A PROPOSED FLUORESCENT MICROSCOPIC METHODOLOGY OF DISTINGUISHING OF DIOXIN-LIKE BIOLOGICAL EFFECTS IN WILD ANIMALS

Vladimir M. Bresler, Lev Fishelson* and Avigdor Abelson.

Institute for Nature Conservation Research and * Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel

Introduction

Polychlorinated aromatic hydrocarbons (PCAH), such as biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-dioxins (PCDDs), are wide-distributed anthropogenic pollutants. Exposure to sublethal PCAHs' concentrations, especially PCDDs', has been observed to produce a variety of species-specific biological effects. They include hepatotoxic, immunotoxic, neurotoxic, embryotoxic and teratogenic, endocrine-disrupting (hypothyroidism, hypoestrogenia and hypoandrogenia), mutagenic, clastogenic, tumor-promoting effects, and induction of detoxifying enzymes [1-3]. Early detection of these effects is highly important for ecological monitoring.

Enzyme induction and many other effects are mediated by aryl hydrocarbon (Ah) receptor that can be assessed indirectly by the determination of cytochrome P450 or monooxygenase (MO) activities. Such examinations are the most known test to detect PCAHs. Using tetrachlorodubenzo-dioxin (TCDD) equivalents can normalize this test for assessment of PCAHs mixtures.

A major problem, however, in assessing the effects of PCAHs, is that these compounds are presented together with other common pollutants like polycyclic aromatic hydrocarbons (PAHs), nonionic surfactants, organotins, heavy metals, and several natural xenobiotics, some of which can produce MO induction, endocrine disrupting, mutagenic, clastogenic and carcinogenic effects as well as modify PCAHs effects [1-3]. In the field, chronic sublethal exposure to pollutants may produce resistant populations that are able to endure concentrations of xenobiotics, which are toxic for normal populations. Therefore, we propose a new approach and methodology for reliable detection of the biological effects of the pollutants mixture on biota and distinguishing possible effects of its main components [4-6]. For this purpose, we integrated data of chemical ecology, physiology, biochemistry and molecular biology concerning origin, structure and ecological functions of various anti-xenobiotic defense mechanisms in diverse organisms [4-11]. The data source is based on studies that used specific fluorescent probes and quantitative contact fluorescent microscopy to detect anti-xenobiotic defense mechanisms' activity and unmask several responses of target cells to chemical actions in various species from protozoa to vertebrates [4-7, 9, 10, 12].

Material and Methods

We examined selected species from sites with different pollution history collected: 1) along the Israeli Mediterranean shore (foraminifera, bivalves *Donax trunculus* and *Mactra corallina*, gastropod *Patella coerulea*) and Red Sea shore (coral *Acropora hyacinthus,* bivalves *Callista florida, Dosinia histrio*, *Lucinia dentifera* and intertidal rocky gastropod *Cellana rotha*); 2) in the

German North Sea (bivalve *Mytilus edulis* and fish *Limanda limanda* and *Platichthys flesus*); 3) along small Israeli stream, Yarqon River (various fish species). Also terrestrial turtle *Emys orbicularis* and tadpoles of *Bufo viridis* from different sites in Israel were used.

These animals were studied by using quantitative fluorescent cytochemistry and cytophysiology as previously described [4-6]. Particularly, permeability of external epithelial and histohematic barriers and activities of carrier-mediated export pumps for elimination of xenobiotics, system of active transport of organic anions (SATOA) and multixenobiotic resistance-mediated transporter (MXRtr), were determined *in vivo* by using corresponding fluorescent probes and specific inhibitors. Activity of MO was examined *in situ* by using fluorogenic substrate 7-ethoxyresorufin (EROD activity). Functional activity of nuclear chromatin, frequency of one-stranded DNA breaks, aneuploidy and chromosome aberrations and breaks (micronucleus test) were measured by using versatile probe, acridine orange. Complete histopathological examination by contact microscopy allowed to detect and assess pathological alterations in different tissues and organs.

Results and Discussion

The investigated animals exposed various anti-xenobiotic defense mechanisms, particularly external epithelial and histohematic barriers, export pumps, SATOA and MXRtr, extracellular and intracellular depot and detoxifying enzymes, the activities of which were species- and population-dependent. For example, first line of the defense formed in external epithelia by barrier, MXRtr, SATOA and glutathione-S-transferase demonstrated the lowest power in species and populations dwelling in clean sites and the highest power in populations surviving in very polluted sites. Thus, freshwater fish *Acanthobrama telavivensis* that have very low power of the first line and other anti-xenobiotic defense mechanisms are detected only in clean part of the Yarqon River. Tadpoles of *Bufo viridis*, which also have low power of these anti-xenobiotic defense mechanisms, are declined in the polluted sites.

Populations of mollusks, *Donax trunculus*, *Patella coerulea* and *Cellana rotha,* from the clean sites exhibited higher variability and lower mean MXRtr and SATOA activities. Populations from the polluted sites demonstrated lower variability and higher mean activities of these pumps. These data testify that higher activity of these pumps may be mediated by phenotype selection. Kinetic analysis showed that decrease of apparent K_M and increase of apparent V_{max} mediates such enhanced MXRtr activity that is typical for different isoforms of enzymes or carriers. Also kinetic data showed that MXRtr and SATOA in freshly collected mollusks from polluted sites are inhibited competitively by environmental xenobiotics. Activities of SATOA and MXRtr in the gills, liver and kidney and MO in the liver of tilapia and catfish from the polluted sites of Yarqon River were higher than those in the same fish species from the clean sites.

Corals, mollusks, marine and freshwater fish, tadpoles and turtles from the polluted sites exposed an enhanced activity of nuclear chromatin, alterations in cell cycle, higher frequency of one-stranded DNA breaks, micronucleus-containing and apoptotic cells. Strong inverse correlation was detected between expression of these signs of environmental genotoxicity and clastogenicity and the activity of anti-chemical defense mechanisms, especially export pumps.

Complete histopathological examination exposed in specimens from the polluted sites various pathological alterations in their organs. In general, mollusks and fishes from the polluted sites exhibited more frequent and expressed pathological alterations as compared to the same species from the clean sites. All animals from the polluted sites demonstrated a strong (>0.9) inverse correlation between expression of these alterations and activity of MXRtr. Marine fish from Elbe estuary and freshwater fish from the polluted parts of Yarqon (especially catfish) exhibit marked proliferative processes in hepatocytes (basophilic cell foci) and bile ducts, hypoplastic processes both in male and female gonads, resorptive and hypoplastic alterations in thyroid gland, hypoplasy of thymus and spleen, hypoplasy of interrenal tissue, brain asymmetry, proliferation of glial and degeneration of nerve cells in brain cortex. Some fish from the polluted sites have also developmental anomalies, particularly skeletal malformation and gonadal dysgenesis.

A comparison of our results and biological effects of TCDD, PCBs and PAHs described in the literature (summarized in Table 1) show that in some polluted sites we detected in fish and turtles some dioxin-like effects. They included male and female hypogonadism and hypothyroidism, developmental abnormalities, immunotoxic and neurotoxic effects. Both dioxin-like compounds and PAHs may produce mutagenic, clastogenic and carcinogenic (or tumor-promoting) effects and induction of Ah locus in fish and mollusks.

Table 1. Biological effects of TCDD, PCBs and PAHs chronic sublethal exposure [1-3, 13-16]

Effect	Our results	TCDD-like compounds	PCBs	PAHs
Induction of MO	+	+	+/-	+
Hypothyroidism and morphological alteration in thyroid gland	+	+	+/-	-
Hypoandrogenia and hypoplasia of male gonads	+	+	+/-	-
Hypoestrogenia and hypoplasia of female gonads	+	+	+/-	-
Developmental abnormalities	+	+	+/-	-
Preneoplastic and neoplastic processes	+	Promoting	Promoting	+
Immunotoxicity and hypoplasia of lymphoid tissues	+	+	+/-	-
Neurotoxicity and morphological alterations in brain cortex	+	+	+/-	-
General toxicity, degenerative and necrobiotic alterations	+	+	+	+

Our data also show that pollutants act on more or less protected specimens and species, and their effects may be partially neutralized by the defense mechanisms. High activity of anti-xenobiotic defense mechanisms mediates the environmental health and stimulates surviving in polluted environment. Low activity of these mechanisms enhances toxic effects of pollutants and promotes dystrophic processes and lethality. Therefore, the proposed examination creates more extensive and realistic picture of biological effects of pollutant mixtures on specimens, populations, species and communities as well as permits to distinguish some effects of its components. The methodology is also a good base for integration of various morphological, biochemical and

toxicological tests [5,6]. Further development and perfection of the proposed methodology may enhance its diagnostic potential.

Acknowledgements
This work was supported by grant from Israeli Ministry of Science and grant MARS1 from Commission for German-Israeli Cooperation in Marine Sciences.

References

1. Ankley G, Mihaich E, Stahl R, Tillitt D et al; *Environ. Toxicol. Chem.* **1998**, 17, 68
2. Gillesby B. and Zacharewski T; *Environ. Toxicol. Chem.* **1998**, 17, 3
4. Matta M, Cairnscross C and Kosan R; *Environ. Toxicol. Chem.* **1998**, 17, 26
4. Bresler V, Fishelson L and Abelson A; *Rapp. Comm. Int. Mer Medit.* **1998**, 35, 338
5. Fishelson L, Bresler V, Yawetz A, Manelis R and Zuk-Rimon Z; *Sci. Total Environ*. **1999**, 152, 231
6. Bresler V, Bissinger V, Abelson A, Dizer H, Sturm A, Kratke R, Fishelson L and Hansen P-D; *Helgolander Meersuntersuchungen*, **1999**, in press
7. Bresler V, Belyaeva E and Mozhaeva M; *J. Insect Physiol.* **1990**. 36, 259
8. Kurelec B; *Crit. Rev. Toxicol*. **1992**, 22, 23
9. Bresler V and Fishelson L; *Dis. Aquat. Org*. **1994**, 19, 193
10. Bresler V and Yanko V; *J. Foram. Res.* **1995**, 25, 267
11. Bresler V, Montgomery W, Fishelson L and Pollak P; *J. Bacteriol.*, **1998,** 180, 5601
12. Harborne J. *Introduction to Ecological Biochemistry*. Academic Press, London, **1993**; NSBN0-12-324685-7.
13. Arcand-Hoy L and Benson W; *Environ. Toxicol. Chem*. **1998**, 17, 49
14. Hontela A; Rev. Toxicol. **1997**, 1, 159
15. Bosveld A, Gradener J, Murk A, Brouwer A, Van Kampen M, Evers E and Van den Berg M; *Environ. Toxicol. Chem.* **1995**, 14, 99
16. Henshel D; *Environ. Toxicol. Chem.* **1998**, 17, 88