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Application of physiological and biochemical assays as ecotoxicological tools for the detection and monitoring of pesticide pollution in earthworms and agroecosystem- A review

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ABSTRACT

The world population has grown exponentially over the last four or five decades and as a result food crisis with respect to the population had become a major problem for the governments of all the countries. As a result, it gradually became inevitable to increase the production of crops to meet the supply and demand ratio. Agriculture became much more advanced both in terms of technology and on field production to grow more genetically viable, disease and pest resistant variety and to grow them faster, application of chemical fertilizers drastically increased respectively with the addition of unmanaged application of pesticides resulting in advanced varieties of crops but more deteriorated soil quality, destruction of the entire ecology of beneficial and non-target soil organisms and also effecting the health of the food crops. Genotoxicity from food grains to humans has increased the prevalence of many dreadful diseases including cancer. Ecotoxicological research over the years has also developed and has helped us understanding the nature of the xenobiotic materials, specially pesticides, their mode of action, pattern of toxicity etc. Thus, researchers and different global organizations, specially Organization for Economic Cooperation and Development (OECD) has formulated different biochemical and analytical methods to study and determine the acute and chronic toxicity of pesticides and other xenobiotics.

1. Introduction

The synthetic pesticides machinery has been rolling since early 1940s when Dichloro diphenyl trichloroethane (DDT) was first introduced, bringing a novel paradigm in man's fight against pests and diseases (Rathore and Nollet, 2012). Unfortunately, this was the beginning of xenobiotic insult of pesticides to environment, non-target animals, man and human society. In order to increase agricultural output pesticides are always been major inputs in addition to seed, fertilizer and water in the modern agro-ecosystems which is reflected in the continuous growth of the global pesticide market especially in the tropical countries (Ecobichon, 2001).Increased food production is essential for mankind because human population is growing at an alarming rate and

is likely to cross the 9 billion mark by 2050 (UN, 2005). It cannot be denied that pesticides play an essential role in controlling harmful pests of crops. Inventions of high yielding varieties of crop, collective farming and specialization leading to the competent use of machinery prompted the farmers to use chemical pesticides. More and more potent pesticides were invented and the application rate continued to increase. The farmers got an immediate return with high yield. But continuous and indiscriminate use of all the kinds of pesticides since last few decades have caused serious damages to ecosystems (Reinecke and Reinecke, 2007) and concern for contamination of the environment gained tremendous importance in recent years (Zhu et al., 2004).Detection of insecticideresidue in fish (Tilak et al., 2003), milk products, vegetables (Lin and Shiau, 2005), food grains (Toteja et al., 2006), meat, groundwater and even in humanblood and breast milk (Strucinski et al., 2006; Furst, 2006; Damgaard *et al*.,2006) became priority research. Scientists throughout the world are engaged inevaluating damages caused by the pesticides (Hafez and Theimann, 2003).Increasing number of ecosystems are being contaminated by huge quantities of pesticides that are released daily into the environment (Sarkar, *et al*., 2006). Agricultural fields are the worst affected ecosystems. Ploughing, clearing, levelling, burning, watering etc. and application of synthetic fertilizer and pesticides make agricultural fields different from natural soil ecosystem. Some soil species cannot withstand such disturbances and may disappear either completely or partially from agricultural soil system and hamper natural equilibrium among organisms (Wallwork, 1970; Lasebikan, 1975; Aleinikova and Utrobina, 1975; Tadors and Saad, 1980).Scientists throughout the world are engaged in evaluating damages caused by the insecticides.Thus, the fate of insecticides in the soil and their effect on nontarget soil organisms are priority research in recent years (Hafez and Theimann, 2003).In this review, we will discuss about these different techniques and methodologies which can be used as ecotoxicological tools to detect and monitor pesticide pollution in soil organisms and agroecosystems.

2. Methodologies

2.1 Acute Toxicity

2.1.1 Lethal Dose (LD_{50})/ Lethal Concentration (LC_{50}) determination

Bioassays are made with age synchronized test specimensin the same small inert polythene boxes. In case of soil organisms, dried, finely ground soil (0.25 mm particle size) are usually laid in the experimental boxes as an experimental medium. Soil moisture of the test soil is maintained at 60–70 % level. Different levels of the pesticides based on their recommended agricultural doses (RAD) are administered into the test boxes with a micropipette (Bostrom and Lofs Holmin, 1982), but the actual doses used are presented in terms of mg / kg soil given in an experimental box. After the application of pesticides, the boxes are left undisturbed for about 30 minutes for

uniform spreading of the chemical in the medium. Each experiment is accompanied by a test box which received no pesticide and is treated as control. Then age synchronized test specimens are transferred into the control and treated test boxes. Finally, the experimental boxes are kept in an Environmental chamber at a constant temperature of 28 ± 0.5°C and 60-70 % moisture level. Mortality of the test specimensis recorded every 24 hours and dead specimens, if any, are removed from the experimental boxes (OECD, 1984).The total mortality of the test specimens obtained after 96 hours of exposure are subjected to probit analysis by EPA probit analysis program, version 1.5 (US EPA, 2006) to determine LC_{50} value and 95 % confidence limit of each insecticide.

2.1.2 Lethal Time (LT_{50}) determination

Bioassays to determine LT_{50} of the pesticide for test specimensare made in the same experimental boxes and experimental conditions as described above for bioassays to determine LC_{50} value. The treatment of a pesticide used for this bioassay is the RAD of that pesticide. The experimental boxes, after treatment of the pesticide, are left undisturbed for about 30 minutes for uniform spreading of the insecticide in the soil medium. Then age synchronized specimens are transferred into the boxes which are kept in an Environmental chamber at a constant temperature of 28 ± 0.5° C and 60-70 % moisture level. Observations were made every hour and the dead individuals were removed (OECD, 1984). The time taken to achieve 50% mortality was noted and expressed as LT_{50} of the insecticide.

2.1.3 Residual toxicity determination

Residual toxicity bioassays are also carried out with the application of RAD of the pesticides tested. A total of 15 treatment boxes are used for each pesticide. Bioassays are done with age synchronized specimensin small inert polythene boxes each containingthe test medium. The test medium is dried, ground and sieved to get a particle size of 0.25 mm before laying in the experimental boxes. Moisture level of the test soil was maintained at 60-70 % level. Recommended agricultural dose of a pesticide is then applied into the test boxes. The boxes are kept in an Environmental chamber at a constant temperature of 28 ± 0.5°C (OECD, 1984). Three boxes are sampled at intervals of 7, 15, 30 and 45 days and age synchronized specimens are transferred into the boxes. Mortality was recorded after a period of 96 h (Chakravorty, 1990). Control sets were maintained for each pesticide.

2.1.4 Filter paper contact toxicity method

Acute toxicity test is performed following the method described in the OECD (1984) guideline for testing of chemicals no. 207. This is a simple screening test to identify the toxic potential of the chemical. The test vial is a petri dish (Wang et al., 2012) of 14cm diameter and 2cm height. Round filter paper (Whatman No. 1) is cut to the suitable size and placed in such a way that sides are lined with filter paper. 5ml test solution was pipetted into each vial in order to wet the filter paper. Blank tests were performed with

5ml of deionized water. Each treatment, consists of one earthworm per vial. Adult specimens,having a standardised wet weight, are selected for testing. Specimensare washed briefly with deionized water, and are kept on moist filter paper for 3h to devoid the gut content, after which it is rinsed again with deionized water, blotted on the filter paper and placed in a test vial. An earthworm is introduced per vial and the vial is covered with plastic film that had been punched with small holes using needles. Tests were done in the dark at 28±2°C for 48 h. After 48 hours the earthworm was monitored for mortality by a gentle mechanical stimulus to the front part.

2.2Chronic Toxicity

The chronic toxicity bioassays are done with the main objectives of assessing the physiological and biochemical stress caused to soil organisms as a result of long-term exposure of the pesticides. The physiological stress assessment is done from the life cycle parameters, like biomass change, clitellum development and cocoon production and biochemical stress was assessed from the activities of certain enzymes and tissue nutrients of the test specimens. Different sub-lethal doses of pesticides are applied.

2.2.1. Life cycle bioassay

The same small and inert polythene boxes are used to carry out the bioassays with age synchronized test specimens as described in the acute toxicity experimental procedure. Standardised weight of dry, finely ground soil (0.25 mm particle size) is being put in the experimental boxes. Soil moisture of the test soil was maintained at 60–70 % level. Different sub-lethal doses of test pesticides are applied based onthe LC50 value of the respective pesticides. In each test box, standardised number of age-synchronized adult specimens is released. Before releasing, the specimens are washed with water, blotted dry on a filter paper and the total biomass is determined. Finally, the experimental boxes are kept in an environmental chamber at a constant temperature of 28 ± 0.5!. Food is provided for the test speimens. Additional food was given when all the food in the test boxes was exhausted. The test soil was checked by weighing the test containers at weekly intervals to determine the loss of moisture and replenished if needed (OECD, 2004).

i) Biomass change

On the 28th day, the weight of the test specimens are taken to determine the biomass change and are removed from the test boxes according to the protocols by OECD (2004).

ii) Reproduction

The rate of reproduction is evaluated to determine the reproductive success of the test specimens. For this purpose, careful examination of the test soil is done with a magnifying glass and the number of cocoons/ nymphs/larvae/eggs is counted every week for four weeks. The reproductive maturity is also studied. After counting, the cocoons/nymphs/ larvae/eggs are removed from the test boxes every week and the boxes were housed inside environmental chamber. The temperature and

moisture were maintained at 28 ± 0.5°C and at 60 – 70 % (OECD, 2004).

iii) Respiration

The adult specimens removed from the test boxes on the 28th day are used to determine the rate of evolution of carbon dioxide to assess the rate of respiration by alkali absorption technique (Aira et al., 2001). Earthworms are taken on moist filter paper in a Petri dish which is covered by an inverted glass beaker. 1N KOH is used to absorb carbon dioxide evolved. An experimental control, without the test organisms, was maintained simultaneously. Rate of carbon dioxide evolution is determined by titrating the 1N KOH used to absorb carbon dioxide evolved against 0.1 N HCl and wasexpressed in mg $Co_2/m^2/h$.

2.2.2. *Biochemical assays*

On the 28th day of the experiment, adult specimens are removed from the test boxes to determine acid and alkaline phosphatase and acetyl- cholinesterase of the earthworms.

i) Acid phosphatase (Walter and Schutt, 1974)

Standardisedamount of tissue is taken and homogenized in 5 ml normal saline and then centrifuged and 0.2 ml of supernatant is taken in a test tube to which 1 ml acid buffer is added thoroughly. A blank is prepared simultaneously by adding 0.2 ml of 0.7% saline to 1 ml of acid buffer. Both the tubes are incubated for about 30 mins at 37°C. At the end of the stipulated period 2 ml of 0.1 N NaoH is added to both the test tubes andmixed thoroughly. The amount of p nitrophenyl liberated in the tissue sample mixture is determined spectrophotometrically at 405 nm in a Systronix UV visible spectrophotometer after adjusting the absorbance of the blank.A standard curve is prepared in the same manner with known amount of paranitrophenyl and the values liberated were determined from the standard curve. The enzymatic activity was finally expressed in µg pnp/mg of protein/30 min after estimation of protein content of the samples.

ii) Alkaline phosphatase (Walter and Schutt, 1974**)**

Standardised amount of tissue is taken and homogenized in 5 ml normal saline and then centrifuged and 0.05 ml of supernatant is taken in a test tube to which 2 ml acid buffer is added thoroughly. A blank is prepared simultaneously by adding 0.05 ml of 0.7% saline to 1 ml of acid buffer. Both the tubes are incubated for about 30 mins at 37°C. At the end of the stipulated period 10 ml of 0.05 N NaoH is added to both the test tubes andmixed thoroughly. The amount of p nitrophenyl liberated in the tissue sample mixture is determined spectrophotometrically at 405 nm in a Systronix UV visible spectrophotometer after adjusting the absorbance of the blank.A standard curve is prepared in the same manner with known amount of paranitrophenyl and the values liberated were determined from the standard curve. The enzymatic activity was finally expressed in µg pnp/mg of protein/30 min after estimation of protein content of the samples.

iii) Acetylcholinesterase (Ellman *et. al*., 1961)

Standardized portions from the surviving

specimensare separated to estimate theacetylcholinesterase activity. They are homogenized in 10% (w/v) 0.1 M, phosphate buffer,pH 7.5 using a homogenizer. The homogenate is centrifuged at 10,000 g for 10 min and thesupernatant was further centrifuged at 10,000 g for 10 minutes at 4° C (Remi cold centrifuge).The resultant supernatants are stored in ice and used for Acetylcholine esterase assay.Kinetic measurements are performed with acetylthiocholine iodide as the substrate. Reactions are performed in 300 µl of 0.1 M Phophate buffer, pH-8 containing-

a. 20 µl of 0.01 M DTNB (5,5'-dithio-(2-nitrobenzoic acid))
b. 20 µl 0f 0.075 M substrate
c. 10 µl tissue extracts

Contents are thoroughly mixed and absorbance was measured at 412 nm in systronics UV-Vis spectrophotometer. Substrates are continued to be added before adding of the substrate till a stable reading was recorded. After addition of the substrate, the change in absorbance is recorded for a period of 10 min at an interval of 2 min. Change in absorbance per minute is thus determined. The enzymatic activity is finally expressed in nmoles/min/mg of protein after estimation of protein content of the samples.

2.2.3. Digestive enzyme and tissue nutrient bioassay

Separate bioassays are made to determine the effects of the sublethal doses of the pesticides on the digestive enzyme cellulase, alpha-amylase, total tissue carbohydrate and proteins under laboratory conditions in natural garden soil. Ten specimens of earthworms were kept inside inert polyethylene boxes each with 192 cm2 area and containing 250g of sieved garden soil. Distilled water was added to maintain 60-70% moisture. In each of the test boxes a small Petri-dish was placed, so that the soil surface and the brim of the Petri-dish are at the same uniform level, with finely cut cashew leaf litter as food for the earthworms during the entire experimental period. The experiment is set following the procedure of open choice experiment as described by Maityand Joy, (1999). The food is contaminated with pesticide in the treatmentboxes. The whole set up is kept inside an environmental chamber and thetemperature (28±0.5°C) and humidity (67%) is maintained. The determinationof cellulase activity,alpha-amylase, total tissue carbohydrate and protein is performedon 3rd, 7th, 15th and 30th day from the setting of the experiment. The testspecimens were kept in starvation before setting of the experiment.

i) Determination of specific activity of cellulase (Sadasivam and Manickam, 2010**)**

Standardized quantity of tissue from test specimen are homogenized in 5ml of normal solution and then centrifuged to get the supernatant with enzyme extract. 0.45ml of 1% carboxymethyl cellulose and 0.05ml of enzyme extract are mixed together and incubated at 55! for 15 minutes and 0.5ml of Dinitrosalicylic Acid Reagent is mixed immediately after removing the mixture from water bath. Then the mixture is heated in boiling water for 5 minutes. While the tubes are worm, 1ml of potassium sodium tartrate is added and let to

cool to room temperature. 5ml of distilled water is added to make the volume up to 5ml. The absorbance is measured at 540 nm. The specific activity of the enzyme is expressed as mg glucose released per minute per mg protein.

ii) Determination of specific activity of á-amylase (Sadasivam and Manickam, 1992)

Standardized quantity of tissue from test specimen are homogenized in 5- 10ml of ice cold 10mM calcium chloride solution and then centrifuged to get the supernatant with enzyme extract. 1ml of starch solution and 1ml of enzyme extract are mixed together and incubated at 27°C for 15 minutes and 2 ml of Dinitrosalicylic Acid reagent is added immediately after taking out the mixture out of the water bath. Then the solution is heated in boiling water bath for 5 minutes and while the test tubes are warm 1ml of sodium potassium tartrate solution is added and cooled under running tap water. The final volume is made to 10ml by adding distilled water. The absorbance of the solution is measured at 560 nm. A unit of á-amylase is expressed as mg maltose produced during 5 minutes of incubation with 1% starch.

iii) Determination of total tissue carbohydrate by anthrone method (Sadasivam and Manickam, 1992)

Standardized quantity of tissue from test specimen are hydrolysed in test tube for 3 hourswith 5 ml of 2.5 N-HCl and cooled to room temperature. The mixture is neutralized with sodium carbonate. The final volume is made up according to the weight of the tissue then centrifuged to get the supernatant with enzyme extract.1ml aliquot is collected and 4ml anthrone reagent is added. The mixture is then heated for 8 minutes in water bath and cooled rapidly. The colour of the solution will go from green to dark green, absorbance of which is measured at 630 nm. The amount of carbohydrate present in the standardized quantity of tissue is expressed in percentage mg of glucose divided by volume of sample.

iv) Determination of total tissue protein (Lowry *et. al*., 1951)

The blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard (Bovine Serum Albumin) are pipetted out into a series of test tubes. 0.5 ml of the sample extract is taken in another test tube. The volume is made up to 1 ml with distilled water. A tube with 1 ml of distilled water served as the blank. 5 ml of alkaline copper sulphate solution was added to each of the test tube, mixed well and allowed to stand for 10 minutes. Then 0.5 ml of diluted (1:2) Folin-Ciocalteau reagent was added to each of the test tube, vortexed and incubated at room temperature preferably in dark for 30 mins. The intensity of blue colour developed was measured spectrophotometrically at 660nm.

2.2.3 Determination of genotoxicty

i) Coelomocyte count method

For the study of toxicity posed by the pesticides on the test specimens in DNA level, free coelomocyte cells of the coelomic fluid are used. Coelomocytes are collected from the coelomic cavity along with coelomic fluid by a non-invasive extrusion technique described by Eyambe et al., (1991). Extracted coelomic fluid in the extrusion medium is then centrifuged in 150 X g at 4°C and cells are collected from pellets and used for all assays. The extrusion medium prepared of 5.0%ethanol and 95% saline supplemented with 2·5 mg/ml EDTA and 10mg/ml of the mucolytic agent guaiacol glycerol ether, and adjusted to pH 7.3 with 1N NaOH.

According to Muangpra and Gooneratne (2011), coelomic fluid is smeared on a set of three glass slides from each concentration. After drying the fluid, the coelomocytes are fixed with a methanolic fixative solution for differential staining of cellular components (Wright Rapid Stain). From three slides of each applied fungicide concentration, a gross of 3000 coelomocytes (1000 cells x 3 slides) are observed and tallied by a compound microscope (Olympus, CH20i) at 100X magnification in oil immersion to determine the micronuclei and binucleate cells frequency.

ii) Comet Assay

Single cell gel electrophoresis or comet assay is done to assess the level of DNA damaged in coelomocytes of the test specimens exposed tosub lethal doses of the pesticides. A standard protocol prepared by Bajpayee et al. (2019), is followed to prepare the conventional slides with isolated coelomocytes from the test specimens in 1% low melting point agarose gel (1% LMPA) on the base layer of 1% normal melting agarose gel (1% NMA). After proper cell lysis in lysing solution, slides are kept in electrophoretic chamber in alkaline electrophoresis buffer and incubated for 20 minutes. Then power supply turned on to 24 volts and adjusted the current to 300 milliamperes and the gels are electrophoresed for 30 minutes. Slides are then neutralized in neutralization buffer and stained with EtBr, and covering with coverslips viewed under fluorescence microscope to capture the images of the damaged nucleuses. The images are analysed by ImageJ software.

2.3 Molecular Toxicity Assessment

2.3.1. Mass Spectrometry (MS)

Risk assessment of chemical effects in the environment requires the understanding of the fate and behaviour of anthropogenic chemicals in natural and technical systems. The exposure data obtained by environmental chemists are in turn used to evaluate the significance of toxicological effects in organisms, as studied by environmental toxicologists. Mass spectrometry-based techniques are frequently applied to monitor the exposure or investigate the effects of chemicals, particularly their mechanism of action.

Interactions between chemicals and living organisms are governed by toxicokinetic and toxicodynamic processes (Ashauer *et al*., 2011),

and contemporary studies in both fields rely heavily on mass spectrometry (Groh and Suter, 2014). Toxicokinetics describes uptake, biotransformation, distribution, and excretion of a chemical by an organism, also referred to as absorption, distribution, metabolism, and excretion (ADME) processes (Kirla et al., 2016; Mottaz *et al*, 2017). Toxicodynamics looks at the actions of a chemical or its metabolite, carried out at the target sites where toxicity becomes manifested (Groh *et al*., 2015). Such actions could include, for example, DNA adduct formation (Madureira *et al*., 2014), oxidation of membrane lipids (Pillai et al., 2014), or binding to a nuclear receptor, which in turn could trigger gene or protein expression changes and metabolite alterations (Oliveira *et al*, 2016; Mottaz *et al*, 2017; Nestler *et al*., 2012a; Nestler *et al*., 2012b; Hidasi *et al*., 2017).

Information on effective internal organismal or tissue concentrations of chemicals and their transformation products is important for toxicokinetic modeling (Kirla *et al*., 2016) and can be obtained with the same approaches as applied to environmental compartments (Ammann *et al*., 2014; Ammann and Suter, 2016; Hidasi *et al*., 2017). Taking samples at different time points, performing depuration experiments, or carrying out non-targeted or targeted metabolite screening allows constructing time-resolved profiles of chemical uptake, biotransformation, and excretion (Kirla *et al*., 2016; Mottaz *et al*, 2017; Di Paolo *et al*., 2015; Kirla *et al*., 2018). MS has also been instrumental in obtaining information that sheds light on the internal distribution of chemicals. This can be done by measuring chemical contents in the dissected body parts or by using MALDI imaging to decipher chemical location on tissue sections (Kirla *et al*., 2016; Kirla *et al*., 2018). With regard to toxicodynamics, MS can provide data on gene expression and cellular signaling cascades, for example through looking at proteins (proteomics) and metabolites (metabolomics) (Sturla *et al*., 2014). The study of metabolomics is rapidly establishing itself in ecotoxicological research (Viant *et al*., 2017), also because it's analytical pipelines are often similar to untargeted environmental chemical analysis (Ammann and Suter, 2016; Viant and Sommer, 2013).

2.3.2. Nanotoxicology

One subfield of toxicology which has received a lot of attention in recent years is nanotoxicology. Research focused on elucidation of fate and effects of nanoparticles in humans and the environment would not have been possible without the MS advances in place (Sigg *et al*., 2014). Inductively coupled plasma MS (ICP-MS), has been routinely applied to study metal-based nanoparticles, with dedicated separation methods such as ultra filtration, used to distinguish between ionic and nanoparticulate forms (Groh *et al*., 2015; Yue *et al*., 2017).

2.3.3. Ecotoxicological risk assessment using DNA chips and cellular reporters

i) Microarray and 'in vivo' testing for risk assessment

Gene expression profiling is a unique way of characterizing the response and adaptation of an organism to changes in the external

environment. The gene profile can be characterized by (eco) toxicogenomics techniques, which can therefore be considered as a technology with high added value for risk assessment purposes (Robbens *et al*., 2007).

Currently, several strategies can be applied to investigate differential gene expression in these – genomically poorly characterized – species, such as heterologous hybridization (Lettieri, 2006; Renn *et al*., 2004) and suppressive subtraction hybridization (SSH) PCR (Moens *et al*., 2006). The recent construction of array platforms based on SSH PCR for several ecotoxicologically relevant organisms has proven to be generally applicable and has promoted the application of microarray gene expression profiling in ecotoxicology– that is, in ecotoxicogenomics (Robbens *et al*., 2007). In ecotoxicology, following exposure to a chemical, gene expression profiling using microarrays is used to address two complementary tasks: to provide information about the mechanism of action of the toxicant and to form a 'phenotypic signature' for the identification of toxic products (Moens et al., 2006). Both types of information can contribute to ecological risk assessment by identifying biomarkers of effect and biomarkers of exposure, and thus lead to being able to assess the effect upon a cell or organism and to monitor the presence of a toxicant (Robbens *et al*., 2007). Microarrays provide a snapshot view of genomic activity; a first factor involves the fact that gene expression data are meaningful only in the context of a detailed description of the conditions under which they were generated, including the state of the experimental organisms, possible perturbations and confounding factors, in addition to the microarray platform and experimental design and processing used. Therefore, an important task is to define the standards for microarray data, to facilitate the effective management, integration, interpretation and sharing of (eco) toxicogenomic data (Ankley *et al*., 2006; Boverhof and Zacharewski, 2006; Pognan, 2007). Keeping this complexity in mind, a minimum standard for information associated with microarray experiments has been defined, known as 'Minimum Information About a Microarray Experiment' (MIAME), ensuring the interpretability of the experimental microarray results and their potential independent verification (Brazma *et al*., 2001). The strategy of ecotoxicogenomics is often to compare gene expression patterns in two samples – one control and one exposed to the toxicants under study (Snell *et al*., 2003). Controls provide an implicit reference collection of 'normal' gene expression levels under a given set of conditions and variables (Robbens *et al*., 2007).

Although the growing list of reports on the application of microarrays in ecotoxicogenomics is proof of the great opportunities for ERA for chemicals, standardization alone will not suffice for a proper implementation of new technologies in ERA. The application of this technology is still in its infancy (Moens *et al*., 2006) and most data are of an exploratory or research nature. Before full implementation in ERA, effort must

be undertaken to overcome several considerations, inherent not only to gene expression data, but also to the use of many other biomarkers in ERA (Forbes *et al*., 2006).

ii) Cellular Reporters in ecotoxicology

A cellular reporter can be defined as a cell that contains a promoter and regulatory sequence in control of a reporter gene. Following interaction with a chemical compound or through a more complex signaling chain, activation of the promoter by the transcriptional regulator leads to the upregulated expression of the reporter gene, yielding an output signal that can be detected, calibrated and interpreted. Light-based and fluorescence-based reporters are the most frequently used reporters (Denison *et al*., 2004). However, many other types of reporters have been used in various studies (Haruyama, 2006; De Coen *et al*., 2004). Cellular reporters can be ranked as general, semispecific or specific (Harms, 2006; Sorensen, 2006). These different types of cellular reporters are used according to the toxicological questions to be answered. General cellular reporters often contain a constitutive or general promoter and produce a decreasing reporter signal as a result of a general toxic stress. Semispecific and specific cellular reporters produce a chemical-specific signal. Semispecific reporters are responsive to a group of related stress-inducing compounds. Well-known examples are the chemical-activated luciferase (CALUX)-based bioreporter, such as dioxin-responsive CALUX (DR-CALUX1), endocrine-responsive CALUX (ER-CALUX1) and thyroid-responsive CALUX (TRCALUX1). DR-CALUX1 responds by emitting light to all chemicals [e.g. dioxin and polychlorinated biphenyls (PCB)] that are able to activate the aryl hydrocarbon receptor (Scippo *et al*., 2004; Pliskova *et al*., 2005). Amore recent version of this reporter uses the green fluorescent protein as a reporter–chemically activated fluorescence expression (CAFLUX) (Denison *et al*., 2004). A dual reporter system has already been developed with different luciferase mutants; however, so far it has not been used in an ERA environment or for monitoring purposes (Branchini, 2007). It is clear that this step will be essential for further validation of cellular reporters for risk assessment purposes.

3. Conclusion

In conclusion the above discussed techniques play very important functional roles in both environmental chemistry and toxicology. These techniques are used in the (i) measurement of chemicals in environmental compartments and biota, (ii) identification of unknown chemicals potentially causing adverse effects in the environment, which and (iii) providence of insights into the internal toxicokinetic processes and molecular mechanisms of toxicity. Thus these methodologies have become essential in both fundamental and applied research in ecotoxicology, supporting risk assessment and management of chemicals.

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