutyltin
MDR/MXR Multidrug/multixenobiotic Resistance
MODELKEY Models for Assessing and Forecasting the Impact of Environmental Key Pollutants on
 Marine and Freshwater Ecosystems and Biodiversity
MONAS Monitoring and Assessment
MS Mass Spectrometry
MS Member State
MSFD Marine Strategy Framework Directive (2008/56/EC)
MT Metallothionein
NPE Nonylphenol Ethoxilates
OECD Organisation for Economic Co-operation and Development
OSPAR Oslo Paris Convention (The Convention for the Protection of the Marine Environment of the North-East Atlantic)
PAH Polycyclic Aromatic Hydrocarbons
PBDE Polybrominated Diphenyl ethers (/penta/)
PCB Polychlorinated biphenyl
PCDD/F Polychlorinated dibenzodioxins/furans
PCP Pentachlorophenol
PEC Predicted Environmental Concentration
PFCs perfluorinated compounds
PFOA perfluorooctanoic acid
PFOS Perfluorooctanesulfonic acid
PHS Priority Hazardous Substances
PICT Pollution Induced Community Tolerance
PNEC Predicted No Effect Concentration
POP Persistent Organic Pollutants
PPAR Peroxisome Proliferator Activated Receptors
PR Progesterone Receptor
PS Priority Substances
QA Quality Assessment
QA/QC directive" (2009/90/EC),
QS Quality Standard
QSR Quality Status Report
RBSP River Basin Specific Pollutant
REACH Registration Evaluation Authorisation and Restriction of Chemicals (EC/1907/2006)
RID Comprehensive Study on Riverine Inputs and Direct Discharges
RBMP River Basin Management Plan
RPSI Relative penis size index
RYA Recombinant Yeast Assay
SCHER Scientific Committee on Health and Environmental Risks
ScorePP Source Control Options for reducing emissions of priority pollutants
SDS Substance Data Sheets
SOCOPSE Source Control of Priority Substances in Europe
SPE Solid Phase Extraction
SPEAR Species At Risk
STP Sewage Treatment Plant
TBT tributyltin
TEF Toxicity Equivalent Factor
TEQ Toxic Equivalent
TIE Toxicity Identification Evaluation
TOC Total Organic Carbon
TR Thyroid Receptor

TU Toxic Unit
UN United Nations
VDSI (Vas Deference Sequence Index)
VTG Vitellogenin
WEA Whole Effluent Assessment
WFD Water Framework Directive (2000/60/EC)
WGBEC Working Group on Biological Effects of Contaminants
WGE Working Group E (Environment)
WHO World Health Organisation
YAS Yeast Androgen Screen
YES Yeast Estrogen Screen

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