IN VITRO BIOASSAYS – VALUABLE TOOLS CONTRIBUTING TO THE CONSERVATION OF ENDANGERED SPECIES

ARNO C. GUTLEB, Centre de Recherche Public – Gabriel Lippmann, Departement, Environnement et Agrobiotechnologies, 41, rue du Brill, L-4422 Belvaux, Luxembourg
ERIK ROPSTAD, Norwegian School of Veterinary Science, POB 8146 Department, N-0033 Oslo, Norway
INGVAR BRANDT, Uppsala University, Department of Physiology and Developmental Biology, Norbyvägen 18A, SE 752 36, Uppsala, Sweden,
ALBERTINKA J. MURK, Wageningen University, Toxicology Section, Tuinlaan 5, NL-6703 HE Wageningen, The Netherlands

ABSTRACT: Exposure assessment usually is performed by means of chemical analytical methods and subsequently reporting the concentration of known and expected pollutants. Effects assessment typically measure the effects of single compounds or mixtures on laboratory experimental animals. Because these compounds often are present in complex mixtures, the difficulties and costs for chemical analysis to assess internal exposure in organisms are rapidly increasing. More importantly, chemical analysis alone often is not sufficient for toxicological risk assessment because mixture effects cannot yet be predicted and unknown compounds and biotransformation products may contribute significantly to the toxicological risk. An alternative approach is to detect and quantify the presence of endocrine disrupting chemicals by means of biological markers to assess exposure and effect. During recent years a range of *in vitro* bioassays have been developed, validated, and applied that are able to detect compounds having a "common mechanism of action." Biological test systems based on a "common mechanism of action" may provide very useful and rapid screening methods by measuring the biological potency of single compounds, mixtures or environmental extracts, with outcomes identifying the effects of hitherto unknown and eventually species-specific metabolites of the compounds in question. Reporter gene assays, also have been used in many studies and the combination of speed, relative simplicity, and ability to integrate effects of complex mixtures also makes it a valuable additional tool for studies related to pollution in endangered species such as otters and their environment.

KEY WORDS: bioassay, complex mixtures, toxicity assessment.

In addition to natural stressors in the environment of wild animals (i.e., temperature, food and water shortage, and predatory stress), man-made chemicals, with a potential to disrupt endocrine systems have been indicated as modulators of the mammalian endocrine function capable of producing adverse health effects in both in adults and offspring (Brouwer et al. 1998, EDSTAC 1998, Vos et al. 2000, Skakkebaek 2002, Legler and Brouwer 2003, Meerts et al. 2004). Altogether there is a complex interaction between biotic and abiotic factors that determine the potential for contaminant exposure and subsequent effects of complex mixtures present in the environment.

Ecotoxicological risk assessment of environmental contaminants is based on exposure assessment comparing environmental levels with no-effect levels determined in toxicological experiments. Exposure assessment is usually performed by chemical analytical methods and concentration of known and expected pollutants are reported. Effects assessment is usually performed using laboratory experimental animals measuring the effects of single compounds or mixtures. Whereas in the past many contaminants were present in high concentrations and crude endpoints such as retarded growth or death were recorded, in the current situation of

decreasing concentrations of many compounds more dedicated approaches are needed. Because many compounds are suspected to act as endocrine disrupting compounds (EDCs) (Tyler et al. 1998, Vos et al. 2000, Charles 2004, Fisher 2004, Wester et al. 2004, Charles et al. 2005) at very low concentrations further aggravates the analytical problems. There are several indications for compounds with subtle but important negative effects on wildlife in the environment, including long-term effects of low-dose exposure, agonistic, potential and antagonistic effects of compounds present in realistic exposure mixtures and different effects of parent compounds and metabolites (Hayes et al. 2006, Gutleb et al. 2007*a,b*, Smith et al. 2007, Foekema et al. 2008).

The number of chemicals suspected to have various affects on wildlife and humans are steadily increasing in the environment and in food, and feed and prey, respectively (Sjödin et al. 2003, Levin et al. 2004, Valters et al. 2005, Weiss et al. 2005, DeLorenzo and Serrano 2006, Fischer et al. 2006, Goksøyr 2006, Kannan et al. 2006, Toppari et al. 2006, Belpomme et al. 2007, Hall et al. 2007, Juhasz and Naidu 2007, Koutsaftis and Aoyama 2007, Strid et al. 2007, Antizar-Ladislao 2008). As such compounds often are present in complex mixtures, the difficulties and costs for chemical analysis to assess internal exposure in organisms are rapidly increasing. More importantly, chemical analysis alone is not sufficient for toxicological risk assessment because mixture effects cannot yet be predicted and unknown compounds and biotransformation products may contribute significantly to the toxicological risk. In addition, the evidence of delayed effects of early life-stage exposure to low doses increases the need to analyse low concentrations of all relevant compounds present (Gutleb et al. 1999, Meerts et al. 2004, Bistodeau et al. 2006, Hayes et al. 2006, Dickerson and Gore 2007, Gutleb et al. 2007*b*, Rhind et al. 2007, Foekema et al. 2008, Ottinger et al. 2008), thus further increasing the need for sophisticated chemical analyses, which are costly and time consuming.

An alternative is to detect and quantify the presence of endocrine disrupting chemicals by means of biological markers for exposure and effect. During recent years a range *of in vitro* bioassays have been developed, validated and applied to detect compounds that have a common mechanism of action. Biological test systems based on a common mechanism of action may provide very useful and rapid screening methods by measuring the biological potency of single compounds, mixtures or environmental extracts with responses including the effects of hitherto unknown and eventually species-specific metabolites of the compounds in question. Bioassays also are able to integrate agonistic and antagonistic effects of complex mixtures on a certain receptor or act via a certain pathway, thereby providing results of high biologically relevance. In case of a high bioassay response, chemical analysis of the mixture then can be applied to identify the relevant causative compounds.

The common aspect of many *in vitro* bioassays is that receptor binding determines if a compound is relevant or not. This binding of environmental contaminants to a specific biological receptor protein can be utilised for bioanalysis. A bioanalytical tool, usually a cell line expressing the receptor, is able to selectively detect specific receptor ligands (Fig. 1). When from *in vivo* research the relationship between toxic potency via such specific receptors and biochemical/physiological and toxic effects is known, it is possible to predict possible consequences of the *in vitro* toxic potencies determined in the environmental samples such as animal tissues or sediments (Fig. 1).

Within the last years a variety of bioassays have proved useful to detect hazardous compounds, to elucidate their modes of action, or to screen for the presence of a wide variety of chemicals. For example, *in vitro* reporter gene bioassays offer fast and reliable detection of compounds that bind and activate arylhydrocarbon (Ah)-receptors (Murk et al. 1996, 1998, Legler et al. 1999, Hamers et al. 2003, Whyte et al. 2004, Vethaak et al. 2005, Manabe et al. 2006, Mortensen and Arukwe, 2007, Narita et al. 2007), estrogen receptors (Murk et al. 2002,

Houtman et al. 2004), or thyroid hormone receptors (Gutleb et al. 2004; Schriks et al. 2006, 2007). The challenge for bioassays is the rapid and cost-efficient detection of single chemicals or mixtures that are potentially responsible for sublethal, chronic toxic effects.

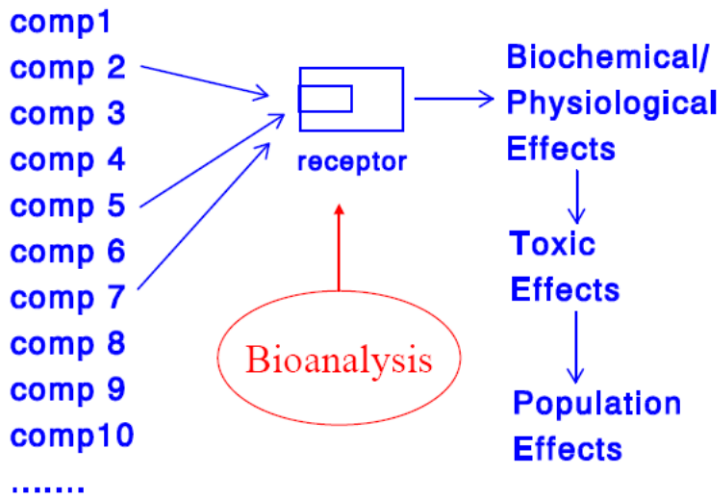

Fig. 1. *Principle of bioanalysis for compounds present in biotic or abiotic environmental samples*

Chemicals of different structural classes have been linked to biological responses through binding and activation of certain receptors (Bainy et al. 2007), the most well known and thoroughly studied example is the binding of certain polychlorinated dioxins (dibenzofuranes, coplanar polychlorinated biphenyls (PCBs), and polyaromatic hydrocarbons (PAHs) to the Ah receptor. In reporter gene assays such as for dioxin-like compounds (Fig. 2) the induction or repression of gene transcription following contaminant exposure is measured. Binding of a ligand to the AhR results in a conformational change that facilitates binding of the activated receptor to specific DNA sequences, with subsequent modulation of gene expression. A marker for AhR dependent gene activation (e.g., luciferase) can be analysed by measuring light after addition of luciferin.

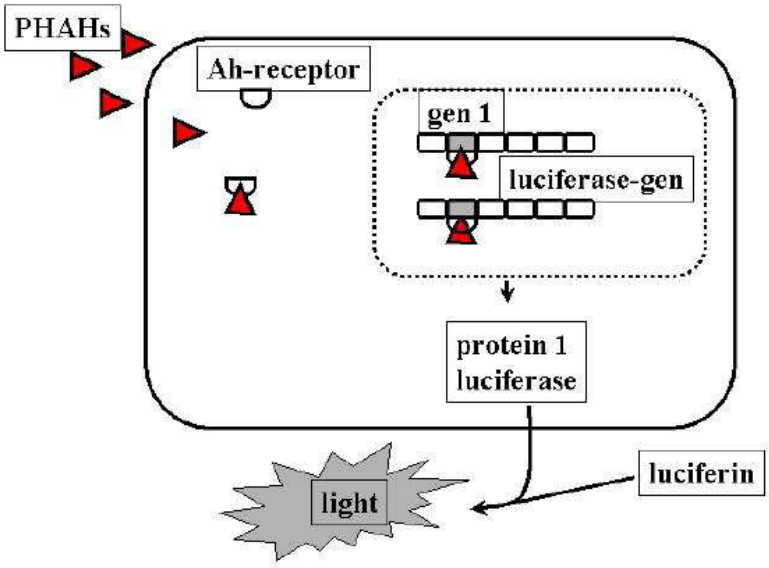

Fig. 2. *Schematic representation of an in vitro reporter gene assay for compounds such as polyhalogenated aromatic hydrocarbons (PHAHs) interfering with the arylhydrocarbon (Ah)-receptor.*

Gene induction assays are advantageous compared with receptor binding assays in that they can distinguish between agonists and antagonists when single compounds are used in an experimental setting, or that the total potential of an extract, integrating agonistic and antagonistic activity can be interpreted. Also, such assays are more sensitive than receptor binding assays as in these assays the ligands have to compete with the (usually radioactive) natural ligand to the receptor, as has been shown for assays for estrogenic compounds (Murk et al. 2002). Generally, reported correlations between chemical analyses and CALUX assay are good (Bovee et al. 1998, Murk et al. 1998, Windal et al. 2005), although bioanalysis tends to indicate higher toxic equivalents than can be explained based on GC/MS analysis in combination with knowledge about relative toxic potencies of the known compounds. This result has been observed with dioxin-like, estrogenic and esterase inhibiting activity (Hamers et al. 2000, Stronkhorst et al. 2002, Hamers et al. 2003, Legler et al. 2003, Keiter et al. 2008). This type of outcome is not an artefact, but a consequence of the contribution from even the lowest levels of compounds, unknown compounds, and mixture effects. For biotic samples total extraction of the compounds from tissues is relevant. For abiotic matrices, however, only a fraction of the pollutants will be biologically available in many cases as some parts are strongly bound to the organic matrix therefore being not “visible” for an organism under natural conditions. An interesting new analytical approach is the combining of *in vitro* bioassays with realistic (non exhaustive) extraction of the biologically available fraction, which results in a more realistic and lower estimated risks posed by the toxic compounds present (Puglisi et al. 2007, Weert et al. 2008).

Reporter gene assays, have been used in many studies and the combination of speed, relative simplicity, and ability to integrate effects of complex mixtures also makes it a valuable additional tool for studies related to pollution with endangefed species, which includes some species of otters. When applied with a proper protocol for sample preparation and quality controls (Besselink et al. 2004, Windal et al. 2005), these types of bioassays can serve as an example for the successful application of *in vitro* bioassays for bioanalysis of relevant contaminants related to wildlife, including otters (Murk et al. 1998). Generally the high biological relevance of the results has importance for the estimation of potential risks of any chemical exposure to endangered species.

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