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New generation of analytical tests based on the assessment of enzymatic and nuclear receptor activity changes induced by environmental pollutants

Anna Bejrowska [a], Błaż ej Kudłak [b] Katarzyna Owczarek [b], Natalia Szczepań ska [b], Jacek Namieśnik [b], Zofia Mazerska [a]

[a] *Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza Str. 11/12, 80-233 Gdańsk, Poland*
[b] *Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza Str. 11/12, 80-233 Gdańsk, Poland*

A B S T R A C T

Analytical methods show great potential in biological tests. The analysis of biological response that results from environmental pollutant exposure allows: (i) prediction of the risk of toxic effects and (ii) provi-sion of the background for the development of markers of the toxicants presence. Bioanalytical tests based on changes in enzymatic activity and nuclear receptor action provide extremely high specificity and sen-sitivity. We describe the application of xenobiotic metabolizing enzymes (i.e., cytochromes P450, glutathione S-transferases, and sulfotransferases), enzymes involved in natural metabolic pathways (i.e., acyltransferases, and N-acetyltransferases), and several other enzymes. We also describe the tests employing changes in nuclear receptor activity, including aryl hydrocarbon receptor, pregnane X receptor, constitutive andro-stane receptor, and retinoid X receptor, as promising tests allowing the prediction of dangerous effects of environmental pollutants a long time after exposure.

Keywords: Biotest, Cytochrome P450, Environmental pollution, Enzyme, Gene-reporter assay, Nuclear receptor assay, PXR receptor, RT-PCR analysis, Trace pollutant, Xenobiotic metabolism

Contents

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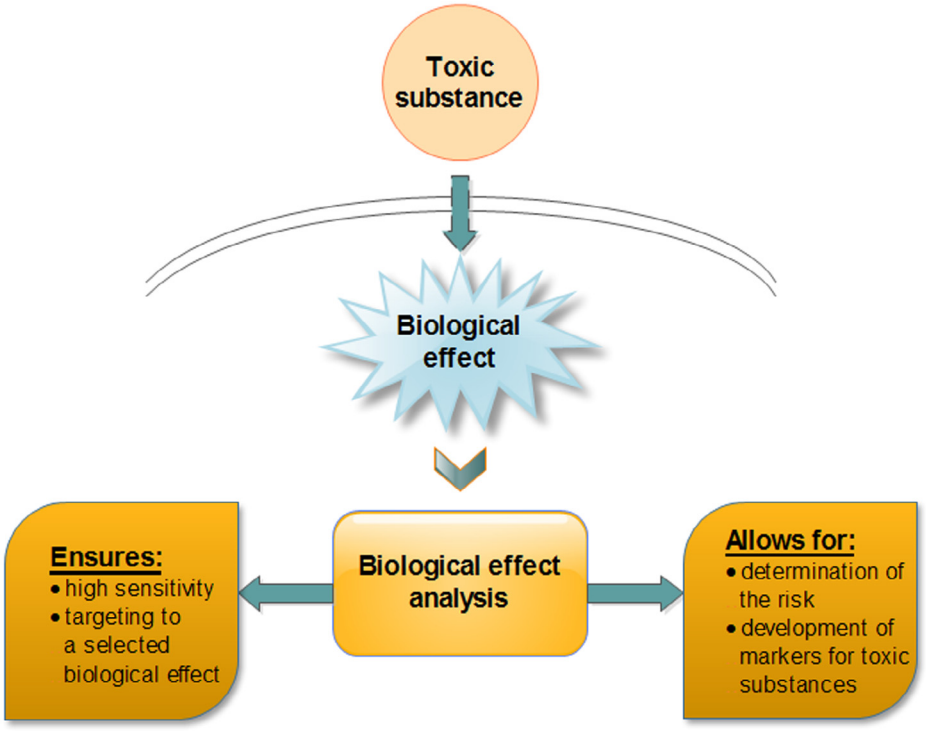
1. Introduction

Exposure of living organisms to external factors results in a complex reaction dependent on the degree of interrelations between the metabolic pathways of endogenous and exogenous compounds and the mechanisms of their regulation. The analysis of biological response being the result of xenobiotic exposure, on the one hand, can determine the risk of toxic effects resulting from the presence of a specific compound, and, on the other hand, can provide the basis for the development of appropriate biological tests – markers for the presence of toxic substances in a given environment (see Fig. 1).

The observed intensive development of the biological assessment methods for the levels of toxic substances in the environment is primarily because of their high sensitivity in comparison to conventional chemical-analysis procedures. These methods use advanced instrumental techniques at the detection, identification and quantification stages. In addition, the tools of molecular biology ensure high specificity of biological methods. Another aspect of the importance of these methods to the analysis of pollution levels is their selectivity toward specific biological effects, no matter what environmental factor is responsible for these effects.

To begin with the history of biological response applied to the assessment of environmental pollutants, numerous improvements in cell and enzymatic tests must have been performed – starting from the culture of HeLa cells at the end of the nineteenth century. Below are most important dates and milestones that enabled cell culture and the development of biotests useful for qualitative and quantitative determination of environmental stressors [1–25]:

- 1958–59 – the culture of HeLa cells performed by Gey and the development of chemically-defined medium by Eagle;
- 1961 – proving that human cell lines in culture have a limited lifespan (by Hayflick and Moorhead (called the Hayflick limit); the improvement of the method for cell growth in a serum-free media by Ham;
- 1963 – development of acid phosphatase activity assay as the cell-damage marker by Bitensky;



ig. 1. A toxic substance in the cell induces a biological effect, analysis of which allows the performance of risk assessment and the development of toxicity markers, and : also ensures high sensitivity and specific targeting of the selected biological effects.

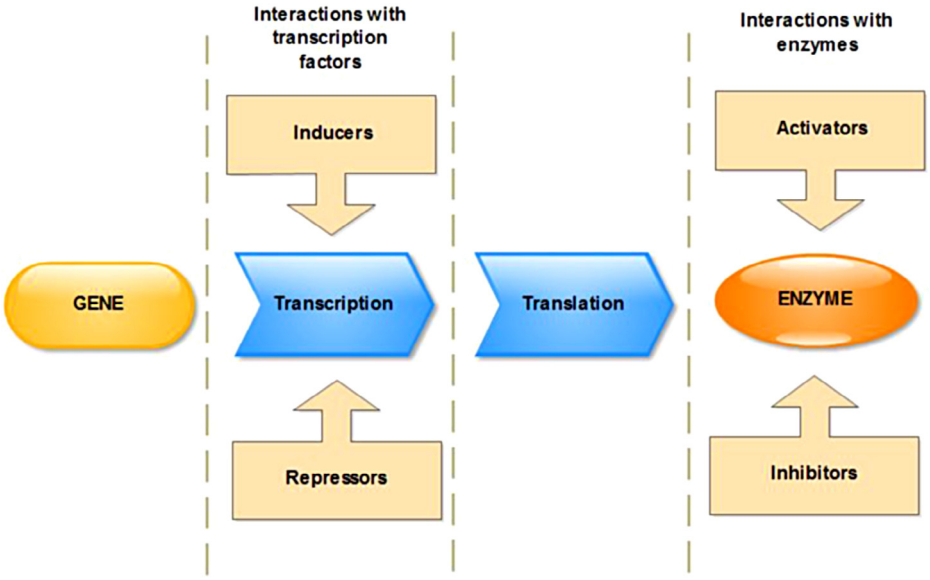


Fig. 2. Modulation-activity pathway of metabolic enzymes occurs directly by interaction with the enzymes and ultimately by the modulation of interaction with transcription factors.

- 1971 – development of the Protein Data Bank (PDB) containing information on proteins (including enzymes) and nucleic-acid structures;
- 1975 – development of adenosine triphosphate (*ATP)*-level determination methodology with luciferine-luciferase assay;
- 1976 – proving that various cell lines require specific mixtures of growth factors and hormones to grow in a serum-free medium;
- 1978–79 – application of lactate-dehydrogenase activity in culture media as the marker of NADH/NAD-dependent conversion of pyruvates into lactates; improvement of the methods for producing antibody-secreting hybridoma cells in serum-free media out of hormones and growth factors;
- 1979 – development of enzymatic O_2 consumption assay;
- 1983 – development of the first PCR methods;
- 1995 – demonstration of vitellogenin assay as an indicator of estrogenic activity determined by direct ELISA with polyclonal antibodies;
- 2003 – development of a gill filament EROD assay based on the pollutant binding to the aromatic hydrocarbon receptor (AhR) responsible for the expression of cytochrome P4501A enzymes;
- 2004 – application of the Hershberger assay based on the detection of toxic chemicals by organ-weight measurement in rodents;
- 2005 – development of the ER-CALUX assay (by Sonneveld and further developed by Houtman); introduction of MVLN transactivation assay based on cells originating from the human MCF-7 breast cancer cell line stably transfected with the luciferase gene and with the corresponding hormone-responsive element.

These new analytical trends utilized the tools of molecular biology to give deep insight into the abnormalities induced in living organism by environmental pollutants. In the cases studied to date, observations improved knowledge about the disruption of the balance in various biological systems induced by environmental factors.

There is a great demand for analytical methodologies directed at detection and identification of endocrine and other hormonally-active compounds, such as 17β-estradiol, estrone, testosterone, dehydroepiandrosterone, progesterone, cortisone and prednisone. Hormonal regulators in living organisms are characterized by sensitivity to very low concentrations of compounds, and, furthermore, changes in their activity can be evaluated indirectly by marking selected effects with enzymatic labels [26].

Other tools for biological analysis of environment status are enzymatic proteins, which act as regulators of many processes. These proteins catalyze the transformation processes of endogenous compounds and xenobiotics to their less toxic metabolites. In both cases, this metabolism is designed to remove toxic substances from the organism and is the mechanism of defending the organism against harmful effects of these substances. We must add that xenobiotics, after penetration, not only undergo metabolic transformations, but may also interact with enzymatic proteins and change their activity. As shown in Fig. 2, change in the enzymatic activity may result from direct interaction of the xenobiotic with the enzyme (activator, inhibitor), but it can also occur through the effect of the xenobiotic on nuclear receptors (inducer, repressor) responsible for the transcriptional regulation of enzyme gene expression. Xenobiotic-induced changes in enzyme activity and in nuclear receptor action can therefore be used as markers of the presence of xenobiotics in the ecosystem.

In view of the above, methodological approaches used to analyze the presence and the concentration of enzymatic modulators in the environment can be classified into two groups:

- methods based on the inhibition processes of enzymatic activity (in cellular and cell-free systems); and,
- experimental procedures employing the ability of xenobiotics to induce changes in the gene expression of enzymatic proteins controlled by nuclear receptors (in cellular system).

Many analytical procedures have emerged in the past decade using the first approach, while the second approach arose as a consequence of the development of molecular biology and genetic engineering, and was slowly introduced into environmental analytics.

2. Changes in enzyme activity as markers of environmental pollutants

The activity of xenobiotic-metabolizing enzymes responsible for phase I, II and III of biotransformation and transport, respectively,

can be monitored and applied for the evaluation of environmental pollution. The majority of xenobiotic-metabolizing enzymes had their primary role in the biotransformation of endogenous lipophilic compounds, such as bilirubin, cholesterol or estradiol derivatives, in order to excrete their excess outside the organism. Over time, these enzymes have undergone evolutionary changes in line with the exposure of living organism to exogenous lipophilic compounds (xenobiotics), including environmental pollutants. Catalytic properties of enzymes that show analytical potential in this respect are shown in Fig. 3. Next, we give a short description of the associated enzymatic tests.

2.1. Enzymes employed for the analysis of environmental pollutants

2.1.1. Cytochromes P450 (EC.1.14)

Cytochromes P450 constitute a large and multi-functional superfamily of enzyme isoforms that catalyze oxidative metabolism. They are present in almost all living organisms, from bacteria, yeast, fungi, plants to animals and humans. In the human body, they are found primarily in the endoplasmic reticulum in hepatocytes, but also in intestines, kidneys, lungs, heart and brain. P450 metabolic products are characterized by better solubility than initial compounds, improving their excretion outside the organism [27]. Due to the wide range of roles known for cytochromes P450 in living organisms [28–30], each of disorders in their function may be a universal test for the presence of a wide variety of xenobiotics in the environment. Furthermore, a relatively well-understood mechanism of their action allowed for methods to be developed for monitoring the P450 dysfunction.

2.1.2. Glutathione S-transferases (GSTs) (Enzyme Commission number EC.2.5.1.18)

GSTs represent a large family of enzymes that catalyze the conjugation reactions of glutathione (GSH) to a wide range of compounds with increased solubility compared to the substrate. The feature that distinguishes GST from other xenobiotic-metabolizing enzymes is the specificity of the catalysis to electrophilic substrates (Fig. 3A). Apart from their enzymatic role, GSTs have "non-enzymatic" ability to bind to compounds of lipophilic character, such as bile acids, fatty acids, bilirubin and certain drugs. In addition, GST co-substrate, GSH is alone an effective deactivator of reactive peroxides, which results in their detoxification catalyzed by GSH peroxidase (GPx) [31].

2.1.3. Sulfotransferases (SULTs) (EC.2.8.2.1)

The mechanism of catalysis with SULTs involves the transfer of a sulfone group from the cofactor that is 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to an acceptor group of the enzyme substrate giving the sulfates (Fig. 3B). SULTs are cytosolic enzymes that catalyze the transformations of endogenous compounds and xenobiotics. The identified SULT isoforms (i.e., SULT1A1, SULT1A3, SULT1A5, SULTs 1B, SULT1E1 and SULT2A1) differ in tissue distribution and substrate specificity. These isoenzymes are traditionally responsible for inactivation and detoxification processes, but many xenobiotics undergo metabolism with SULT, resulting in reactive metabolites, because of the electron-acceptor capability of the sulfate group [32,33]. However, changes in SULT-mediated detoxification of xenobiotics, (phase II reactions) have greater analytical potential.

2.1.4. Acyltransferases (ATATs) (EC.2.3)

ATATs belong to the class of transferases that transfer acyl groups from various coenzymes to other organic compounds. They are involved in the endogenous metabolism of fatty acids and steroid hormones or biosynthesis of mono-, di- and tri-glycerides. They also catalyze the biosynthesis of neurotransmitter acetylcholine and acetylcarnitine, the carrier of acetyl group across mitochondrial membranes [34]. ATAT activity is used as a marker of endocrine disruptors in the environment because it conjugates testosterone, expression of which is high in invertebrate species [35].

2.1.5. Arylamine N-acetyltransferases (NATs) (EC.2.3.1.5)

NATs create a specific subgroup of the ATATs, which catalyze N-acetylation and O-acetylation reactions of aromatic and heterocyclic amines (Fig. 3C). There are two functional isoenzymes in humans: NAT1 and NAT2 found in most tissues, whereas NAT2 is mainly in the liver and the intestinal epithelial tissue. NAT substrates are medicines and various types of contaminants, which are often products of chemical treatment, combustion and pyrolysis. Some of them, also present in cigarette smoke, exhibit carcinogenic and genotoxic properties [36]. There are reports about changes of NAT1 activity by organic and inorganic mercury compounds. It is also suspected that oxidative stress may affect catalysis of NAT1 enzymes in different types of lung epithelial cells, including alveoli and bronchi. Changes in NAT1 activity are therefore potential markers for analytical applications [37,38].

2.1.6. Catechol-O-methyltransferases (COMTs) (EC.2.1.1.68)

COMTs catalyze phase II conjugation reactions, which consist in the introduction of methyl group by its donor S-adenosyl-L-methionine (SAMe) into substrate (Fig. 3D). The primary function of the methylation catalyzed by COMT is to deactivate biologically active endogenous catechols and the secondary role is to metabolize endogenous catecholamines as well as steroid and thyroid hormones. Two forms of COMT enzymes were recognized in the human organism: (i) the cell membrane-bound and (ii) the soluble in the cell cytoplasm. These enzymes perform also important functions in the metabolism of xenobiotics as catechol derived drugs (e.g. levodopa, apomorphine, isoprenaline) [39]. The application of COMTs as environmental markers for analytical tests seems to be limited in comparison to other enzymes characterized above. However, their specificity is very useful in selected cases [32,40].

2.1.7. Glycine N-methyltransferase (GNMT) (EC.2.1.1.20)

After COMT, the second methyltransferase with analytical potential is GNMT, which plays a key role in the process of DNA methylation. GNMT catalyzes the transfer of the methyl group from S-adenosylmethionine (SAMe) to glycine, which results in the formation of S-adenosylhomocysteine (SAH) and N-methylglycine (sarcosine). Maintaining the proper ratio of SAMe to SAH determines good physiology of the organism. Disturbance of this balance may favor a number of diseases, such as hypermethioninemia or hepatomegaly, and may be a marker of the presence of environmental pollutants. It has also been proved that compounds from the group of carcinogenic polycyclic aromatic hydrocarbons (PAHs) reduce GNMT activity [41,42], which encourages analytical application of this test.

2.2. Enzymatic tests in analytical applications

The enzymes described above are the most popular in the analysis of environmental pollutants. Changes in enzymatic activity of these enzymes can give two different conclusions:

- a specific biological disorder was induced by some xenobiotic; or,
- the selected xenobiotic was found in the environment.

In the second case, change in enzymatic activity plays the role of the pollutant biosensor (marker) and can be applied in analytical assays. Selected methods of detection of these enzymatic changes are described below and summarized in Table 1.

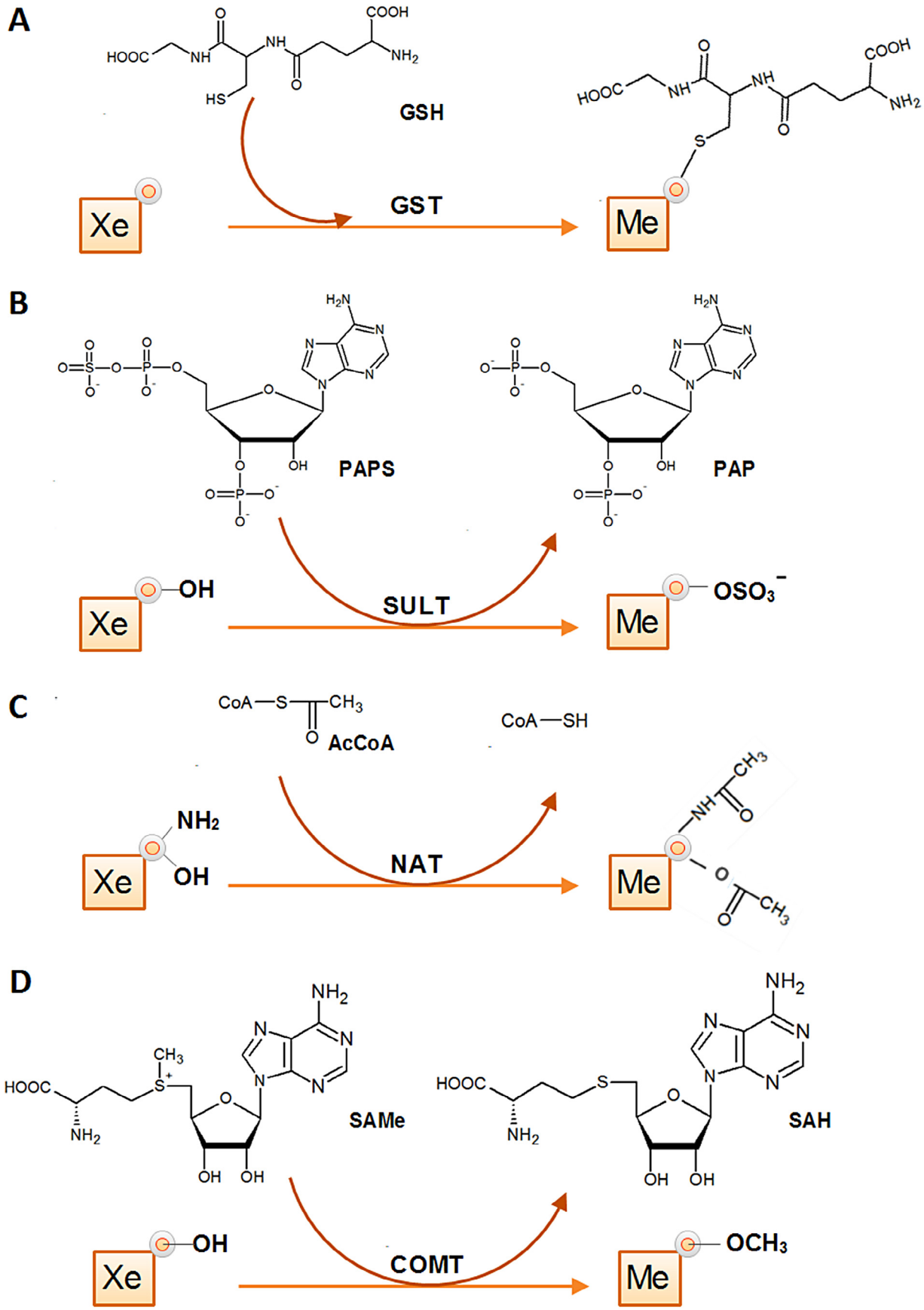


Fig. 3. Xenobiotic (Xe) transformations catalyzed by: A) glutathione S-transferase (GST) with GSH cofactor, where metabolite (Me) is applied as the marker of GST inhibition; B) sulfotransferase (SULT) with PAPS cofactor, which is the marker of SULT inhibition; C) N-acetyltransferase (NAT) with AcCoA, which is applied to monitor NAT inhibition; D) catechol O-methyltransferase (COMT), where S-adenisine-L-methionine (SAMe) is applied as the donor of methyl group and the methylation is monitored as the marker of COMT inhibition.

Table 1
Examples of enzymatic tests applied as the pollutant biosensors (the markers)

Enzyme	Marker of enzyme dysfunctions	Examples of applications
Cytochromes P450	Disruption of 7-ethoxycoumarin metabolism to 7-hydroxycoumarin detected by the fluorescence intensity Inhibition of 1β-H^3-androstenedione transformation to estrone measured by radiation intensity	P4501A2 inhibition as a marker of mosquito-larvae exposure to toxic leaf litter [30] Changes in activity of brain cytochrome P450 in relation to the reproductive behavior of the guppy [43]
Glutathione (GSH) S-transferases (GSTs)	Conjugation of GSH with 1-chloro-2,4-dinitrobenzene monitored by the changes of pH value or of the absorbance at 340 nm	GST activity as an element of the control system for the toxicity of the soluble fraction of biodiesel [44] GST activity for monitoring the changes in antioxidant defense system of earthworm *Eisenia fetida* [45] Changes in GST activity as a marker of environmental stress in the gills of ribbed mussels [46].
Sulfotransferases (SULTs)	Inhibition of SULT activity measured by the disruption of thyroid hormone sulfation monitored with LC-MS/MS SULT-mediated testosterone conjugation monitored by radioactivity of ^{14}C sulfated testosterone	SULT activity inhibition by flame retardants: polybrominated diphenyl ethers, 2,4,6 trihalogenophenol and bis-phenol A [33] Changes in SULT activity as a detector of endocrine disruptors found in invertebrate species: the gastropod, the amphipod and the echonoderm [36]
Acyltransferases (ATATs)	Inhibition of testosterone acylation by ATAT measured by HPLC with UV-vis detection or by radioactivity of ^{14}C-acylated testosterone	Disruption of ATAT activity as a marker of tributyltin, which inhibited testosterone metabolism in zebrafish [34]
Arylamine N-acetyltransferases (NATs)	Inhibition of NAT-dependent acetylation of aromatic amine substrate monitored by HPLC or spectrophotometric analysis	Inhibition of NAT1/Nat2 activity in bronchial epithelial cells as a biotest of lung dysfunction induced by oxidants: H_2O_2 or peroxynitrite [38] NAT1 irreversible inhibition as a marker of the presence of inorganic (Hg^{2+}) and organic mercury (CH_3Hg^+) toxicants [39]
Catechol-O-methyltransferases (COMTs)	Inhibition of COMT activity measured by the methylation of hydroxyestradiol and monitored by the radioactivity content	Inhibition of the COMT-mediated methylation of 2- and 4-hydroxyestradiol in human liver and placenta by coffee polyphenols as a potential marker of estrogen-induced tumors [35]
Glycine N-methyltransferases (GNMTs)	Inhibition of GNMT catalyzed metabolism of S-adenosylmethionine to S-adenosylhomocysteine measured by scintillation analyzer	Changes in GNMT activity in mummichog embryos and zebrafish development as a biotest of benzo[a]pyrene exposure [41]

2.2.1. Tests based on the activity of cytochrome P450 enzymes

Induced by toxicant disorders in P450, enzymatic functions are usually determined by changes in the metabolic rates of standard substrates of selected P450 isoenzymes. The analyzed probe can be added directly to the incubation mixture of an enzyme or to the cell culture, or, e.g., insect larvae previously exposed to the tested toxic substance can be put there. The concentration of the analyzed compound is determined on the basis of a calibration curve created by the fluorescence intensity that monitors the concentration of the standard metabolite of P450 isoenzyme [30,43].

2.2.2. Tests based on GST activity

Studies on the effective biosensors based on the inhibition of GST activity have usually employed insecticides of vegetable origin [47]. The ability of these compounds to inhibit the reaction catalyzed by the GSTD6 isoenzyme was tested by spectrophotometric analysis or iodometric titration [48]. More recently, it was suggested using a mutant form of GST1 isoenzyme obtained from corn seeds that catalyzes the conjugation of GSH with 1-chloro-2,4-dinitrobenzene [44]. The product of the reaction, hydrochloric acid, changes the pH of the reaction environment depending on the rate of the tested metabolism. The reaction can therefore be monitored by measuring pH. This method was tested on a wide variety of xenobiotics {e.g., insecticides (e.g., DDT, aldrin and endosulfan), fungicides (e.g., penconazole and tebuconazole) and herbicides (diuron, fluorodifen and tribenuron-methyl) [44]}.

The intertidal copepod, *Tigriopus japonicas,* was also recognized as a potential model species, where changes in GSTs gene expression were regarded as a biomarker of exposure to a number of endocrine-disrupting chemicals and trace metals [49]. GST was also described as an element of a multi-biomarker antioxidant system of terrestrial and aquatic organisms that employed to monitor toxic effects of chemical pollutants, including reactive oxygen species (ROS) [45,46].

2.2.3. SULT activity in analytical assays

The impact of activity of SULTs is usually tested in cytosolic fractions of animal or human liver cells. The suspension of enzymatic proteins is placed in a buffered solution with the addition of cofactor PAPS and a specific compound standard for SULT catalysis. After pre-incubation, the tested xenobiotic is added and incubated for about 1 h. Next, all samples are analyzed for concentration of a standard substrate. Changes in SULT activity are defined as the ratio of the concentration of the standard substrate in the control and in tested samples. The above method was used, e.g., to specify the inhibition of SULTs involved in metabolic detoxification of thyroid hormones by environmental pollutants [33]. The activation of this enzyme by polybrominated ethers used as substances slowing down the burning process have also been specified [32].

2.2.4. ATATs

The activities of testosterone ATAT were identified in the microsomal fraction of animals exposed to various types of pollution [e.g., organotin compounds, in particular tributyltin (TBT), used for the protection of ships hulls against fouling]. Microsomal proteins were incubated with a xenobiotic in acetate buffer in the presence of ATAT cofactor, palmitoyl-CoA. After 30–60 min of incubation with [^{14}C]testosterone, the reaction was stopped by the addition of ethyl acetate. Afterwards, multiple extractions with this solvent were performed and the organic fraction was analyzed [34].

2.2.5. NATs

Changes in NAT enzymatic activity can be defined in a cellular system and a cell-free system:

- the *cell-free assay* is based on the activity of isolated or recombinant NAT isoenzyme. Apart from the enzyme, the incubation mixture must include an acetyl group donor [e.g., p-nitrophenyl acetate (PNPA)] and the tested compound. After incubation for 15–60 min, an SDS detergent is added to stop the reaction, and

analysis of the inhibition of enzyme activity is performed. Usually spectrophotometric analysis is applied, as colored p-nitrophenol is a product of the tested reaction.

- In *cell-based assays*, the cell monolayer is exposed to the tested xenobiotic solution or environmental sample of unknown composition for 10–60 min. To initiate the reaction, an acetyl group donor is added [e.g., acetyl coenzyme A (AcCoA)]. After the incubation period, the cellular extract of compounds is analyzed with reversed-phased high-performance liquid chromatography (RP-HPLC). The change in enzymatic activity is determined by comparison with the sample that was not exposed to the xenobiotic. Different types of human and animal cells were used in the tests to measure NAT activity {e.g., bronchial and alveoli epithelial cells, including human cell lines A549 and 16HBE and mouse lung epithelial cells, Clara cell line (mtCC1-2) [38,39]}.

Using the tests mentioned above, it was proved, e.g., that metabolic detoxification of aromatic amines catalyzed by NAT is significantly inhibited in cells exposed to other environmental pollutants, including oxidative stress inducers [38] and heavy metals [39].

2.2.6. COMTs

The marker in testing COMT activity may be 4-nitrocatechol undergoing methylation in the presence of the enzyme. Thawed granulocyte cells were pre-incubated in a buffer solution, and next with 4-nitrocatechol for 1 h. The cells incubated with the selective inhibitor of the tested COMT (i.e., 3,5-dinitrocatechol) constituted the negative control [32].

2.2.7. GNMT

Tests employing GNMT are based on the results of studies showing that PAHs may be GNMT protein ligands. They also point to the parallel activity of the GNMT enzyme and the AhR nuclear receptor. The background of the test is the analysis of changes in GNMT activity in embryos of the mummichog (*Fundulus heteroclitus*) or the zebrafish induced by carcinogenic benzo[a]pyrene (BaP). Embryos were exposed to BaP, and then GNMT expression and enzymatic activity in the cytosol of embryonic cells were marked [41,42].

3. Changes in the action of nuclear receptors as a sensitive marker of environmental pollutants

The activity of metabolic enzymes, involved in the first and second phases of metabolism is regulated by several factors. On the one hand, the level of appropriate enzymes is genetically determined; on the other hand, it is influenced by external factors (see Fig. 4). These factors alter the rates of catalyzed reactions because of the affinity to enzyme molecules and/or direct interaction with these enzymes. The regulation of enzyme activity can also be performed indirectly at the level of gene transcription of selected proteins known as nuclear receptors [50,51].

The activation of nuclear receptor in the cell may lead to an increase in the transcription level of a certain gene. There exists a complex relationship between the metabolic pathways of chemicals and the signaling pathways of nuclear receptors [52,53]. That is why any disturbance in this relationship can be reflected in significant changes in cell physiology, which, in turn, can be analyzed when a selective marker of such changes is found [54].

Fig. 5 illustrates the induction of gene transcription. It occurs as a result of ligand (L) binding to the nuclear receptor. The additional coregulators (coactivators and corepressors) support this process. Coactivators induce the modification of histone proteins, which loosen the DNA structure and allow genetic material to interact with the receptors. Among the receptors involved in the regulation of gene

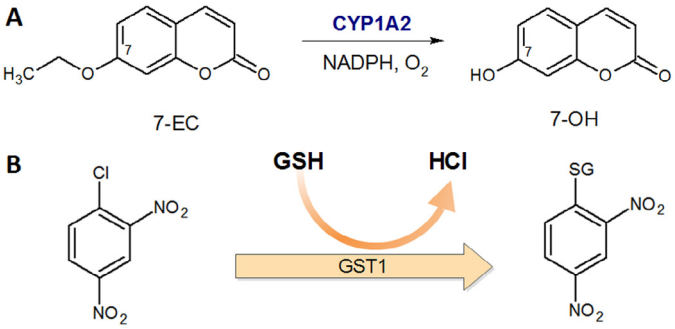


Fig. 4. Chemical-reaction schemes of: A) 7-ethoxycoumarin (7-EC) metabolized by cytochrome P450 1A2 to 7-hydroxucoumarine (7-OH), which is applied as the marker of P450 1A2 inhibition; and, B) chloronitrobenzene transformed to adduct with GSH by GST1, the excreted HCl (pH value) being the marker of the inhibitory properties toward GST enzyme, which can be measured by potentiometric method.

expression of enzymes responsible for the metabolism of xenobiotics, AhR, pregnane X receptor (PXR), constitutive androstane receptor (CAR) and retinoid X receptor (RXR) are the most common [52,55].

3.1. Receptors considered with respect to their analytical potential

3.1.1. AhR receptor

The AhR factor is a member of the extended family of transcriptional regulators, basic helix-loop-helix PAS (bHLH-PAS), where the PAS domain was named after the three proteins in which it was first discovered:

- **P**er – period circadian protein;
- **A**rnt – aryl hydrocarbon receptor nuclear translocator protein; and,
- **S**im – single-minded protein

The bHLH-PAS family is involved in the regulation of the expression of several P450 enzymes, including P4501A1 and P4501B1. Among its ligands, there are many xenobiotics and toxic substances, including dioxins and biphenyl derivatives. Other compounds (e.g., PAHs, bacterial metabolites and flavonoids) also modulate AhR activity. Upon ligand binding, the protein is transferred with protein kinases to the cell nucleus, where the appropriate transcription factor binds with the ARNT protein (AhR nuclear translocator). AhR expression takes place in all tissues of the body [56,57].

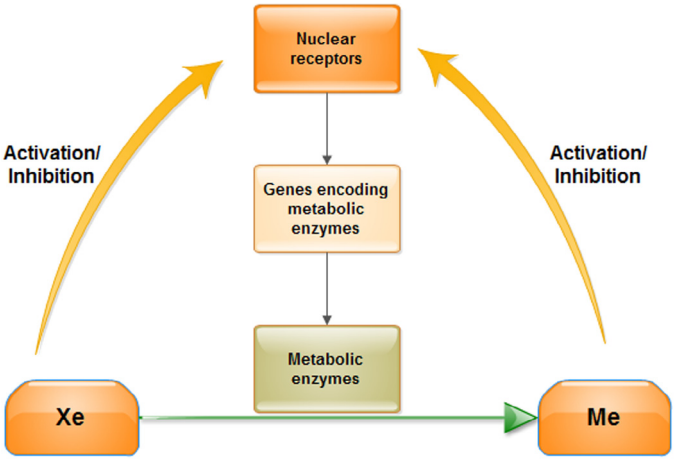


Fig. 5. The cross-talk between metabolic enzyme activity and the activation/inhibition of nuclear receptors, by xenobiotics (Xe) and metabolites (Me).

3.1.2. PXR nuclear receptor

The PXR and CAR receptors belong to the group of the so-called orphan receptors (of unknown natural ligands). Their mechanism of action is based on the heterodimer formation with an RXR receptor. This heterodimer binds to the xenobiotic-response element (XRE), which results in the activation of DNA transcription [30]. The PXR with a mass of approximately 50 kDa is the best known orphan receptor. The highest expression of PXR was identified in liver, intestine and kidney tissues and smaller quantities in lungs, ovaries, the heart, bone marrow and certain areas of the brain. Ligands of PXR include a range of xenobiotics in addition to a lot of endogenous substrates. Vitamin and lipid metabolism, steroid hormone homeostasis, bile-acid and bilirubin detoxification are regulated by PXR. Furthermore, the PXR receptors influence the activation of selected ATP-binding cassette (ABC) proteins responsible for the transport outside the cell of endogenous metabolites and xenobiotics [30,51,58].

These versatile physiological effects induced by PXR result from its effect on the increased expression of cytochrome P450 isoenzymes of the P450 3A, P450 2B, P450 2C families [30,53]. Carboxylesterases and aldo-keto reductases are also regulated by PXR. Phase II enzymes modulated by PXR are UDP-glucuronosyltransferases (UGT1A), SULTs and GSTs [51,58]. Thus, the PXR protein has relatively low ligand selectivity.

It was demonstrated that the function of PXR ligands is also fulfilled by the following medicines: warfarin, dexamethasone, taxol or rifampicine, and some pesticides (e.g., chlordane) and brominated flame retardants (e.g., hexabromocyclododecane) [51,53,59,60]. However, PXR ligands induce different expression of P450 enzymes in various living organisms and it is difficult to predict the effect on humans on the basis of *in vitro* studies [61].

3.1.3. CAR nuclear receptor

In terms of structure and operation, this receptor is very similar to PXR. Differences occur at the first stage of receptor activation. CAR does not require direct binding of the ligand. In addition, the CAR receptors exhibit a stronger response under stressful conditions in the cell [51]. CAR exhibits increased expression in the liver and intestine epithelial cells. Many known CAR inductors do not bind with the receptor, but influence only pathways necessary for translocation of CAR to the nucleus [55]. Among natural CAR ligands are the androstane metabolites (androstenol and androstanol). A certain number of chemicals express CAR-affinity dependent on the species of the experimental animals. So far, it has been proved that CAR induces cytochrome P450 expression (especially P450 2B), and UGT, SULT and transporter proteins (e.g., OATP2), thereby speeding up excretion of metabolites outside the organism [51,53].

3.1.4. RXR receptor

RXR belongs to a family of ligand-activated transcription factors. It regulates the retinoid signaling pathway and is also identified as a "coregulator" necessary for efficient binding of retinoid acid receptors (RARs) to their response elements. A class of nuclear eceptors, PXR, PPAR, VDR and RAR, requires RXR as a ıeterodimerization partner for their function. Thus, RXR finds itself ıt the crossroads of many distinct biological pathways. RXR-elective ligands activate multiple cellular pathways. Natural RXR igands are retinoic acids derived from vitamin A, which play es-ential roles in growth, reproduction, retinal development, vision ınd control of neurogenesis [62,63].

Environmental contaminants are able to interfere with RXR-egulated gene expression. For example, organotins used in marine ınti-fouling paints on ships alter the level of human chorionic go-ıadotropin and aromatase activity in human placental cells through nteraction with RXRs. Over the past decade, trace levels of a few RXR ligands were detected in several river systems in Taiwan in biotests for tributyltin and tetrachlorobisphenol A in water and sediment samples [64].

3.2. Development of analytical procedures based on nuclear receptor activity

Although the knowledge of the mechanism of ligand-receptor interactions is still at the stage of basic research, the ability of environmental chemicals to interact with AhR and nuclear receptors PXR, CAR and RXR has promising potential for fast development of new analytical methods for (i) toxicant detection and (ii) identification of environmental toxic effects. The task is not easy because only living organisms have the ability to induce nuclear receptors, so cell cultures and/or whole organisms are necessary at the stage of designing new analytical methods (refer to Fig. 6).

Development of new analytical methods for pollutant detection, which are based on the mechanisms of gene expression are linked with a new scientific field – ecotoxicogenomics. High diversity of biological markers may be applied in this way. The procedures were performed in many marine organisms concentrated in sediments [50,57]. The organisms used were also invertebrates characterized by reduced gene transcription of metabolizing enzymes and reduced mechanisms of gene-expression regulation. For example, the family of cytochrome P450 4 isoenzymes fulfils functions in invertebrates different from those in higher organisms, and changes in gene expression of these enzymes can be used as the marker of insecticide levels in environmental samples [54].

Most of analytical tests related to ecotoxicogenomics are currently at the design and verification stages, and are based on the use of selected molecular biology techniques, among which there are several groups:

- the real-time polymerase chain reaction (RT-PCR);
- gene-silencing procedures (siRNA); and,
- methods based on the reporter genes introduced into the cell.

3.2.1. Results based on the development of RT-PCR techniques

RT-PCR methods are based on the isolation of cellular RNA followed by the synthesis of complementary DNA *via* reverse transcriptase. As a result of replication (amplification) of particular DNA fragments, in fact, the RNA is analyzed, and the change in the level of gene transcription is monitored. RT-PCR analysis is the basis for the comparison of transcription profiles of selected biomarkers of environmental pollution [50,65].

Marine benthic polychaetes are an example of organisms employed as bioindicators, where the RT-PCR technique was used for the detection of harmful agents. The tests developed consist in the evaluation of benzo[a]pyrene (BaP)-induced carcinogenic effects on benthic polychaetes *Perinereis nuntia*. A comparison of transcriptional profiles of this seawater organism exposed to BaP proved that the presence of the carcinogen affected the transcription of the genes responsible for xenobiotic-metabolizing enzymes: P450 4 family and aldehyde dehydrogenase [50]. It was also shown that gene expression of new P450 isoenzymes of four families in *Perinereis nuntia* incubated with BaP in water increased evidently [57].

Expression changes of proteins involved in metabolic and hormonal pathways in vertebrates were also applied to determine the effect of TBT in the aqueous environment [53,66]. The organism tested was juveniles of salmon, *Salmo salar*. With the use of RT-PCR, transcriptional profiles of these fish exposed to xenobiotics indicated changes in expression of many proteins, including estrogen receptors (ER-α) and vitellogenin (VTG) involved in the production of egg yolk. An indicator organism for TBT in an aqueous environment can also be *Chironomus riparius* from the

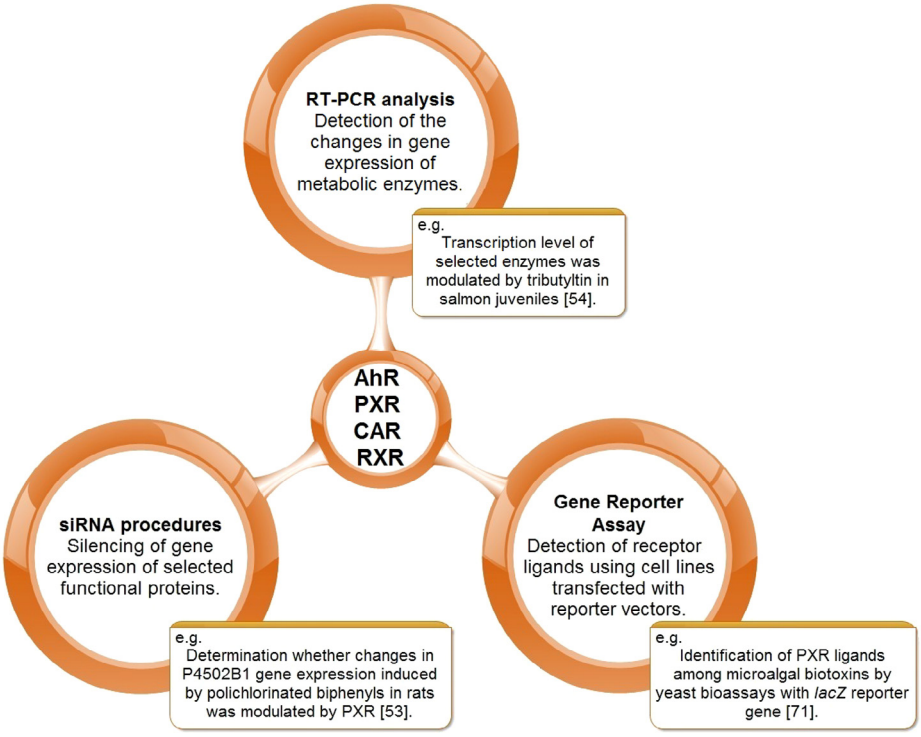


Fig. 6. Examples of the proposed analytical procedures based on the activity of nuclear receptors evaluated by RT-PCR, siRNA and gene-reporter assays.

family of *Chironomidae*. Contact with TBT causes an increase in the isoenzyme P450 4G gene transcription in these organisms [51].

3.2.2. Gene=-silencing procedures using siRNA

siRNAs are methods based on a naturally-occurring cellular mechanism of gene-expression regulation. This methodological approach of gene silencing applies RNA sequences complementary to the genes to be silenced [67].

In ecotoxicogenomics, siRNA techniques are still in the initial phase of use. However, a few examples can be found. For example, rodent liver cells were treated with siRNA targeted to the PXR/CAR receptors in order to determine whether the increased expression of isoenzymes P450 2B1 and 3A1 induced by polychlorinated biphenyls (PCBs) in rats was mediated by these receptors. After treating experimental animals with Aroclor 1254, reduced expression level of sex hormones, testosterone and estradiol, were indicated in their serum. Moreover, lower gene expression of the antioxidant enzymes – SOD (superoxide dismutase), CAT (catalase), GR (GSH reductase), GPX (GSH peroxidase), GST or P450 isoenzymes – was also exhibited [52]. Results of the studies on rat hepatocytes exposed to PCBs, where siRNA targeted to the PXR and CAR receptors were used, also showed that a decrease in the level of selected cytochrome P450 depends on the PXR/CAR action [68].

3.2.3. Tests based on the introduction of a reporter gene into the cell (reporter-gene assay)

A reporter-gene assay is able to determine whether environmental chemicals are ligands of the nuclear receptors, so it allows testing of whether the pollutant disrupts the action of a selected nuclear receptor. For this procedure, vectors of the tested gene and reporter gene are constructed. Cells are transfected with the vectors, and then analyzed for reporter-gene expression, which is a marker of changes in the expression of the tested gene [56]. Gene-encoding luciferase very often fulfils the function of the reporter gene, because this enzyme induces luminescence after oxidation of specific substrates [69]. PXR is the best-known target for xenobiotic ligands, and reporter-gene assay was usually applied to test the impact of xenobiotics on the PXR-regulated signaling pathway.

RXR is also an example of employing reporter genes to analyze the impact of environmental pollutants *via* nuclear receptors. A number of organic compounds, including 2-tert-butylphenol and hexachlorobenzene, are RXR agonists. A variety of retinoid-acid (RA) derivatives (e.g., 13-cis RA) and all-trans RAs expressing RXR-ligand properties are known for their teratogenic effects by binding to the RXR nuclear receptors [56].

The development of gene-reporter bioassay was based on the functional expression of fusion proteins, including PXR, in yeast strains. Expressed fusion proteins were coupled with the *lacZ* reporter gene that facilitates detection of probable ligands for the PXR receptor of tunicate. Such recombinant yeast bioassays suggest that they can find applications in robust and inexpensive screens for microalgal biotoxins and for novel marine bioactive chemicals in general [70].

We recommend that gene-reporter assays should be developed as the standard analytical bioassay as soon as possible, as it would be useful for detecting a great number of environmental chemicals.

4. Summary

After determination of hormonal activity, measurement of the activity of xenobiotic-metabolizing enzymes appears to be the second type of analytical methods, whose speed of development is trying to keep up with demand [26]. The undeniable advantage of enzymatic markers for the presence of xenobiotics is their high specificity and sensitivity. Specificity derives from the nature of the biological material used and the specificity of the molecular biology methods. High sensitivity results from the use of more and more efficient fluorescent dyes and the intensive development of

advanced methods for visualization of biological objects (e.g., two-photon confocal microscopy). Another aspect of the importance of this approach for environmental analytics is the possibility of focusing on a selected biological effect, regardless of the agent causing this effect.

The current possibilities of the experimental methods of molecular biology make it possible to determine the impact of xenobiotics on not only the activity of selected crucial enzymes, but also the regulators of gene expression of many functional proteins. This possibility, indeed, greatly complicates the interpretation of results, but, on the other hand, it allows prediction of a broader spectrum of harmful effects of xenobiotics in order to assess risk in a more comprehensive manner. In addition, the precise detection of harmful effects gives the background for the development of precise analytical bioassays for detection of chemicals at extremely low concentrations.

The new procedures of the methods described above are being continually developed. Standard methods include the analysis of changes in the activity of: cytochromes P450, GSTs and NATs. By contrast, so far, untapped potential lies in analytical methods based on changes in the activity of nuclear receptors and other transcription factors. As indicated above, the strongest potential seems to be in methods employing markers of gene-expression modulation (e.g., the gene-reporter assay). A genetically-constructed recombinant organism can be designed to express the appropriate receptor, and fast screening of the receptor ligands in probes can be a standard analytical test. There are promising reports on this aspect [70,71].

The field where biological tests have a significant advantage over instrumental methods is their potential to determine overall biological effects of very complex chemical mixtures (e.g., environmental samples). It is the basic reason for intensive development of such analytical protocols. The future of biological tests is probably in the combined use of bioassays, biomarkers and other biological indices to detect and to identify toxicants in various living organisms and in various elements of the environment. In addition, we expect such multidimensional "environmental hazard identification" to be performed *in situ* [72]. We should add that this approach demands application of advanced statistical analysis.

However, the specific position and the role of the development of a variety of biological assays in the future are reserved for methods based on monitoring gene expressions – ecotoxicogenomics. Currently, the greatest analytical potential seems to lie in:

- RT-PCR analysis, which detects the changes in gene-expression levels modulated by environmental pollutants; and,
- the gene-reporter assay employed for screening of xenobiotics with properties of nuclear receptor ligands.

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