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In vitro Environmental Toxicology - Concepts, Application and Assessment

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In vitro Environmental Toxicology - Concepts, Application and Assessment

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Springer

Editors
Georg Reifferscheid
Federal Institute of Hydrology
Koblenz, Germany

Sebastian Buchinger
Federal Institute of Hydrology
Koblenz, Germany

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Preface

In recent years, considerable technological progress has been made in the development of in vitro bioanalytical methods and instruments for the elucidation of toxic effects of compounds of both natural and anthropogenic origin.

Such methods, which are progressively applied in toxicologyand environmental science, allow the detection of cytotoxicity as well as the investigation of specific, sublethal effects of chemicals and chemical mixtures of different complexity. In toxicology, in vitro bioanalytical tools have so far mainly been used to generate scientific knowledge, to elucidate the chemical causes of effects, and to provide data in support of environmental monitoring.

It is widely accepted that in vitro methods add substantial value to the field of ecotoxicology because of their efficiency, their high throughput capacity, and their ability to obtain mechanistic information about toxicity and basic data on possible toxicological risks in different environmental compartments. However, the use and interpretation of test results in regulation is challenging and still under discussion. Although reduction, replacement, and refinement of in vivo toxicologyis always being called for by society and regulatory stakeholders, one main reason for regulatory obstruction is that options for in vitro/in vivo extrapolation of effects are still missing.

This book gives an overview of the current state of the art of in vitro bioassays in the field of (eco)toxicology with special focus on effects of very high concern and reasonable areas of application. Furthermore, selected chapters address topics related to the application of in vitro bioassays in environmental sciences, such as passive sampling/passive dosing and effect-directed analysis. A special chapter describes the possibilities of linking results of in vitro assays to in vivo effects by making use of physiologically-based pharmacokinetic modeling. According to the basic test principles, the underlying concepts of the various techniques are shown.

The book exemplifies the use of in vitro approaches in different fields of application. It discusses the potential, current limitations, research needs,and regulatory perspectives of some selected bioanalytical tools and of in vitro bioassays in general.

Georg Reifferscheid
Sebastian Buchinger

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Endocrine Disruption and In Vitro Ecotoxicology: Recent Advances and Approaches

Martin Wagner, Cornelia Kienle, Etiënne L.M. Vermeirssen, and Jörg Oehlmann

Abstract Endocrine-disrupting chemicals (EDCs) are man-made compounds interfering with hormone signaling. Omnipresent in the environment, they can cause adverse effects in a wide range of wildlife. Accordingly, Endocrine Disruption is one focal area of ecotoxicology. Because EDCs induce complex response patterns in vivo via a wide range of mechanisms of action, in vitro techniques have been developed to reduce and understand endocrine toxicity. In this review we revisit the evidence for endocrine disruption in diverse species and the underlying molecular mechanisms. Based on this, we examine the battery of in vitro bioassays currently in use in ecotoxicological research and discuss the following key questions. Why do we use in vitro techniques? What endpoints are we looking at? Which applications are we using in vitro bioassays for? How can we put in vitro data into a broader context? And finally, what is the practical relevance of in vitro data? In critically examining these questions, we review the current state-of-the-art of in vitro (eco)toxicology, highlight important limitations and challenges, and discuss emerging trends and future research needs.

Keywords Bioanalytical tools, Bioassay, Effect-directed analysis, Endocrine-disrupting chemical, Mechanism of action, Risk assessment

M. Wagner (✉) and J. Oehlmann
Department Aquatic Ecotoxicology, Goethe University Frankfurt, Max-von-Laue-Str. 13, Frankfurt 60438, Germany
e-mail: wagner@bio.uni-frankfurt.de

C. Kienle and E.L.M. Vermeirssen
Swiss Centre for Applied Ecotoxicology, Eawag-EPFL, Dübendorf, Switzerland

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1 Introduction[1]

1.1 Endocrine Disruption: What Is It All About?

The Metazoan endocrine systems essentially and intricately regulate physiological processes in both the short and the long term, including behavior, development, metabolism, reproduction, and stress response. Endocrine systems are highly diverse, with neurotransmitters and neurohormones as ancestral mechanisms of paracrine/endocrine communication in invertebrates [1]. As more complex organizations evolved, endocrine systems were complemented with endocrine cells (annelids) and glands (mollusks, arthropods) secreting 'true hormones' [1]. In spite of their diversity, some components of the invertebrate endocrine systems are remarkably conserved and resemble their vertebrate counterparts [2]. Steroid receptors, for example, have evolved from a common ancestor present before the origin of bilaterally symmetric animals. These receptors presumably already controlled reproduction [3, 4].

Ecotoxicological research has demonstrated in field and laboratory experiments that chemicals can interfere with the endocrine systems, a phenomenon known as Endocrine Disruption (ED). Effects resulting from such disruption and causative compounds, are manifold and have been extensively reviewed elsewhere [2, 5–14]. This includes a comprehensive discussion of ED in mollusks [15], fish [16],

[1]This is an extended and updated version of the introduction published in Wagner [368].

amphibians [17], reptiles [18], and birds [19]. Recent advances in ecotoxicology cover ecotoxicogenomics [20], early exposures [21], population and ecosystem sustainability [22], and the impact of additional stressors, such as climate change [23, 24]. In the light of the wealth of available studies on ED in wildlife species, only selected, well-established examples are discussed here.

1.1.1 Invertebrates

Organotin-induced imposex[2] development in mollusks is one of the best-documented incidences of ED in the field [25]. In the early 1970s, Blaber [26] and Smith [27] observed penis-like structures in female gonochoristic marine caenogastropods from the field. A decade later, tributyltin (TBT) used in naval antifouling paints was implicated in the occurrence of imposex [28, 29]. Numerous field and laboratory studies have conclusively linked organotin exposure to imposex in more than 250 mollusk species (see reviews [15, 25, 30]). Because imposex is irreversible and may result in female sterility, some of the gastropod populations have been locally eradicated (see [31]). However, legislatory action has resulted in declining organotin levels and slow recovery of some caenogastropod populations [32–36]. Despite the clear-cut evidence of TBT toxicity, scientific debate about the underlying mechanism is ongoing. Amongst several available theories (reviewed in [30, 37]), disruption of androgen signaling[3] [38–42] and the retinoid pathway[4] [43–49] appears to be the most promising. Putting aside the putative dispute, a recent mechanistic study suggests that both pathways might be involved in imposex induction [50].

ED in caenogastropod mollusks is not restricted to organotin compounds but is induced by several estrogenic, androgenic, and antiandrogenic substances (for review see [15]). One prominent example is the plastic monomer Bisphenol A (BPA)[5]: In a molluskan model, BPA exposure induced so-called superfemales, that is, females with additional reproductive organs, enlarged glands, and escalated reproduction [51]. Because effects were observed at the lowest concentration studied, a follow-up study established BPA effects at even lower, nanogram per liter levels [52]. Heavily criticized by industry-funded scientists [53], Oehlmann and colleagues [54] replicated the original findings and reported that inadequate experimental conditions (elevated temperatures) masked the BPA effects.

[2]Imposex is defined as the imposition of male reproductive characteristics, for example, penis development, on female individuals.

[3]For example, female testosterone levels might be increased by aromatase inhibition. See Fernandes et al. [369] for a comprehensive review on molluskan steroid biosynthesis.

[4]Organotin compounds have been shown to be potent agonists of the retinoid receptors RXR and RAR.

[5]BPA found to be estrogenic in the 1930s [370] but was abandoned as synthetic estrogen in favor of the more potent diethylstilbestrol (DES). Today, it is mainly used as building block of polycarbonate plastics to produce food and beverage containers [130].

Unsurprisingly, subsequent studies conducted at a higher temperature regime were unable to reproduce the observations [55, 56].

1.1.2 Fish

A masculinization of mosquito fish by paper mill effluent was reported from the U.S. in the 1980s [57]. In the same decade, feminization of teleost fish was first observed in the River Thames [58]. This phenomenon has been termed 'intersex' because affected individuals developed hermaphroditic gonads containing both male and female parts [58]. A decade later, the intersex phenomenon was experimentally linked to exposure to sewage treatment effluents [59].[6] As elevated vitellogenin (VTG)[7] levels were observed in exposed male fish, estrogen-like chemicals have been suggested as causation. Since then, numerous field studies have corroborated this connection with several species being affected worldwide (see [60]). For instance, almost all phenotypic male fish caught at polluted sites in England were intersex [61, 62].[8] In a recent study, 98% of fish exposed to sewage treatment effluent were phenotypic females. In a competitive breeding study, sex-reversed males from the effluent did reproduce as females but with a very low success. Moreover, none of the sex-reversed males contributed to the offspring under competitive conditions [63, 64].

Kidd and coworkers approached the issue of ED at population level in a long-term, whole-lake experiment [65]. Over a period of 3 years, an experimental lake was dosed with a low concentration of a synthetic estrogen mimicking the environmentally relevant estrogenic exposure via sewage treatment effluents.[9] Following the second season of exposure, the population of one fish species collapsed almost completely and did not recover in the first 3 years after the removal of the exposure.[10] However, in the spring of the 4th year, adult size-frequency distribution

[6]Given the almost universal contamination of freshwater ecosystems with (treated) wastewater, the issue of feminization became the focal point for research on ED in fish and EDCs in wastewater.

[7]VTG is an egg yolk protein precursor. Synthesized in the female liver, it is transported via the bloodstream for incorporation into oocytes. Its expression is estrogen-dependent [371]. Naturally only produced by mature females, its prevalence in juvenile or male fish is considered a biomarker of exposure to estrogenic substances.

[8]Intersex males had increased VTG and estradiol levels, delayed spermatogenesis, and malformed reproductive ducts as well as reduced milt production, sperm motility, and fertilization rates.

[9]A nominal concentration of 5 ng L^{-1} 17α-ethinylestradiol (EE2), the synthetic estrogen from the birth-control pill, was dosed to a lake in Ontario, Canada [372, 373].

[10]During the exposure period, VTG levels in male fathead minnow (short life cycle) were three orders of magnitude higher than in the reference. In addition, the testicular and ovarian development was arrested [372]. Similar effects have been observed in pearl dace. While in this longer-lived species a clear impact on population size has not yet been observed, population structure was affected as indicated by the loss of the 1-year-old size class [374]. In another species (rainbow trout), fertility was unaffected [375]. This highlights considerable species differences [376].

and abundance had returned to pretreatment levels. Genetic analyses showed that postrecovery fish were descendants of the original EE2-treated population [66]. The lesson learnt from this whole-lake experiment is that chronic exposure to environmentally relevant levels of estrogens clearly impacts the sustainability of wild fish populations.

The intersex phenomenon in fish has long been attributed to estrogen-like compounds. In contrast, recent research provides a strong argument for an additional contribution of antiandrogenic chemicals producing phenotypic effects similar to estrogens [67, 68]. In an innovative approach, Hill et al. [69] analyzed the antiandrogenic activity of tissue extracts from fish exposed to sewage treatment effluents. Combining a fractionation approach with analytical techniques, the authors identified the antimicrobials chlorophene and triclosan as predominant environmental antiandrogens bioavailable to fish [70]. These are excellent examples that research on EDCs in wildlife is shifting from analyzing a few well-established chemicals to the effect-directed identification of unexpected, 'emerging' pollutants [71–75]. Pursuing such approaches provides a more holistic picture of wildlife exposure to EDCs.

1.1.3 Amphibians

The global decline and loss of amphibian biodiversity [76] is of special concern because amphibians appear to be more threatened than either birds or mammals [77]. With a complex causation involved, climate change, pathogens, and habitat loss have been proposed as global drivers [78, 79]. In this picture, the role of chemical pollution is far from clear, but pesticides have been associated with the amphibian decline [17]. Similar to fish (see Sect. 1.1.5), feminization of male frogs, characterized by testicular oocytes and intersex, has been observed in the field as well as in the laboratory (reviewed in [80]). A retrospective analysis of museum specimens suggests an association between intersex and the use of organochlorine chemicals [81].[11] Atrazine, one of the most commonly applied pesticides worldwide, serves as prototypic EDC in amphibians [82]. It induces demasculinization of male frogs at very low, ecologically relevant concentrations (e.g., [83]). The mechanism of atrazine toxicity in vertebrates is well-documented. In brief, the pesticide reduces androgen levels by inhibiting androgen-simulating hormones, enzymes of the androgen biosynthesis, and binding of dihydrotestosterone (DHT) to its target proteins (see [82]). In addition to atrazine, effects of other EDCs (especially pesticides) have been observed in several amphibian species in the field and laboratory [2].

[11] Polychlorinated biphenyls (PCBs) and *p,p*-dichlorodiphenyltrichloroethane (DDT).

1.1.4 Reptiles

Among reptiles, the American alligator is the best-studied species in terms of ED. Here, the case of Lake Apopka in Florida is of special interest because it provided a clear indication of ED in wildlife vertebrates (reviewed in [18]). Experiencing a pesticide spill from a nearby chemical company in 1980, the lake's alligator population subsequently suffered from population decline [84]. Egg viability and post-hatch survival were compromised, most probably because of high concentrations of organochlorine pesticides in the eggs [85, 86].[12] This is supported by laboratory studies in which pesticide exposure caused infertile eggs and increased embryonic mortality (e.g., [87]). Moreover, female hatchlings from Lake Apopka developed polycystic ovaries that resemble symptoms of DES exposure in other vertebrates [88]. Male alligators from Lake Apopka had decreased phallus size and testosterone levels compared to reference populations [85, 89, 90].[13] Mechanistically, several pesticides found in Lake Apopka disrupt steroid signaling and biosynthesis (review in [91, 92]).[14] Recent transcriptomic analyses indicate a loss of sexually dimorphic gene expression in specimen from Lake Apopka, mirroring the morphologic findings. Interestingly, concomitant interference with non-steroidal pathways might also be involved in the disruption of alligator reproduction [93, 94].

1.1.5 Birds

In the middle of the last century, a dramatic decline of raptor populations was observed in Great Britain and North America [95]. For instance, the bald eagle nearly vanished from the Great Lakes during the 1950s through the early 1970s [96, 97]. The phenomenon concurred with eggshell thinning and was supposed to be caused by pesticide (e.g., DDT) exposure [98]. The levels of organochlorine insecticides and PCBs are associated with a number of reproductive outcomes, including eggshell thinning, embryonic malformations, hatchling mortality, and population productivity [99]. Additionally, a number of compounds (e.g., DDT, PBDE, PCBs, TCDD) are experimentally linked to effects observed in the wild [100–103]. Many of these chemicals have been proposed to mediate their toxicity via an estrogenic mechanism [97].[15] However, DDT has a different mechanism for

[12]Newer studies indicate that the pesticide levels have not appreciably decreased in the early 2000s [87, 377]. Interestingly, associations between organochlorine pesticides and reproductive performance have also been reported for caimans [378].

[13]Similar effects can be induced by other endocrine disruptors, including 17β-estradiol, atrazine, and Bisphenol A [379].

[14]Several reptilian estrogen receptors have been cloned and display a differential responsiveness to estrogens and pesticides [293, 380].

[15]Interestingly, *in ovo* exposure to estrogenic chemicals feminizes the male gonad in birds [381] as it does in rodent models.