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Workshop 3.3

Biomonitoring: Integration of biological endpoints into chemical monitoring*

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Abstract: Biomonitoring is currently performed at two levels, assessing exposure to pollutants and effects monitoring by bioassays. As an example for the first approach, vitellogenin (VTG) in male fish of *Abramis brama* as an endpoint for estrogen exposure is discussed. However, similar changes of VTG or VTG-like proteins in the hemolymph of mussels could not be detected. Enzyme-linked receptor assays for monitoring estrogenic effects at the molecular level serve as an example for the second category. Applications of the enzyme-linked receptor assay (ELRA) developed in our laboratory are presented. Detection limits of 0.02 μg/l 17β-estradiol were recently achieved with the chemiluminescent format. Although effect monitoring provides information in terms of toxicity equivalents, it is not possible to relate the signals to specific pollutants and their concentrations. For this purpose, chemical analysis is required. New approaches are reported for the direct coupling of bioassays and chemical analysis. This concept is defined as bioresponse-linked instrumental analysis. It combines biomolecular recognition, initiating a biological effect, and chemical analysis. In addition to the classical bioanalytical approaches, new strategies in genomics and proteomics have been developed. This may lead to multimarker approaches opening this area to environmental analytics.

INTRODUCTION

Biological responses can be exploited for environmental monitoring. Especially programs in the assessment of water quality monitoring take advantage of this approach. Biomonitoring is carried out at several levels of complexity: the level of populations and species as well as the suborganismic and molecular level. Two different strategies have been used in the past: the application of (1) bioindicators for assessing exposure to pollutants and (2) biotests, biosensors, and reporter organisms for monitoring the effects of pollutants. Endocrine disruptors (EDCs) are used in this chapter for highlighting current approaches to biomonitoring at the suborganismic level. Although test systems with whole organisms currently provide the only means to measure integral effects, their disadvantages are due to the high variability of the results resulting from the heterogeneity of organisms, high costs, and the difficulty to relate damage to individual pollutants. In contrast, suborganismic tests can provide more specific and faster responses. But it is clear that structure and concentration of pollutants are only revealed by chemical analysis. Therefore, new approaches are required for a tighter coupling of bioassays and chemical analysis. Bioresponse-linked instrumental analysis combines biomolecular recognition, initiating a bi-

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ological effect, and chemical analysis. In addition, it is necessary to learn more about effects and mechanisms of EDCs in living organisms. One of the main problems of bioanalytics is due to the fact that test systems with whole organisms do not provide specific information on the mechanism of the effect. In contrast, suborganismic tests can only cover a limited range of toxicity classes. This gap can be bridged by the application of multi-endpoint approaches, which can be realized in the form of DNA microarrays and 2D gel electrophoresis.

VITELLOGENIN AS A BIOINDICATOR FOR ENDOCRINE DISRUPTION

Biomarkers are valuable tools for estimating the exposure of organisms to environmental pollutants. A well-known example is the use of vitellogenin for assessing estrogenic effects in male fish [14]. VTG is an ancient transport and storage protein that serves as a ligand for the delivery of nutrients to the egg yolk. In male fish, the levels are very low, but they rise dramatically in the presence of estrogenic compounds. For instance, plasma levels in the mg/ml range are detected in male as well as immature fish after injection of 17ß-estradiol [13,15]. Figure 1 illustrates this effect in bream *(Abramis brama)* where an exposure of male fish was carried out with ethinylestradiol (EE2). The SDS gel shows an additional band of 120 kDa that is present in untreated females, but missing in male controls.

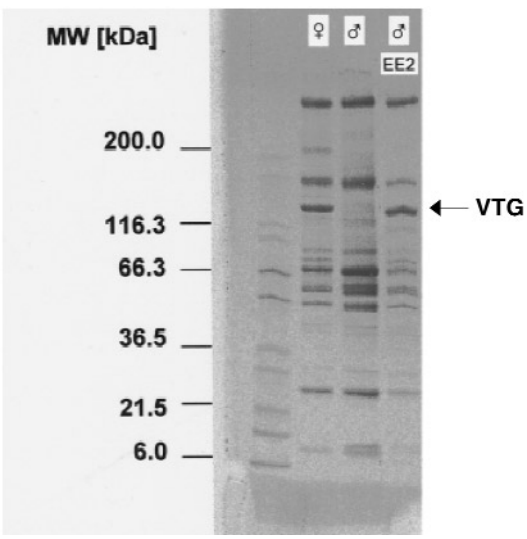

Fig. 1 Effect of ethinylestradiol on male fish *(Abramis brama)*. The animals were exposed to EE2 and compared to untreated female and male fish. After separation of serum proteins by SDS gel electrophoresis, an additional band at 120 kDa is seen.

There is considerable interest in the use of biomarkers from bivalve species for estrogens. Mussels are sessile and filter huge amounts of water. They should therefore be main targets for estrogenic contaminants. However, it is still under debate whether there is a dose–response relationship for estrogens and VTG in mussels. If the situation was similar to fish, VTG or VTG-like proteins in mussel hemolymph would be an ideal endpoint for an exposure to estrogenic compounds and allow the monitoring of a large number of samples, for instance by enzyme-linked immunosorbent assay (ELISA). Therefore, experiments were carried out to examine whether hemolymph is a carrier of VTG or VTG-like proteins or other proteins under estrogenic control that could be targets for indicator assays. For this purpose, 1D and 2D polyacrylamide gel electrophoresis (PAGE) of mussel plasma were performed. First, it was found that the protein concentration of mussel plasma prepared from the hemolymph is very low compared to other animals (0.36 mg/ml for *Anodonta* and 0.40 mg/ml for *Mytilus* plasma).

This means that mussel plasma contains 50–200 times less soluble protein than fish. Even when compared to other mollusks (snails and octopus), mussel plasma has a very low protein concentration.

Mussels were either injected or exposed to water (hormone concentrations up to 2 μg/l 17ß-estradiol, E2). Control experiments with male fish showed clearly that the parameters chosen were suited to strongly induce VTG in plasma. However, no significant change of proteins in response to E2 in the plasma of treated mussels could be detected. Figure 2 shows the results for the freshwater mussel (*Anodonta cygnea*).

Even 2D electrophoresis did not reveal any protein, which was induced by estrogen [10].

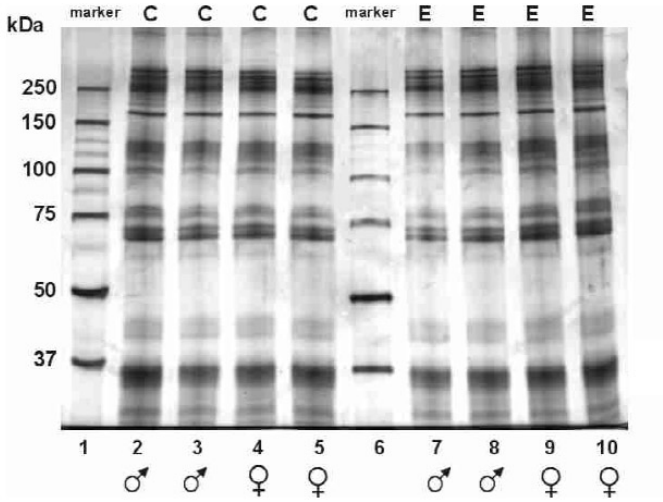

Fig. 2 SDS-PAGE (7.5%) under reducing conditions of the pooled plasma ($n = 5$) from male and female *A. cygnea* after exposure to 2 μg/l E2 for three weeks (E) and compared to control mussels (C).

USE OF GENE EXPRESSION PATTERNS FOR THE MEASURING OF MULTIPLE ENDPOINTS

Biomarkers like VTG are suitable tools for the detection of estrogenic compounds in environmental compartments. However, the mechanisms of action in target organisms are not entirely clear. For example, it is open whether different estrogenic compounds cause the same effect or trigger a variety of effects.

For this reason other approaches have to be applied, which provide information on effect mechanisms. As estrogenic compounds act by altering the gene expression in different tissues, one option is to examine gene expression patterns. This can be done in different ways. For the mRNA level, rt-PCR and DNA microarray techniques can be applied. For the protein level, 2D gel electrophoresis combined with mass spectrometry is used. To carry out gene expression experiments, zebrafish as a common model organism were exposed to xenobiotics, and the resulting alterations of the gene expression patterns in different tissues are mapped. DNA microarray techniques are based on the hybridization of two complementary nucleic acid strands. The probe consists of an oligonucleotide with the sequence of a specific gene and is immobilized on a suitable surface such as a glass slide. The mRNA from a target tissue is reverse transcribed to cDNA. In this process, a fluorescent dye is incorporated into the cDNA. The cDNA is then hybridized to the probe. After readout with a fluorometer, the gene expression pattern can be analyzed.

On the protein level, gene expression patterns can be determined by 2D gel electrophoresis giving information on the proteins expressed at the time the sample is collected. After picking differentially expressed spots, an identification of the protein can be carried out by mass spectrometry, e.g.,

MALDI-TOF. Using massive parallel approaches of DNA microarrays and/or 2D gel electrophoresis, a global view of the gene response in an organism can be gained.

For the detection of estrogenic effects in zebrafish (*Brachidanio rerio*) reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA microarray approaches are developed in our group. Fish exposures, were carried out in 20 l glass tanks for five days. A flow-through system with a flow-rate of 14.4 ml/min fresh water in the tank provided stable conditions for the fish. Plants, gravel, or plastic (e.g., filtering unit) was omitted in the tank. For the exposures the concentration was kept at 1µg/l 17ß-estradiol. To control the endocrine activity in the water, samples were taken three times a day and measured by the ELRA (cf. below).

The RT-PCR method starts with a phenol-extraction of RNA from gonads and liver of female and male fish. Then mRNA was transcribed to cDNA by reverse transcription (RT). Specific sequences of VTG gene 3 (vg3), aromatase (ovary form), and estrogen receptor α (ER) were used as primers for the PCR. The products are 304 bp (vg3), 410 bp (ER), and 610 bp (aromatase) and were electrophoresed in an 1 % agarose gel (Fig. 3).

In the control fish, vg3 was found mainly in liver tissue of the female fish. Also in the gonads of female fish vg3 was expressed, but weaker than in the liver. In the male fish no vg3 expression could

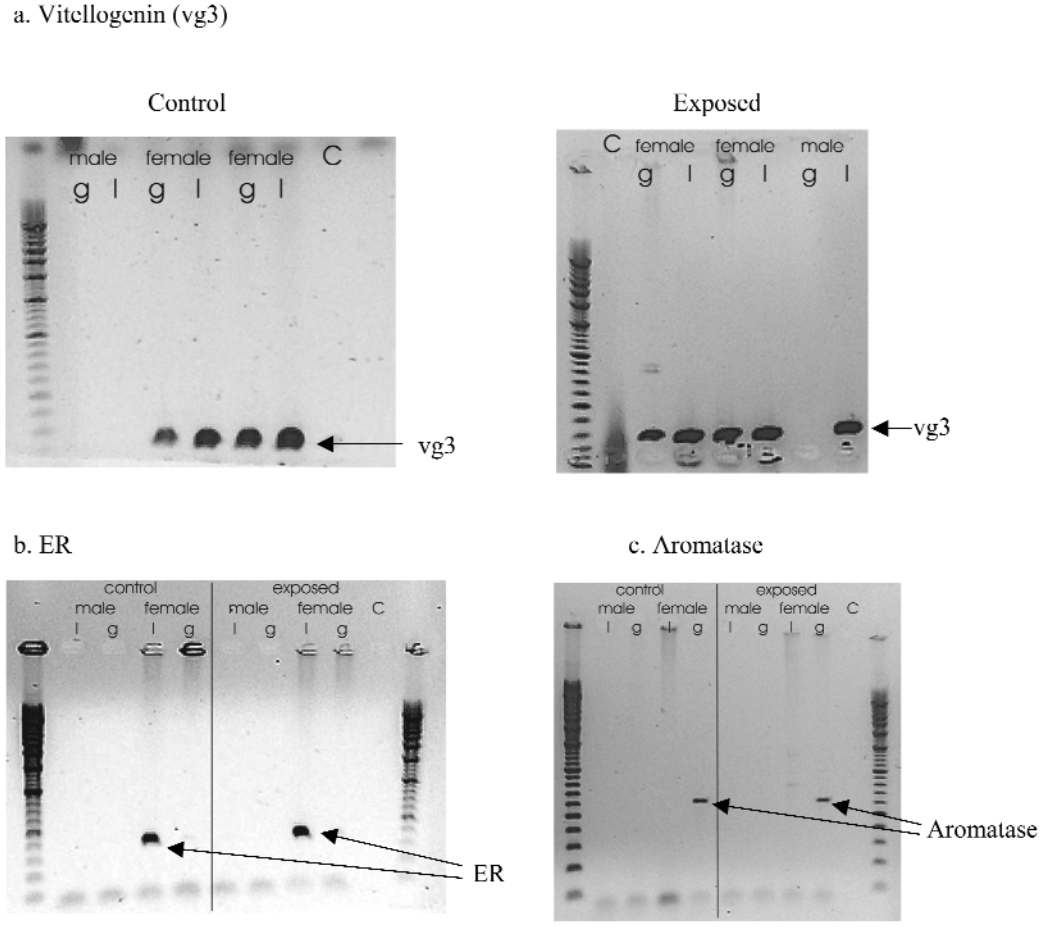

Fig. 3 RT-PCR of zebrafish mRNA of exposed fish and controls (l: liver; g: gonads; C: controls without template).

be found. Since VTG is a vertebrate egg yolk protein, only female zebrafish should express the vitellogenin gene under normal conditions.

In the exposed group, female fish show vg3 mRNA in gonad and liver tissue. Due to 17ß-estradiol in the water, also male fish show a strong expression of VTG in the liver, but not in the gonads.

The second gene we looked at codes for the ERα. We found expression of ER mainly in the liver and much weaker in the gonads of female fish. No expression could be detected in the male individuals. After exposure, no change in the expression pattern could be detected. The amount of ER expressed in females seems to be slightly increased. This is currently under investigation.

The third primer pair we used was complementary to aromatase (ovary form), an enzyme that catalyzes the conversion of androgens to estrogens. Again, no mRNA could be found in male fish, no matter whether they were exposed or not. Therefore, the expression of aromatase does not seem to be under positive control of 17ß-estradiol in male zebrafish.

The exposed and nonexposed females show aromatase expression in the gonad tissues, but not in liver, which illustrates the importance of the gonads for the conversion of androgens.

Further investigations on gene expression patterns are currently carried out in our lab using mainly DNA microarray techniques with a largely expanded number of different zebrafish genes.

USE OF ESTROGEN RECEPTORS FOR THE DETERMINATION OF ESTROGENIC COMPOUNDS

In the environment, estrogenic compounds not only occur as residues of natural sexual hormones of humans and animals, but also as constituents of plants and fungi (phyto- and mycoestrogens). Also, pharmaceuticals and xenobiotics can act as estrogens. The wide variety of potentially estrogenic compounds makes it difficult to predict the estrogenicity of xenobiotics on a structural basis [9]. Thus, monitoring strategies cannot be based exclusively on chemical analysis of environmental samples for known or suspected structures. In addition, an analytical system should be capable of detecting estrogenic compounds, even if they are yet unknown. There are different ways of measuring estrogenic compounds in the environment. The most common ones are cell proliferation assays (E-screen assay with hormone-dependent growth of MCF-7 breast cancer cells), reporter-gene assays and receptor-ligand interaction assays. For receptor-ligand interaction assays, hormone receptors have to be available. This can be achieved by laborious purification from tissues containing the receptor or, more elegantly, by recombinant techniques.

As an example, estrogen receptor α (ERα) can be produced in a recombinant yeast expression system. Briefly, yeast (strain BJ3503) was transformed with an expression plasmid (kindly provided by Prof. D. P. McDonnell, Duke University, NC) containing the gene for the human ERα. After induction with Cu^{2+}, the receptor was expressed as an ubiquitin fusion protein. After removing the cell walls by enzymatic digestion, the cells were lysed by hypotonic shock to liberate the receptor. The cell extract was centrifuged (at 22 000 × g) and the supernatant was used for further purification. The receptor was partially purified by affinity chromatography with heparin agarose. The recombinant receptor was undegraded, soluble, and biologically active. After affinity purification, receptor yields in the range of 10 μg were obtained routinely from 5 l yeast batch cultures.

The receptor properties, especially the affinity characteristics, were determined with a surface plasmon resonance sensor (Biacore). The affinity data obtained for the produced receptors is well comparable to literature data (Table 1).

Table 1 Comparison of K_D values reported for the human estrogen receptor α.

Receptor	K_D	Method	Ref.
Recombinant human estrogen receptor α	2.3×10^{-10}	SPR-sensor	[11]
Human estrogen receptor α isolated from mammary tumor cytosol	4.2×10^{-10}	Radioreceptor assay	[4]
Human estrogen receptor α isolated from MCF-7	2.0×10^{-10}	Radioreceptor assay	[8]

A simple and cost-effective test format to measure biomolecular interactions is the enzyme–linked receptor assay (ELRA). The ELRA is a microwell-based receptor-binding assay using the human ERα. Measurements are carried out in 96-well microwell-plates. In the first incubation step, an estradiol-BSA conjugate is absorbed to the walls of the microwells. In the second (competition) step, an estradiol solution of defined concentration is added together with the ERα. After the receptor binding reaction, a biotinylated mouse antiestrogen-receptor antibody is added. A streptavidin-peroxidase-biotin enhancement system, is applied. By using the luminescent substrate luminol, a significant improvement of the detection limit of estradiol (0.02 µg/l) could be achieved recently (Fig. 4), compared to the standard assay (0.1 µg/l, [12]).

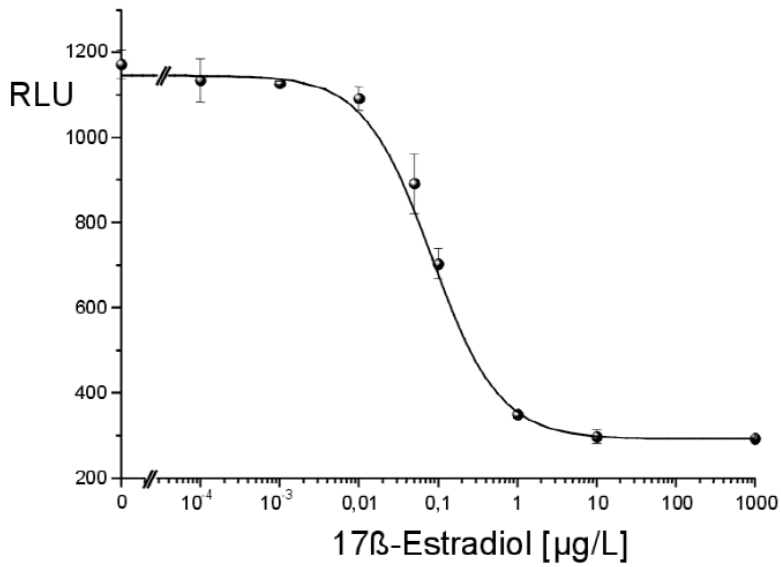

Fig. 4 ELRA calibration curve, using the luminescent substrate luminol. The detection limit is 0.02 µg/l.

The ELRA approach has proven to be robust and highly suited for the detection of natural and synthetic estrogens as well as xenoestrogens in field studies. Figure 5 shows the comparison of spiked lake water measurements, which were carried out with the ELRA and HPLC as reference method. A close correlation ($r = 0.987$) could be achieved.

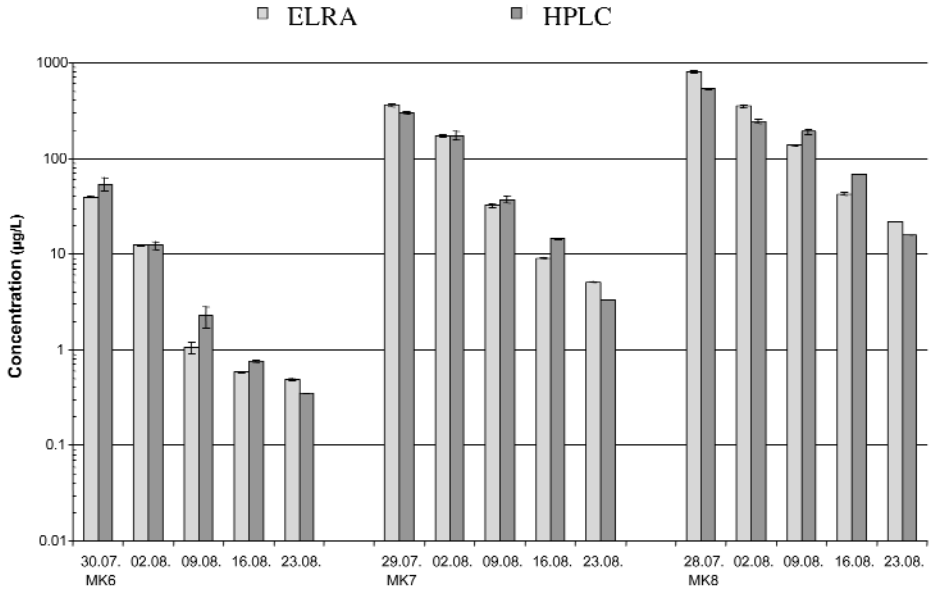

Fig. 5 ELRA applied for real water samples. The results show the measurement of spiked lake water from a mesocosm (MK) experiment. Reference analyses were carried out by HPLC. The mesocosm experiment and HPLC measurements were performed by the Institute of Ecological Chemistry, GSF (Neuherberg, Germany).

BIORESPONSE-LINKED INSTRUMENTAL ANALYSIS

A comparison between biological and chemical approaches to environmental analysis reveals that they are clearly complementary. Bioanalysis provides information on the (potential) biological activity of a sample, chemical analysis on its composition. Therefore, it would be advantageous to combine both principles—the goal of bioresponse-linked instrumental analysis (BLIA). The proper application of this strategy reduces chemical analysis to those samples or fractions of samples that contain bioeffective compounds. The concept has been put forward by Bilitewski et al. [1] and Hock [5].

The biomolecular components serve as targets for bioactive substances. Although binding assays can already be performed on this basis providing toxicological or pharmacological equivalents, only chemical analysis yields information on the responsible substances. Chemical analysis is therefore performed with those substances that are bound by the biological recognition element and are therefore bioactive.

There are principally two strategies for BLIA (Fig. 6): (1) Sample separation followed by bioassays; biological active samples are automatically analyzed by instrumental analysis. An excellent example is given by Schobel et al. [11] who report the identification of estrogenic compounds by bioresponse-linked instrumental analysis (BLIA). (2) Binding assays providing toxicity equivalents are followed by the separation of bound and free binding proteins. Bound ligands are analyzed by MS. Obst and Brenner-Weiß [7] report the further development of this approach using EDCs as an example.

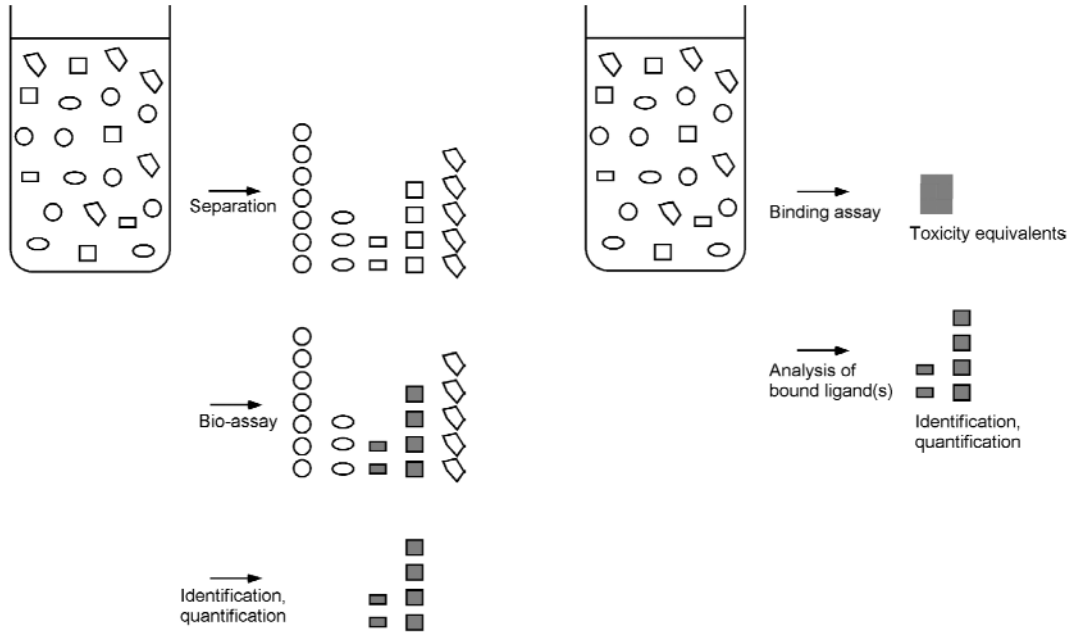

Fig. 6 Two approaches to BLIA. Left: Sample separation, e.g., by LC, followed by bioassay and MS. Right: Binding assay followed by analysis of bound analyte, e.g., by MS.

DISCUSSION

Approximately 5 million chemicals are presently known and 80 000 in use; 500–1000 are added per year [3]. This creates a problem for environmental analysis when relevant pollutants are to be identified. Since usually mixtures of substances are present that can attenuate or enhance individual effects, chemical analysis is not sufficient to estimate a potential damage. For this purpose, bioanalysis is additionally required. BLIA is considered a breakthrough as it links bioanalysis with chemical analysis. Advances in instrumental analysis as well as a better access to biological target structures have contributed to realize this concept. BLIA in parallel obtains chemical and toxicological data and focuses analytics on relevant samples.

A disadvantage of subcellular and related tests is due to the fact that they cover only a small section of the total spectrum of possible bioeffects. This creates a dilemma between tests with whole organisms, which register integral effects, but usually do not link them to specific classes of toxicity, and suborganismic tests, which detect selective effects but do not provide information on effects on the whole organisms. A compromise is offered by two approaches, the analysis of gene expression patterns as well as multifunctional tests.

A change of gene expression patterns not only indicates general stress situations, but also specific effects of pollutants on the organism. In other words, multiple effects can be detected. An important task for the forthcoming years is to expand the list of suitable marker genes, whose expression indicates toxic effects. Available genes include the gene for vitellogenin, aromatase, cytochrome P456. This strategy may be particularly useful in exposition studies, especially in ecosystems. A most attractive aspect is the possibility to detect chromic effects.

Multifunctional tests are the opposite of multianalyte methods, as they are used in immunochemistry [2,6]. Multifunctional tests combine several subcellular bioassays. However, it is not clear, yet, how powerful these measuring systems have to be in order to mirror effects on the whole organism. This question is related to the classical philosophical dispute between reductionism and holism and deals

with the problem, to which extent analytical methods provide information on complex processes in organisms. It is expected that bioinformatics and systems biology will considerably promote this field.

Progress in science in the last decades has increasingly shed light on risks for public health as well as the environment involved in endocrine disruptors. Unfortunately, scientific ignorance for the unintended consequences will always be behind the development and application of new substances that might have an influence on the hormonal system of animals and humans. It is therefore essential to implement a durable and adequate risk assessment and to ensure that reassessment of the issue will always take place when new knowledge that might influence the previous risk estimation becomes available.

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