Review

(Special Topic)

Applications of genomic technologies to the study of organochlorine pesticide-induced reproductive toxicity in fish

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Microarrays and real time PCR are two molecular methods that are gaining acceptance for ecotoxicology. They will help sort out the complex biochemical pathways that are targeted by exposure of organisms to organochlorine pesticides (OCPs) and other toxicants and may point to the ways that these compounds affect development, reproduction and susceptibility to disease. OCPs are a large group of structurally diverse compounds that are present around the globe and are known to be highly persistent pollutants, for which mechanistic toxicity information is lacking. Genomic technologies such as these will help identify and monitor toxicity and help develop subsequent environmental policies. © Pesticide Science Society of Japan

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Introduction

The environment is being saturated by an increasing number of stressors of many different types: the release of all sorts of chemicals, the loss of habitat, changes in the equilibrium of ecosystems, decreased survival of some species and climate change, among others. The release of chemical compounds to the different compartments of the environment (air, water, soil and sediments) represents a complex risk to wildlife and human health. The toxicity of chemicals is very complicated and difficult to assess. The fact that chemicals are always present in the environment as complex and varied mixtures complicates enormously the ability to analyze and predict their effects. Researchers are increasingly applying genomics to environmental monitoring, trying to elucidate the hazards of environmental pollutants. The term “omics” embraces different technologies that focus at different levels: genetics, transcriptomics, proteomics, and metabolomics to examine gene polymorphisms, mRNA transcripts, proteins and metabolites, respectively.

This review explores how transcriptomics can be applied to fish ecotoxicology. Environmental pollutants especially affect fish, as they are constantly exposed to them. Fish live in the

water, which is one of the final compartments where pollutants tend to accumulate, and into which discharges are enormous and constantly fluctuating. The fact that fish are exposed to pollutants can affect not only fish, but also the whole equilibrium of the ecosystem, and particularly higher levels of the trophic chain, including humans. We will focus as an example on only one group of environmental pollutants, the organochlorine pesticides (OCPs), as those are very persistent compounds that tend to accumulate in the aquatic environment and also in organisms. First of all, we will summarize the different types of toxicities related to exposure to OCPs, revealed by different molecular techniques. Next, we will focus on transcriptomics techniques, specifically real-time PCR and microarrays, and their use in fish ecotoxicology.

Organochlorine Pesticides

OCPs, a large group of structurally diverse compounds, are some of the most analyzed and studied environmental pollutants. OCPs can be found in great concentrations in the environment. For instance, in muck farms and contaminated lakes in Central Florida, Marburger *et al.*¹⁾ found concentrations in the flooded soils as high as $385 \pm 241 \mu\text{g/kg}$ of dieldrin, $7173 \pm 1037 \mu\text{g/kg}$ of ΣDDTs (DDT+DDE+DDD) and $39,444 \pm 11,284 \mu\text{g/kg}$ of toxaphene. Furthermore, the toxic effects of OCPs, their high chemical stability and their lipophilicity allows them to accumulate in animal tissue and makes them one of the most widely distributed types of pollu-

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nants worldwide.

Even though most OCPs have been banned for decades, they are still highly persistent in the environment. They were widely used from the 1950s to the 1970s, and as one might expect, they persist in the soils and also in the sediments at the bottom of lakes, even in remote areas, bioaccumulating and biomagnifying in wildlife.^{2–7)} The presence of OCPs in remote areas is related to their physicochemical properties, which result in their long-range transport. The air masses that arrive to remote areas have already traveled long distances. Consequently, the pollutants inside them are very variable and it is almost impossible to determine their source, as the air masses may have passed by many different sources and the pollutants may have suffered many degradation processes. In the atmosphere, pollutants are either in the gaseous phase or attached to particles. HCHs (hexachlorocyclohexanes) and DDTs together with some other low molecular weight compounds tend to be in the gaseous phase, rather than in the particle phase, in relatively clean environments.⁸⁾ Besides, the concentration of OCPs in remote areas is enhanced by the global distillation effect that involves their migration from temperate to cold areas, where they can become trapped. This effect occurs both on the planetary scale⁹⁾ and at regional levels, where high elevations serve as cold traps.^{10,11)}

There are many different types of chemical structures within the OCPs: compounds from the cyclodiene group such as aldrin and dieldrin; halogenated ethane derivatives such as DDT, DDE and methoxychlor; or cycloparaffins such as hexachlorocyclohexane. Many studies have reported the ability of OCPs to act as endocrine disruptors, leading to impaired development and reproduction in wildlife.^{12–18)}

Toxicity Mechanisms of OCPs

OCPs are known to affect multiple biochemical pathways leading to general toxicity in fish, wildlife and humans. Many of these pathways have been elucidated, but there is still a lack of information on the mechanisms of toxicity, suggesting that novel approaches to understand these pathways are necessary. The toxic effects of OCPs can be manifested into broad areas such as immunotoxicity, neurotoxicity, dioxin-like toxicity and endocrine-related toxicity. Included in these broad areas are hepatic, developmental, and reproductive toxicity. Although the majority of the studies have been done on mammals, some effects have also been reported in fish. Table 1 summarizes the toxic effects of the OCPs reported in this review.

1. Immunotoxic effects

Several research groups have shown that OCPs can affect the immune response. Harford *et al.*¹⁹⁾ reported that endosulfan resulted in the modulation of phagocytic responses in crimson-spotted rainbowfish (*Melanotaenia fluviatilis*), golden perch (*Macquaria ambigua*) and Murray cod (*Maccullochella peelii*), three native Australian freshwater fish. Milston *et*

*al.*²⁰⁾ found that a short-term exposure of Chinook salmon (*Oncorhynchus tshawytscha*) to *o,p'*-DDE during early-life stages caused long-term humoral immunosuppression. Lahvis *et al.*²¹⁾ correlated reduced immune response in bottlenose dolphins (*Tursiops truncatus*) with blood concentrations of *p,p'*-DDT, *o,p'*-DDE and *p,p'*-DDE. The biochemical pathways that these mechanisms utilize are diverse and inter-related. A genomic approach could help to show both the commonalities and the differences in gene expression on patterns that contribute to these effects.

2. Neurotoxic effects

OCPs can also act as neurotoxic compounds. Epidemiological studies suggest a relationship between dieldrin concentrations in the brain and the neurodegenerative disorder Parkinson's disease (PD).²²⁾ PD provokes a progressive and selective dopaminergic neuronal degeneration in the *substantia nigra pars compacta* (SNc), the brain region that controls motor activity.²³⁾ The loss of dopaminergic neurons results in depletion of striatal dopamine, resulting in irreversible motor dysfunction.^{24,25)} Oxidative stress, mitochondrial dysfunction, protein aggregation, and apoptosis are key mediators of nigral dopaminergic neuronal cell death in PD (reviewed by Kanthasamy *et al.*²⁶⁾). Dieldrin induces apoptosis, alters dopamine levels, mitochondrial dysfunction, and protein aggregation, by activating a series of cell death signaling molecules.^{27–29)} Other studies show that the OCPs γ -hexachlorocyclohexane, α -endosulfan, and dieldrin can have neurotoxic effects by interacting with the GABA_A receptor.^{30–33)} It is possible that some of these disorders share common biochemical pathways with the immunotoxic effects, which could only be demonstrated by a systems biology approach with microarrays.

3. Dioxin-like compounds

The aryl hydrocarbon receptor (AhR) belongs to a family of transcriptional factors known as bHLH-PAS (basic helix-loop-helix periodicity/ARNT/single-minded).³⁴⁾ Many of the known genes regulated by AhR, like CYP1A, are related to the metabolism of xenobiotic compounds.^{35,36)} AhR also regulates other genes involved in cellular proliferation (TGF- β , IL- β and PAI-2), cell cycle regulation (p27 and jun-B) and apoptosis (Bax).^{37–41)} Dioxins are a group of halogenated organic compounds, the most toxic of which is TCDD (2,3,7,8-tetrachloro dibenzene-*para*-dioxin). The compounds that have toxic effects similar to those of TCDD are known as dioxin-like compounds. Those effects include liver toxicity, embryonic toxicity, teratogenicity, immunotoxicity, dermal toxicity, lethality, and carcinogenesis and tumor induction in many species even at very low concentrations.^{42–44)} It has been shown that many of those toxic effects are through the AhR,^{45,46)} and the toxicity degree depends on the degree of binding between the compound and the AhR.⁴⁷⁾ AhR activation induces expression of some genes, like P4501A (CYP1A), a key enzyme in xenobiotic compound metabolism.

Table 1. Summary of the toxic effects of some OCPs found in the literature, including the species where they have been found, the dose, the technique used for the analysis, the reported effects and the reference.

Pesticide	Model	Dose	Technique	Effect	Reference
Dicofol	Carp (<i>Cyprinus carpio</i>)	100 μ M	Enzymatic activity assays	Activation of 20 β -HSD, and inhibition of 17 β -HSD, 5 α -Reductase and T-UGT	Thibaut and Porte, 2004 ⁸⁶⁾
Dieldrin	Mice cerebellar granule cells	0.2 μ M	Cl ⁻ flux	Interaction with GABA _A	Vale <i>et al.</i> , 2003 ³³⁾
Dieldrin	Human hepatoma cells	1 μ M	CALUX assay	AhR antagonist	Long <i>et al.</i> , 2003 ⁴⁹⁾
Dieldrin	Human cells	30–100 μ M	Caspase activity assay	Neurotoxin	Kanthasamy <i>et al.</i> , 2003 ²⁹⁾
Dieldrin	Largemouth bass (<i>Micropterus salmoides</i>)	0.2 μ g/g	Q-PCR, RIA	Effects on mRNA expression of Vtg, ERs, AR, StAR, CYP19, CYP1A and CYP3A; changes in plasma hormone levels.	Garcia-Reyero <i>et al.</i> , 2006 ⁷⁶⁾
Endosulfan	Human hepatoma cells	1 μ M	CALUX assay	AhR antagonist	Long <i>et al.</i> , 2003 ⁴⁹⁾
Endosulfan	Mice cerebellar granule cells	0.4 μ M	Cl ⁻ flux	Interaction with GABA _A	Vale <i>et al.</i> , 2003 ³³⁾
Endosulfan	Freshwater fish	10 mg/L	Flow cytometry	Immunotoxicity	Harford <i>et al.</i> , 2005 ¹⁹⁾
Endosulfan	Mice	3.8–15 mg/kg body weight /day	Enzymatic activity assays	Increased testosterone metabolism	Wilson and LeBlanc, 1998 ⁸⁵⁾
Lindane	MA-10 Leydig tumor cells	50 μ M	Northern blot /Western blot	Reduced StAR protein expression	Walsh and Stocco, 2000 ⁷⁴⁾
Lindane	Mice cerebellar granule cells	6 μ M	Cl ⁻ flux	Interaction with GABA _A	Vale <i>et al.</i> , 2003 ³³⁾
<i>o,p'</i> -DDE	Chinook salmon	10 ppm	Blastogenesis and surface immunoglobulin expression. Lysozyme activity.	Immunotoxicity	Milston <i>et al.</i> , 2003 ²⁰⁾
<i>o,p'</i> -DDE	Bottlenose dolphin	1–2 ng/g	Lymphocyte proliferation assay	Reduced immune response	Lahvis <i>et al.</i> , 1995 ²¹⁾
<i>p,p'</i> -DDE	Largemouth bass (<i>Micropterus salmoides</i>)	1–10 μ g/g	Q-PCR, RIA	Effects on mRNA expression of Vtg, ERs, AR, StAR, CYP19, CYP1A and CYP3A; changes in plasma hormone levels.	Garcia-Reyero <i>et al.</i> , 2006 ⁷⁶⁾
<i>p,p'</i> -DDE	Bottlenose dolphin	100–500 ng/g	Lymphocyte proliferation assay	Reduced immune response	Lahvis <i>et al.</i> , 1995 ²¹⁾
<i>p,p'</i> -DDE	Carp (<i>Cyprinus carpio</i>)	1 mM	Enzymatic activity assays	Activation of 20 α -HSD and 20 α -HSD	Thibaut and Porte, 2004 ⁸⁶⁾
<i>p,p'</i> -DDE	European common frog	10 mg/kg body mass	Q-PCR/ ELISA	Decreased CYP26 gene and protein expression	Leiva-Presa <i>et al.</i> , 2006 ¹⁰³⁾
<i>p,p'</i> -DDE	Largemouth bass	100 mg/kg	Gene arrays	Several gene expression changes	Larkin <i>et al.</i> , 2002 ⁸⁹⁾
<i>p,p'</i> -DDT	Bottlenose dolphin	10–20 ng/g	Lymphocyte proliferation assay	Reduced immune response	Lahvis <i>et al.</i> , 1995 ²¹⁾

CYP1A induction is used as a biomarker to detect fish exposure to dioxin-like compounds.⁴⁸⁾ Recently, some pesticides have been reported to act as dioxin-like compounds. Long *et al.*⁴⁹⁾ analyzed the AhR antagonistic effects of dieldrin and endosulfan on human or rat hepatoma cell lines.

4. Endocrine-disrupting effects

In vertebrates, normal reproduction is controlled by the hypothalamic-pituitary gonadal (HPG) axis through a cascade of hormones whose concentrations are tightly regulated by hormonal action and feedback control mechanisms. Any chemical compound that alters the concentration of endogenous hormones or mimics their action by binding or interfering with the binding of hormones to their natural receptors can disrupt this process. Those compounds are known as endocrine-disrupting compounds (EDCs). Transient or permanent disruption in normal reproductive function has been linked to chemicals that interrupt normal functioning of the endocrine system, but the mechanisms involved are still unclear.^{13,15,50–52)} There are three main pathways of endocrine disruption: (1) the interaction of EDCs with the hormone receptors; (2) the alteration of processes involved in steroid synthesis and (3) the alteration of processes involved in sex steroid metabolism. All these pathways are very important for the long-term sex hormone homeostasis, as well as proper development and reproduction in fish.

(1) Receptor-mediated effects

EDCs can interact with sex hormone receptors, such as the estrogen receptors (ERs), the androgen receptor (AR), or other sex hormone receptors, by acting as agonists or antagonists. Some can also change their mode of action depending on their concentration.⁵³⁾ By interacting with the hormone receptors, EDCs can disrupt the receptor's programmed gene activation cascade by inducing or inhibiting gene expression at inappropriate times or levels. For instance, exposure of fish to estrogenic compounds increases the expression of vitellogenin (Vtg), the egg-yolk precursor protein, and ER α in the liver of both sexes through the ER-mediated signaling pathway.^{54–56)} It has been shown that fish living in waters polluted with considerable amounts of estrogenic compounds can present different levels of feminization, from the induction of Vtg synthesis to the finding of intersex fish, that is fish with gonads containing both female and male gonadal tissue.^{57–62)} Liney *et al.*⁶⁰⁾ exposed roach (*Rutilus rutilus*) to effluents from wastewater treatment works at two different life stages, either as embryos from fertilization up to 300 days posthatch or as postspawning adult males. They found a correlation between Vtg synthesis at both life stages and the effluent concentration of estrogens. In addition, feminization of male gonads occurred also in a concentration-dependent manner with estrogenic compounds, although only if exposure occurred in early life stages.

Some OCPs can interact with estrogen or androgen receptors, and can interfere in many physiological processes

through different receptor signaling pathways. In fish, *o,p'*-DDE has been found to be able to bind to the ER.^{63–65)} Methoxychlor, dieldrin and endosulfan are also able to bind to the ER in fish.^{65,66)} OCPs can also bind to the AR, as has been shown in several species, and work mostly as antagonists preventing the transcription of androgen-regulated genes (*e.g.*, prostatein subunit C3 in rats) resulting in demasculinization.^{67–70)}

Environmental pollutants can also disrupt the endocrine system by altering hormone homeostasis, and that can happen when they affect either hormone synthesis or hormone metabolism.

(2) Steroid hormones synthesis

The first and rate-limiting step in steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane by StAR (steroidogenic acute regulatory protein).^{71–73)} Through a series of enzymatic reactions, cholesterol is converted into testosterone, which can be then converted either to estradiol (E₂) by the enzyme aromatase (CYP19) or to 11-ketotestosterone (11-KT), the major androgen in fish, by the enzyme cytochrome P450 11 β hydrolase (CYP11 β). Each one of these steps can be affected by EDCs. For example, StAR has been shown to be affected by several pesticides. Walsh and Stocco⁷⁴⁾ showed that lindane (γ -hexachlorocyclohexane) exposure reduced StAR protein expression in mouse Leydig cells. Also, the OCPs *p,p'*-DDE and dieldrin increased StAR mRNA expression in largemouth bass (*Micropterus salmoides*).^{75,76)}

(3) Steroid hormones metabolism

The metabolism of steroids occurs by P450 mediated reactions. CYP1A and CYP3A have been shown to metabolize endogenous steroids and detoxify drugs and environmental chemicals.^{77–83)} The liver is the main site for steroid metabolism, although some activity can also be found in other tissues.⁸⁴⁾ Many studies have shown that compounds that affect the synthesis or metabolism of steroids are responsible for changes in circulating steroid levels. Wilson and LeBlanc⁸⁵⁾ reported that endosulfan was able to induce hepatic CYP-dependent testosterone metabolism and increased the urinary elimination of testosterone in female mice. Exposure of largemouth bass to either *p,p'*-DDE or dieldrin resulted in an increased expression of CYP3A as well as a decrease of CYP1A mRNA, leading to an overall decrease of E₂ and 11-KT levels in plasma in the dieldrin treatment, whereas in the *p,p'*-DDE treatment there was an increase of 11-KT levels and a decrease of E₂ levels in the females.⁷⁶⁾ Thibaut and Porte⁸⁶⁾ analyzed the interactions of estrogenic (dicofol) and anti-androgenic (*p,p'*-DDE) compounds with key enzymatic activities involved in both synthesis (17 β -HSD, 5 α -reductase, 20 α / β -HSD) and metabolism (E₂-UGT, E₂-SULT, T-UGT) of sex hormones. 17 β -HSD (hydroxysteroid dehydrogenase) is involved in the conversion of androstenedione to testosterone, while 5 α -reductase is involved in the conversion of testosterone to 5 α -dihydrotestosterone. 20 α and 20 β -HSD are in-

volved in the ovarian synthesis of maturation-inducing hormones. On the other hand, E₂-UGT, E₂-SULT and T-UGT (UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase) are involved on the sulfation and glucuronidation of testosterone and E₂, two important routes of clearance of active hormones. Dicofol activated 20 β -HSD, and inhibited 17 β -HSD, 5 α -reductase and T-UGT. *p,p'*-DDE activated 20 α -HSD and 20 β -HSD, but did not affect the enzymes associated with metabolism.

Genomic Technologies

Exposure of an organism to chemical stress, or for that matter any environmental stress, will be translated into a series of changes in gene expression, either as a direct (*e.g.*, interaction with hormone receptors) or indirect (*e.g.*, compensation) mechanism.⁸⁷⁾ The challenge of ecotoxicology is to define the gene expression pattern induced by a certain contaminant, known as the 'compound signature,' in order to be able to analyze and predict wildlife exposure to that compound. That information would subsequently be extremely useful for ecological risk assessment.

An increasing number of studies are using genomic technologies in fish ecotoxicology. The ultimate goal of the genomics approach is to link changes in gene expression with effects observed in fish. That means the "omics" approaches should be linked to physiological responses and tissue structure changes. This has been termed 'phenotypic anchoring' by the National Center for Toxicogenomics at NIEHS.⁸⁸⁾ Real-time PCR and microarrays are two transcriptomics technologies that have been repeatedly proven to be extremely useful in the study of fish ecotoxicology.^{75,76,89-93)}

1. Real-time PCR

Polymerase chain reaction (PCR) is a method that allows the logarithmic amplification of short DNA sequences. Quantitative PCR (Q-PCR) is probably the easiest and most accurate method to analyze and validate differential gene transcription. In this method, the PCR data is analyzed in real time through the logarithmic expansion phase of the reaction. Primer pairs specific for the gene of interest are designed using specific software (*e.g.*, Primer Express, from Applied Biosystems) such that they will amplify short amplicons of less than 150 nucleotides. There are two methods by which one can follow the amplification: SYBR Green and Taqman. SYBR Green is a dye that fluoresces only when bound to double-stranded DNA, whereas the TaqMan probe is an oligonucleotide that is complementary to a sequence within the amplicon, which contains a fluorescent reporter dye molecule on one end and a quencher dye on the other end. When the two dye molecules are close together, there is little fluorescence; however, this changes when the molecules are separated from each other, as happens from the 5' nuclease activity of the DNA polymerase.

To analyze the results obtained from Q-PCR, one can use

either relative or absolute quantification. Standard curves are used for absolute quantification, as well as to check the efficiency of PCR. Absolute quantification requires a standard curve that is constructed with a plasmid that contains the cloned PCR product, or RNA that is synthesized and quantified from a plasmid. The standard curve is generated by plotting the threshold values (first significant increase in the amount of PCR product) determined from the PCR amplification *versus* the log of the concentration of the standard. The slope of the standard curve should be -3.30 to ensure that doubling occurred in every cycle. It is extremely important to ensure good quality of the starting RNA and to add a $-RT$ control (untranscribed RNA) to make sure there was no DNA contamination.

Relative quantification allows us to analyze the fold change between samples. A widely used method to analyze fold changes in gene expression between treated and untreated samples is the Comparative Threshold Method (or $\Delta\Delta C_t$ method), where the results are normalized to a control gene. The control gene has to be a highly abundant and constantly expressed gene. The most commonly used are 18S ribosomal RNA or housekeeping genes like β -actin.

To understand the results obtained from Q-PCR, we are going to look closer to an example of fish treated with the OCPs. Largemouth bass (*Micropterus salmoides*) were exposed subchronically through the diet to either *p,p'*-DDE or dieldrin for 120 days.⁷⁶⁾ Both compounds have been described as weak estrogens and weak androgens by a variety of *in vivo* and *in vitro* assays.^{67,94-102)} In order to elucidate if the pesticides were acting as an estrogenic compound through the receptor-mediated pathway, we analyzed the expression of Vtg and ER α in the liver. Figure 1(a) shows a typical Q-PCR result for quantitation of Vtg in females and males, either untreated or treated with *p,p'*-DDE. We can see the differences among both sexes and both treatments. Females treated with *p,p'*-DDE express the most Vtg, as their amplification curve starts earlier. Untreated females have the second highest initial Vtg concentration. The difference we see between treated and untreated females shows that *p,p'*-DDE is activating Vtg mRNA expression. We also find this activation in males treated with *p,p'*-DDE.

Fig. 1(b) shows the relative quantities of Vtg and ER α obtained by the $\Delta\Delta C_t$ method for the liver of female and male largemouth bass treated with *p,p'*-DDE and dieldrin. The treatment with *p,p'*-DDE increased the expression of Vtg and ER α in both females and males, confirming that *p,p'*-DDE is acting as an estrogenic compound through the ER-mediated signaling pathway.^{55,56)} Dieldrin, on the other hand, acted as a very weak estrogen, but only in females, where Vtg was up regulated, but there were no significant effects on ER α . Typically, ER α is the more sensitive gene for estrogens. The fact that it was not up regulated in females suggested that dieldrin might be acting through a different pathway than E₂.

Fig. 1. a) Q-PCR graph showing the results for the mRNA expression levels of Vtg in liver from female and male largemouth bass treated with p,p' -DDE (45.9 $\mu\text{g/g}$). Females treated with p,p' -DDE show the highest Vtg expression, followed by untreated females, males treated with p,p' -DDE and, finally, untreated males, which show the lowest expression levels. These results indicate that p,p' -DDE is activating Vtg mRNA expression in both females and males. b) Changes in gene expression for Vtg and $\text{ER}\alpha$ in the liver of female and male largemouth bass treated with either p,p' -DDE (45.9 $\mu\text{g/g}$) or dieldrin (0.81 $\mu\text{g/g}$). Results are plotted as $\log_2 \Delta\Delta\text{Ct}$, in order to see both the up regulation and the down regulation. Following p,p' -DDE treatment, Vtg and $\text{ER}\alpha$ are up regulated in both females and males, with the higher up regulation for $\text{ER}\alpha$ in females (>32 fold). Following dieldrin treatment, Vtg is up regulated (>8 fold) in females and down regulated in males (2 fold). $\text{ER}\alpha$ only changes in males, where it is down regulated by 4 fold.

In another example, Leiva-Presa *et al.*¹⁰³⁾ analyzed the role of p,p' -DDE as a retinoid (vitamin A) disrupter in European common frog (*Rana temporaria*). One of the main systems of reproduction and immune function control in adult amphibian are vitamins and especially vitamin A. Retinoids are metabolized by the cytochrome P450 system, mainly by CYP26.¹⁰⁴⁾ Exposure of European common frog to p,p' -DDE decreased CYP26 mRNA expression in the liver, as well as hepatic CYP26 protein levels, resulting in an increase of liver retinol concentration.

2. Microarrays

A DNA array consists of a large number of either cDNAs or oligonucleotides organized on a glass slide (microarray) or a nitrocellulose membrane (macroarray). mRNA from the tissues or cells of interest is converted to cDNA and labeled with either fluorescent dyes or radioactive nucleotides and hybridized to the DNA to obtain a semi-quantitative measure of expression for each of the genes present on the array. The hybridization can be performed with one single fluorescently labeled sample (Affymetrix type), or with a competitive para-

dig where the two samples to be compared are labeled with two different dyes (Agilent type). The use of a reference sample design allows the comparison of treated and untreated samples to the same reference.

Microarrays allow one to analyze gene expression from hundreds to thousands of genes at a time, generating a huge amount of data that has to be carefully analyzed. While the technique is not very hard, the enormous number of data points that can be generated makes the bioinformatics and statistical analysis involved with the array data increasingly important. For statistical significance at least four biological replicates are required. Data has to be filtered, transformed, normalized, and then analyzed. The data filtering includes the elimination from the dataset of features that exhibit artifacts and those detected at or below background level under all conditions. Also, background subtraction is usually applied across the dataset. Typically, the data is $\log_2$ transformed, but other transformations like the shift-log transformation, curve fitting or variance stabilizing transformations can also be used (reviewed by Cui *et al.*¹⁰⁵⁾). The shift-log transformation¹⁰⁶⁾ adjusts log ratios by adding a constant to the signal values of one channel and subtracting it from the other channel prior to

the logarithmic transformation in order to minimize the curvature-causing background differences. The most common curve fitting transformation is the Lowess method,¹⁰⁷⁾ with a freely available implementation in the statistical software package R.¹⁰⁸⁾ The Lowess method performs robust locally linear fits. In particular, it will not be affected by a small percentage of differentially expressed genes, which will appear as outliers.¹⁰⁹⁾ When the assumption of uniform variance is needed, it is common to use a transformation of the data that stabilizes the variance, like the *Arsinh* or the *Linlog* transformation. The *Arsinh* transformation¹¹⁰⁾ is based on the assumption of a quadratic relationship between variance and intensity of microarray signals at the original scale. The *Linlog* transformation¹⁰⁵⁾ combines the linear and logarithmic transformations through a smooth transition to take advantage of both.

The normalization will compensate for any difference in RNA preparation, labeling, probe purification, and hybridization. Some commonly used forms of normalization are the variance normalization or the normalization to the sum of squares. There are several methods of analysis. We can perform an unsupervised analysis in order to identify an expression profile in non-predefined subsets of samples. Among unsupervised analyses, we find the Principal Component Analysis (PCA),¹¹¹⁾ and various forms of clustering analyses.^{112,113)} The PCA is designed to analyze the variance in a dataset in terms of principal components, reducing the dimensionality of the data to find the most influential components. PCA can be used on datasets to capture the cluster structure prior to clustering analysis. The clustering analysis allows the identification of higher order relationships among the genes by grouping genes that exhibit similar expression patterns, assuming that those similar patterns are likely to be functionally related. Some of the most used clustering algorithms are hierarchical clustering,¹¹²⁾ self-organized maps (SOMs),¹¹⁴⁾ support vector machine,¹¹⁵⁾ *k*-means,¹¹⁶⁾ and Bayesian statistics.¹¹⁷⁾

The supervised analyses allow us to identify characteristic expression profiles from predefined subsets of samples. Among those, one would use statistical tests like *t*-tests, *S*-test, *f*-tests or ANOVA. The *t*-test is a simple method for detecting differentially expressed genes between paired conditions. The *S*-test is a version of the *t*-test included in the Significance Analysis of Microarrays (SAM)¹¹⁸⁾ that adds a small positive constant to the denominator of the gene-specific *t*-test. This modification removes the *t*-test stability problem due to a lack of consistency in the variance of the genes, as the genes with small fold changes will not be selected as significant (reviewed by Cui and Churchill¹¹⁹⁾). The *f*-tests are generalized *t*-tests that allow the comparison of more than two samples. ANOVA is used to perform intra and inter-group comparisons. Once the statistical methods have been applied, the statistical significance needs to be determined. There are several methods to analyze the statistical significance: nominal *p*-values, family-wise error-rate control and false-discov-

ery-rate control (as reviewed by Cui and Churchill¹¹⁹⁾).

3. Use of microarrays in fish ecotoxicology

Microarray technology is relatively new for the aquatic toxicology field, but there are already some research groups that have been working on developing and using them. As we have not found many studies focusing on OCPs, we include a few examples of how microarrays can be used for fish ecotoxicology related to different aspects or compounds. Larkin *et al.*^{89,90)} exposed largemouth bass (*Micropterus salmoides*) to estradiol, nonylphenol (NP) and *p,p'*-DDE. They used a membrane macroarray to compare the effects of the two contaminants to estradiol, concluding that they had similar, but not identical, genetic signatures. There were a total of 28 differentially expressed genes. For instance, transferrin was down regulated by E₂, NP and *p,p'*-DDE in females, but was not affected in males treated with *p,p'*-DDE. On the other hand, vitellogenin 2A was up regulated by E₂ and NP, down regulated by *p,p'*-DDE in females, and was not affected by *p,p'*-DDE in males.

Williams *et al.*⁹¹⁾ used a cDNA microarray to study toxic stress response from European flounder (*Platichthys flesus*) caught in the much polluted Tyne estuary (UK) compared to fish from the relatively unpolluted Alde estuary (UK). Seven transcripts were found to be significantly higher in the Tyne male fish: CYP1A, UDPGT, α -HSD-glycoprotein, dihydropyrimidine dehydrogenase, Cu/Zn SOD, aldehyde dehydrogenase and paraxonase. On the other hand, four transcripts were found to be significantly less abundant in the Tyne male fish: elongation factor 1 (EF1), EF2, Int-6 and complement component 3. Selected genes were assayed by real-time PCR and then normalized to α -tubulin. These assays confirmed the significance of the array results for CYP1A, UDPGT and EF1. Q-PCR is a very useful and widely used method for the verification of microarrays results.

In another example, Hook *et al.*¹²⁰⁾ exposed rainbow trout (*Oncorhynchus mykiss*) to a series of model toxicants with varying modes of action, including ethynylestradiol (xeno-estrogen), 2,2,4,4'-tetrabromodiphenyl ether (BDE-47, thyroid active), the aquatic herbicide diquat (oxidant stressor), chromium VI, and benzo[*a*]pyrene (BaP) for a period of 1–3 weeks. They found that each exposure caused between 64 and 222 genes to be significantly altered. Most of those genes responded to only one of the toxicants and relatively few were co-expressed in multiple treatments. For example, BaP and Diquat, both of which exert toxicity *via* oxidative stress, up regulated 28 of the same genes. Their results suggested a link between gene expression profiles and mode of toxicity. Also, their array results showed good agreement with Q-PCR results, demonstrating that the arrays were an accurate measure of gene expression.

Oleksiak *et al.*¹²¹⁾ used microarrays to address how individual variation affected cardiac metabolism and related this to changes in patterns of mRNA expression in *Fundulus hetero-*

clitus. They found extensive variation in both cardiac metabolism and the expression of metabolic genes among individuals from natural out bred populations raised in a common environment. Metabolism differed among individuals by a factor of more than 2, and expression levels of 94% of genes were significantly different ($P < 0.01$) between individuals in a population. This high variation in metabolic gene expression explained much of the variation in metabolism, suggesting that it was biologically relevant. The patterns of gene expression that were most important in explaining cardiac metabolism differed between groups of individuals. Nevertheless, the magnitude of differences in gene expression in those groups was not important: large changes in expression had no greater predictive value than small changes. In conclusion, their data suggested that variation in physiological performance was related to the subtle variation in gene expression and that this relationship differed among individuals.

In another study, van der Meer *et al.*¹²²⁾ used microarrays to analyze the long-term adaptive responses to hypoxia in adult zebrafish (*Danio rerio*) that survived severe hypoxic conditions for three weeks and showed adaptive behavioral and phenotypic changes. They identified 367 differentially expressed genes in the gills of zebrafish of which 117 showed hypoxia-induced and 250 hypoxia-reduced expressions. They observed enhanced expression of the monocarboxylate transporter and of the oxygen transporter, myoglobin. The hypoxia-induced group also included the genes for Niemann-Pick C disease and for Wolman disease, both of which lead to a similar intra- and extracellular accumulation of cholesterol and glycolipids. Their data suggested a novel adaptive mechanism to hypoxia, the induction of genes for lysosomal lipid trafficking and degradation.

In another example, Tilton *et al.*¹²³⁾ analyzed the toxicogenomic profiling of the hepatic tumor promoters indole-3-carbinol (I3C), 17 β -estradiol (E_2) and β -naphthoflavone (BNF) in rainbow trout (*Oncorhynchus mykiss*). They examined hepatic gene expression profiles after dietary exposure to I3C and 3,3'-diindolylmethane (DIM), a major *in vivo* product of I3C, and compared them to the transcriptional signatures of two model hepatic tumor promoters: E_2 and BNF. They demonstrated that I3C and DIM acted similarly to E_2 at the transcriptional level based on correlation analysis of expression profiles and clustering of gene responses. Of the genes regulated by E_2 , most genes were regulated similarly by DIM (87–92%) and I3C (71%), suggesting a common mechanism of action. Some of the upregulated genes were associated with signaling pathways for cell growth and proliferation, vitellogenesis, and protein folding, stability, and transport. Other genes including those involved in acute-phase immune response were downregulated by E_2 , and also by DIM and I3C. They confirmed gene regulation by Q-PCR and Western blot. Their data indicated that I3C promoted hepatocarcinogenesis through estrogenic mechanisms in trout liver and suggested that DIM might be an even more potent hepatic

tumor promoter than the parent compound in rainbow trout.

Conclusions

In conclusion, genomic technologies can be extremely useful in the study of fish ecotoxicology. Gene expression changes are likely to be the initial response to toxic exposure, and different types of stressors might be affecting different genes and/or pathways. Genomics can help in the characterization of genes/pathways affected by a type of stressor, even to the level of 'compound signature.'

Although genomics technologies are very useful, they do have some very important limitations. One of the most important disadvantages of microarrays is the very expensive price, not only of the slide itself, but also of all the needed reagents. In addition, an experiment using microarrays will need a minimum of four slides per condition, in order to get significant results, increasing the price of the experiment enormously. However, it is likely that these costs will come down in the future as the technology becomes incorporated more generally into various laboratories.

For microarrays to be used in ecotoxicology, it will be important to find robust biomarkers that relate to contaminant exposure. This will undoubtedly occur, as has already been demonstrated by several studies.^{89–91,120–123)} However, this is made difficult by the high variability of gene expression both intra- and interspecies. For instance, gene expression may vary by tissue, reproductive status, season, exposure time, dose or sex. Furthermore, a compound might affect different species in different ways. Those complexities of gene expression analyses should be taken into account when analyzing sub-lethal toxicity of compounds, emphasizing the impor-

Fig. 2. The 'omics' paradigm is a diagram showing the integration of the different organizational levels (molecular and cellular, organism, population and ecosystem) into the 'omics' technologies. The intersections between the different levels show the classical approaches. The diagram shows that while the 'omics' technologies can be used to study all levels, any factor affecting any of the different levels will also affect the results. That makes the 'omics' technologies both very powerful, in the sense that they allow the acquisition of an enormous amount of information that cannot be obtained otherwise; and also fragile in the sense that 'omics' technologies are very susceptible to changes at any level.

tance of robust experimental designs.

Figure 2 explains what we called the 'omics' paradigm, which is a diagram showing the integration of the different organization levels (molecular and cellular, organism, population and ecosystem) into the 'omics' technologies. The diagram shows that while the 'omics' technologies can be used to study all levels, any factor affecting any of the different levels will also affect the results. That makes the 'omics' technologies both very powerful, in the sense that they allow the acquisition of an enormous amount of information that cannot be obtained otherwise; but also fragile in the sense that 'omics' technologies are very susceptible to changes at any level.

The large amount of existing and new chemical compounds that are either known or suspected to be toxic, as well as the enormous number of combinations that can be found in the environment, makes it impossible to analyze them one by one. That makes it imperative to use genomic technologies to find some additional biomarkers, or gene expression patterns, which correlate with their possible toxic effects. Genomic technologies are state-of-the-art methods to understand the effects of pollutants on wildlife, in order to monitor their use and continue proper environmental policies.

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