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ENDOCRINE DISRUPTORS – ECOTOXICOLOGICAL ASSAYS

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ZWIĄZKI ZAKŁÓCAJĄCE PROCESY HORMONALNE – BADANIA EKOTOKSYKOLOGICZNE

Związki zakłócające procesy hormonalne (EDs – endocrine disruptors) zmieniają funkcje hormonów regulujących przemiany fizjologiczne u ludzi, kręgowców i bezkręgowców. Do tych związków należą zarówno ksenobiotyki jak i substancje pochodzenia naturalnego między innymi o charakterze estrogenów. W piśmiennictwie jest niewiele danych o systemach hormonalnych u bezkręgowców i zaburzeniach w ich funkcjach pod wpływem zanieczyszczeń. W artykule podano informacje mające na celu przybliżenie problemów metodycznych w badaniach ekotoksykologicznych wybranych ED na niektórych grupach bezkręgowców.

Summary

Endocrine disrupting compounds (EDCs) have the potential to alter hormone pathways that regulate life processes in humans, vertebrates and invertebrates. Besides xenobiotics having endocrine effects, there are naturally occurring estrogenic compounds. The limited number of studies with EDCs in invertebrates is partially due to the fact that their hormonal systems are rather poorly understood in comparison with vertebrates. It is also important, but difficult to discriminate between hormone – mediated and other toxicological modes of action. Data of the potentially adverse impact of EDCs on wildlife species are reviewed.

INTRODUCTION

It has been a decade now since the presence of endocrine disrupting chemicals (EDCs) in the environment was noticed. The interest in EDCs addressed the issue of human health protection, and first of all the hypothesis that EDCs, including synthetic hormones, are responsible for the development of breast and prostate cancers and disrupting male reproductive functions – the quantity and quality of sperm. In parallel to research on EDCs with regard to humans, a search for a method to assess the influence of these chemicals on fish and aquatic invertebrates was launched [6, 18]. Endocrine disrupting chemicals belong to various groups as for the structure and physical-chemical properties. Among EDCs one should differentiate among substances of natural origin – phyto- and mycoestrogens and xenobiotics – pesticides (mainly chloroorganic), polychlorinated biplenyls (PCBs), dioxins, alkyl phenols, phthalates, bisphenol A, aniline dyes, heavy metals (Table 1 and Table 2).

Table 1. Examples of chemicals suspected of causing endocrine disruption listed according to suspected mechanism of action [6]

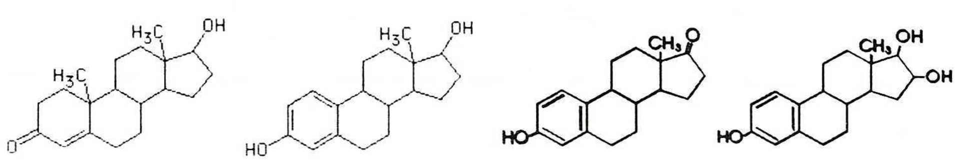
Environmental estrogens: estrogen receptor mediated
Chlordecone
Polichlorinated biphenyls (PCBs)
o,p'-DDT
Environmental antiestrogens
Dioxin
p,p'-DDT/DDE
Endosulfan
Environmental antiandrogens
Vinclozolin
Procymidone
Kraft mill effluent
Toxicants that alter circulating steroid hormone levels
Dioxin
Endosulfan
Aroclor 1254
Toxicants that act via the CNS
Dithiocarbamate pesticides
Carbon disulfide
Manganese
Other mechanisms
Dibutyl phthalate
Benzidine-based dyes
Vinylcyclohexene
Antithyroid endocrine disruptors
PCBs
Herbicydes, e.g. nitrofen
Phthalic acid esters
Adrenal endocrine disruptors
Aniline dyes
Ketoconazole fungicides
PCBs

Table 2. Chemicals suspected of causing endocrine disruption in invertebrate species [6]

Herbicides
Diquat bromide
Atrazine
Simazine
Diuron
Metals
Cadmium
Selenium
Zink
Mercury
Lead
Tributyltin (TBT)
Vertebrate steroids
Diethylstilbestrol (DES)
Testosterone
Estradiol
PCBs/alkylphenols
Clophen A50
Aroclor 1242
Nonylphenol
Pentylphenol
Insecticides
Pyriproxypen
DDT
Endrin
Methoprene
Diflubenzuron
Kelthane
Complex mixtures
Tannery and Kraft mill effluent
Sediment extracts
Sewage effluent

It is known that EDC can mimic the effects of hormones (agonism) or influence antagonistically natural hormones, alter the pattern of synthesis and metabolism of hormones as well as disturb signal functions.

A majority of scientific papers raise the problem of sexual processes disruption in organisms with respect to the functions of steroid hormones – estrogens and androgens.



Testosterone 17β-Estradiol Estron Estriol

Fig. 1. Testosterone, 17β-estradiol, estriol, and estron structures

The research on EDCs with regard to humans is carried out on rodents; while the research with regard to ecotoxicity is performed on fish. Little data is given about the influence of EDCs on hormonal processes in invertebrates despite the fact that they represent more than 95% of all known animal species and play an important role in the structure and functioning of ecosystems.

The research on EDCs ecotoxicity should take into account the following:

- functioning of hormonal systems in fish and invertebrates and mechanisms of hormonal processes regulation,
- EDC effects and other effects of toxicants,
- environmental factors,
- properly chosen criteria for the EDCs risk assessment

Learning the mechanisms of the functioning of natural hormones and EDCs in invertebrates is complicated by the hormone – receptor complexes. It is known that one hormone (or EDC) may bind with various receptors, which results in the synthesis of functionally diverse proteins. The places of binding in the area of receptors display non-specify. The number of receptors depends on the course of catabolic processes and the synthesis speed; whereas the EDC concentrations decide on a bind with a particular receptor. The knowledge of biochemical reactions of these chemicals is of primary importance to assess the influence of EDCs on organisms, for example PCB metabolites – polychlorinated hydroxybiphenyls, disturb hormonal functions.

ENDOCRINE DISRUPTING EFFECT IN FISH AND INVERTEBRATES

Among the researches on the influence of EDCs on aquatic organisms, the majority of experiments were connected with the measurement of vitellogenin (Vtg) in fish, the production of which is regulated by estradiol – a female sexual hormone. Vtg is a protein which is present in the yolk of the eggs laid by animals in the environment. Vtg receptors in the liver are possessed not only by fish females but also males and fry; however only females produce enough estrogen to induce synthesis of this protein. The content of Vtg in fish blood plasma may be marked by means of the ELISA test (enzyme linked immunosorbent assay) with the protein detection level of 10^{-9} g. Vtg can be measured also by radioimmunoassay (RIA) and Western Blot methods.

Fossi *et al.* [8–10] carried out a research on fish, mainly swordfish catched from the Mediterranean Sea, which proved the presence of vitellogenin in males. De Metrio *et al.* [5] observed also female egg cells – oocytes in the nuclei of fish males from the Mediterranean Sea. The authors concluded that wastewater containing EDCs of estrogen type flow into the sea waters.

Changes of sex in fish may be caused by inhibiting the P-450 aromatase enzyme, which converts testosterone to estradiol. Female and male gonads secrete testosterone; hence inhibiting the P-450 aromatase may result in the masculinisation of females.

Wester *et al.* [24] performed a comparative analysis of the effects on fish and rats in the presence of numerous EDCs in search for an alternative method of research on EDCs which would replace tests performed on mammals. Significant similarity of mechanisms of the EDCs influence on the examined fish was found, despite the fact that Vtg has no equivalent in mammals, in the case of which the assessment of changes in the area of endometrium is performed (Table 3).

Table 3. Comparison fish-rat for some endocrine disrupters [24]

	Guppy	Medaka	Rat
β-HCH	VTG ↑; gonad changes; pit; gonadotrophs ↑	VTG ↑; testis-ova; thyroid↑	Endometrium transform; gonad changes; pituitary; prolactin cells ↑
TBT/DBT	Thymus atrophy; glycogen storage; local irritant; retina changes	Glycogen storage; local irritant; retina changes; thyroid stimulation	Thymus atrophy; local irritant; thyroid inhibition
Bromide	Goiter, locomotor disturb	Goiter	Goiter, locomotor disturb

Not numerous researches on EDCs refer to invertebrates, including crustaceans, insects, echinoderms and molluscs as regards the functions of hormones taking part in the desquamation and regulation of developmental stages, and first of all sexual hormones. It was proved that non-ionic surfactant 4-n-nonylphenol caused a reduced fecundity of the females *Daphnia*. Few clear examples of EDCs in invertebrates exist *in situ*. One of the best documented examples is that of the "intersex" in gastropod molluscs exposed to antifouling paints containing tributyltin (TBT). Females exposed to TBT develop a penis--like structure, a vas deferens and a convoluted gonoduct [6].

Hahn *et al.* [14] applied the ELISA test to determine the yolk protein contents – vitellogenin and its precursor – vitellin in the aquatic insects *Chironomus riparius*. Despite the fact of obtaining satisfactory results it was concluded that it was necessary to improve the research methodology by preparing purified *C. riparius* vitellin for the generation of standard curves.

An interesting method of research on EDCs, based on the measurement of the activeness of enzymes taking part in binding testosterone (II phase of transformation), was presented by Janer *et al.* [17]. The research tests were performed on gastropods, amphipods and echinoderms in the presence of triphenyltin (TPT), tributyltin (TBT) and fenarimol by estimating acyltransferase and sulfotransferase activities. The results of the tests demonstrated the existence of interphyla differences in testosterone conjugation. TPT and TBT inhibited acyltranferase by 68% and 42% respectively in echinoderms, and TBT by 20% in molluscs. Fenarimol did not affect the activeness of this enzyme in any of the bioindicators.

Generally the biomarkers of endocrine disruption in crustaceans are: intersex individuals, vitellogenin in males, reduced egg vitellogenin, excess males in population, mixed sex broods of offspring, larval – juvenile intermediates, incomplete molting, altered cuticle constituents [19].

Most endocrine studies of the Crustacea have been performed with classes Malacostraca and Branchipoda (with *Daphnia magna* as a model bioindicator).

Soin and Smagghe [23] reported results of EDCs effects on aquatic insects as: midge (*Chironomus dilutus, C. riparius*), mosquito species (*Aedes aegyptii, Anopheles gambiae, Culex quinquefasciatus, C. pipiens*) and mayfly species (*Rhithrogena semicolorata, Ephemerella iquita*). Endpoints of test reactions are: survival, cell proliferation, vitellogenesis, molting, ecdysteroid titers, mouthpart deformities induction, median emergence time, larval wet weight, ecdysteroid synthesis.

Effects of potential EDCs on free living nematodes (mainly on *Caenorhabditis elegans* as a model organism) cover the following parameters: growth, reproduction, fecundity, germ cell number, vitellogenin expression, egg hatching, estrogen binding, and motility [15].

Recently, examples of freshwater invertebrate species used for assessing developmental or reproductive endpoints in current on potential future standard protocols have been tabulated by Hutchinson [16] (Table 4).

Table 4. Examples of freshwater invertebrate species used for assessing developmental or reproductive endpoints in current or potential future standard protocols [16]

Taxa	Species and endpoint
Annelida	Oligochaeate (*Lumbriculus variegatus*) – reproduction up to 28 d
Arthropoda-Crustacea	Water flea (*Daphnia magna*) – neonate production up to 21 d
Arthropoda-Insecta	Non-biting midge (*Chironomus riparius*) – reproduction in two-generation test (aprox. 45 d)
Cnidaria	Hydroid (*Hydra vulgaris*) – development and budding up to 17 d
Mollusca-Bivalvia	Zebra mussel (*Dreissena polimorpha*)– spawning and embryo development after 2 d
Mollusca-Prosobranchia	Freshwater mudsnail (*Potamopyrgus antipodarum*) – embryo production up to 56 d
Mollusca-Pulmonata	Pondsnail (*Lymnaea stagnalis*) – reproduction up to 56 d
Rotifera	Rotifer (*Brachyonus calyciflorus*) – fertilisation and reproduction up to 4 d

The OECD Test Guidelines Program is involved in the international harmonization and validation of the test methods to evaluate effects of chemicals, including potential endocrine active substances. Table 5 shows the substances and their concentrations used (or planned) in various tests, under the OECD (pre-)validation studies [13].

Table 5. Substances and their concentration used (or planned) in the various tests, under the OECD (pre-) validation studies [13]

Substances (CAS No.)	Putative mode of action	Copepods	Mysids	Daphnids	Chironomids
Fipronil (120068-37-3)	GABA-inhibitor (neural toxicant)	–	1.0, 2.5, 6.0, 16, 40 ng/dm^3	–	–
Fenoxycarb (79127-80-3)	JH*) agonist	–	1.0, 2.5, 6.0, 16, 40 μg/dm^3	0.37, 1.1, 3.3, 10 μg/dm^3	–
3,5-DCP (591-35-5)	General toxicant	5.6, 18, 56, 180, 560 μg/dm^3	13, 32, 76, 200, 500 μg/dm^3	Planned (validation study)	0.0313–2.0 mg/dm^3
Pyriproxyfen (95737-68-1)	JH agonist	–	–	Planned (validation study)	0.16, 0.8, 4.0, 20, 50, 100 μg/dm^3
3,4-DCA (95-76-1)	General toxicant	–	–	10, 20, 40, 80 μg/dm^3	–

*) juvenile hormone

ENVIRONMENTAL FACTORS AS ENDOCRINE DISRUPTORS

Environmental factors such as availability of food, light and temperature, as well as oxygen may significantly affect the hormonal functions of organisms. Barata *et al.* [3] noticed that neglecting food issues and energy budget leads to false conclusions formed basing on the experimental studies. The authors presented a comparison of sub-lethal effects in *Daphnia* obtained in the presence of various toxicants by categorizing them into general-physiological, hormonal and food-basis – related. It was concluded that the research does not take into account the demand for food and energy processes. Only in 4 cases the effects of reproduction and growth were related to the hormonal metabolism, and in 3 – no changes were observed as regards the growth and reproduction along with the simultaneous disturbance to the hormonal processes. According to the authors it is necessary to integrate toxic effects on energy intake into toxicity assessment.

Along with diet there appeared problems connected with the presence of natural phyto- and mycoestrogens causing EDCs effects. For example, beta-sitoserol binds poorly to the estrogen receptor but it is able to stimulate vitellogenin in fish males. Isoflavones inhibit beta- hydroxysteroid dehydrogenase, an enzyme involved in the steroid metabolism in the transformation of pregnenolon to progesterone and testosterone, which results in decreasing the quantity and activity of sperm. Estrogenic components in 400 different species of plants were identified [6].

Along with the research on single EDC, the monitoring of their mixtures present in wastewater, surface waters and drinking water is performed. Sapozhnikova *et al.* [22] applied a procedure of extracting various fractions of wastewater (from New York, USA) in ethanol. All fractions were tested for estrogenic activity and the most active fraction was finally analyzed for unknown compounds by using gas and liquid chromatography. As biomarkers the yeast test (in vitro) YES while the test (in vivo) on Medaka fish (*Oryzias lutipes*) was used for the Vtg assays. The YES test was described by Rutledge and Sumpter in 1996. The rule for the test is based on introducing genes encoding human estrogen receptor into the yeast cells *Saccharomyces cerevisiae*. The strain also contains a plasmid carrying the gene which encodes the enzyme beta-galactosidase, under the transcriptional control of human estrogen responsive sequences. EDCs after binding with the estrogen receptor activate the expression of beta galactosidase which converts the added substrate chlorophenol-red-beta-D-galactopyranoside to red chromogen. The quantity of the product corresponds with the level of estrogenity of the sample. It is possible to introduce a gene of the fish estrogen receptor e.g. from rainbow trout [20]. Tests on the content of the EDC in wastewater [22] proved the presence of 19.3 ng/dm^3 of estradiol equivalent (YES test) and of 100 ng/dm^3 (fish test) in 100% ethanol fraction. The presence of nonylphenol, triclosan, phenanthrene and pyrene in concentrations of 19 ng/dm^3 to 7.5 μg/dm^3 was noticed. These results point to a mixture of estrogenic compounds with varied polarities, which are not potent estrogen receptor ligands within wastewater effluent from the New York treatment facility. Chemical methods identify the EDCs and quantify their concentration. Several methodologies for steroid estrogens in solid and aqueous environmental samples (GC/MS, GC/MS/MS, LC/MS(/MS) are given by Gomes *et al.* [12].

Apart from availability of food also other environment conditions may cause ED effects. Wu *et al.* [25] proved that aquatic hypoxia may significantly influence the hormonal changes in males and females of the carp (*Cyprinus carpio*). The authors tested the levels of testosterone, estradiol and triiodothyronine after exposure to normoxia (7.0 mg O_2/dm^3) and hypoxia (1.0 mg O_2/dm^3). Fish were fed with commercial feed (2% body wt daily) and 50% of water in the systems was renewed once every 2 days (Table 6).

Table 6. Serum hormone levels [ng/cm^3] of *Carp* after exposure to normoxia (7.0 mg O_2/dm^3) and hypoxia (1.0 mg O_2/dm^3) for 8 weeks [25, modified]

	Male		Female	
	7.0 mg O_2/dm^3	1.0 mg O_2/dm^3	7.0 mg O_2/dm^3	1.0 mg O_2/dm^3
Testosterone	9.83	2.22	8.23	0.73
Estradiol	0.033	0.063	0.99	0.19
Triiodothyronine	3.37	1.6	3.59	3.08

It was concluded that in the conditions of hypoxia concentrations of hormones in blood decreased except for estradiol in males. These hormonal changes were associated with retarded gonadal development in both male and female carp, reduced spawning success, sperm motility, hatching rate and larval survival.

Aneck-Hahn *et al.* [2] paid attention to a possibility of the presence of EDCs in sterile or distilled water. The authors discovered the presence of EDCs in water used for preparing reagents in YES test. Falconer *et al.* [7] in a review article highlighted the fact that surface water and drinking water contain EDCs; however, insufficient information about the concentrations of the chemicals and consequences for the human makes it difficult to estimate the health risk.

CONCLUDING REMARKS

In literature there appeared papers in which the authors search for causes (other than the influence of EDCs) of the development of cancer in humans, pointing at the lifestyle and nourishment as well as type of food. Safe [21] analyzed the results of a research on the human sperm count and concluded that the level varies accordingly to the demographic changes or season of the year. Ames and Swirsky Gold [1] undermined the generally approved assumption that "what is natural is harmless" proving that the human in his or her food takes in 1500 mg of pesticides produced by plants and residues of synthetic pesticides amount to only 0.09 mg.

Independently of the difference in opinion on issues addressing the EDCs problems in the environment, EU directives point at the need of estimating the risk of chemical factors including endocrine disrupting chemicals. Integrated systems for risk assessment should consider the influence of EDCs on humans and organisms inhabiting ecosystems. To achieve these goals it is necessary to work out simple and cheap tests or biomarkers and to create suitable database for toxicological and ecotoxicological data [4, 11].

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