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Genes and environment — Striking the fine balance between sophisticated biomonitoring and true functional environmental genomics

Christian E.W. Steinberg[a,*], *Stephen R. Stürzenbaum*[b], *Ralph Menzel*[a]

[a]*Humboldt University, Institute of Biology, Laboratory of Freshwater & Stress Ecology, Arboretum, Späthstraße 80/81, 12437 Berlin, Germany*
[b]*School of Biomedical & Health Sciences, Pharmaceutical Sciences Division, King's College London, 150 Stamford Street, London SE1 9NH, United Kingdom*

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ABSTRACT

This article provides an overview how the application of the gene profiling (mainly via microarray technology) can be used in different organisms to address issues of environmental importance. Only recently, environmental sciences, including ecotoxicology, and molecular biology have started to mutually fertilize each other. This conceptual blend has enabled the identification of the interaction between molecular events and whole animal and population responses. Likewise, striking the fine balance between biomonitoring and functional environmental genomics will allow legislative and administrative measures to be based on a more robust platform. The application of DNA microarrays to ecotoxicogenomics links ecotoxicological effects of exposure with expression profiles of several thousand genes. The gene expression profiles are altered during toxicity, as either a direct or indirect result of toxicant exposure and the comparison of numerous specific expression profiles facilitates the differentiation between intoxication and true responses to environmental stressors. Furthermore, the application of microarrays provides the means to identify complex pathways and strategies that an exposed organism applies in response to environmental stressors. This review will present evidence that the widespread phenomenon of hormesis has a genetic basis that goes beyond an adaptive response. Some more practical advantages emerge: the toxicological assessment of complex mixtures, such as effluents or sediments, as well as drugs seems feasible, especially when classical ecotoxicological tests have failed. The review of available information demonstrates the advantages of microarray application to environmental issues spanning from bacteria, over algae and spermatophytes, to invertebrates (nematode *Caenorhabditis elegans*, crustacea *Daphnia* spp., earthworms), and various fish species. Microarrays have also highlighted why populations of a given species respond differently to similar contaminations. Furthermore, this review points at inherent limits of microarrays which may not yet have been properly addressed, namely epigenetics, which may explain heritable variation observed in natural population that cannot be explained by differences in the DNA sequence. Finally, the review will address promising future molecular biological developments which may supersede the microarray technique.

* *Corresponding author*. Tel.: +49 30 6322 4715; fax: +49 30 6369 446.
E-mail address: christian_ew_steinberg@web.de (C.E.W. Steinberg).

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1. Introduction

The '-omics' trend has found its way into environmental sciences which has resulted in a reciprocal fertilization of two highly contrasting disciplines. The disparities derive not only from the intrinsic differences in scale, but also from their paradigmatic backgrounds and practical approaches. On the one hand, the machinery and conceptional approaches of molecular biology become, when merged with ecotoxicology, visible at the individual, community or even population level. One the other hand, the interactions of organisms with their environment can be traced to the molecular level. This paper examines how the application of molecular biological tools, such as microarrays, can be used to study issues of environmental importance, with a strong focus on non-human organisms exposed to chemical stressors. This approach is of critical importance to identify major and minor pathways of toxic action and decipher what drives the interaction between environment and the organism (and vice versa). By doing so, the molecular biological approach will undoubtedly provide a robust platform for legislative and administrative purposes.

Environmentalists traditionally deal with the protection and casual restoration of landscapes, rivers, lakes, and the sea. One may ask if it is necessary to understand the molecular basis of, for instance, an endocrine disrupting chemical, if the adverse effect in the impacted population has already been established. It appears self-evident that these chemicals do not belong in the environment and, *per se*, should be abolished in the first place. Although this so called 'precautionary principle' builds on an ethic, convivialistic rather than a scientific base, several environmental regulations incorporate it. Furthermore, the traditional ecotoxicological approach is more chemical compound, rather than mechanisms orientated and considers organisms in their environment as somewhat sophisticated monitors of chemical burdens and effects. It may be trivial, but it is certainly worth mentioning: The presence of natural endogenic and exogenic chemical stressors have been instrumental for, and in fact have driven, the development of stress defense systems, such as the antioxidant or biotransformation systems, expression of stress proteins or metal-binding proteins. Consequently, anthropogenic chemical stress, though sometimes severe or even lethal, is one of several stressors that impacts on organisms. It therefore may be argued that the use of gene expression experiments in environmental studies is only another fashionable and sophisticated means to identify potentially adverse effect of chemicals in the environment, not unlike a set of highly developed biomarkers.

According to Selye (1936, amended by various authors), a stress response includes three different phases: the bipartite alarm phase, the resistance phase, and the exhaustion phase (Fig. 1). The alarm phase corresponds to modifications of biochemical and genetic parameters in the absence of reduced vital activities and growth. These physiological reactions terminate a primary disturbance and enable restitution. An exposure that is too strong and/or fast will result in acute damage and cell death. The resistance phase is characterized by the activation of defense mechanisms (e.g. antioxidant defense, protein repair, biotransformation) that are concomitant with first signs of reduced vital activity and growth. The exhaustion phase becomes apparent by a collapse of vital cellular functions (e.g. photosynthesis, membrane integrity, reproduction), leading to chronic damage and ultimately death. Especially at the molecular level, the differences between chronic vs. acute effects as well as low- vs. high-concentration exposures are typically neglected. We assume that specific differentially expressed genes can be used to characterize and distinguish between the three phases of stress and possibly the differentiation between natural and anthropogenic stressors (see section 2).

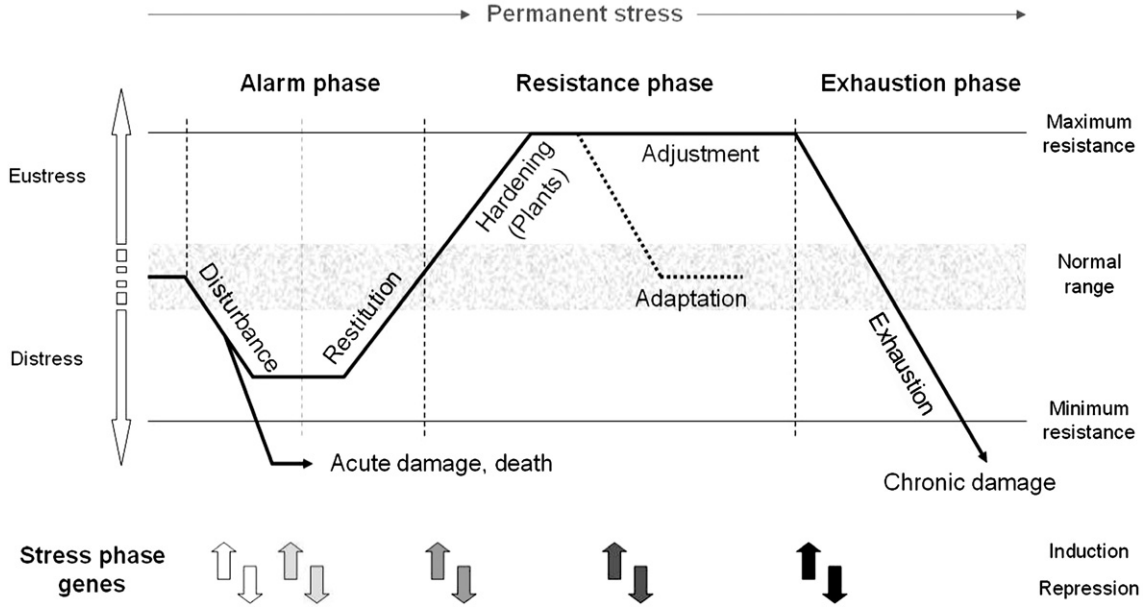

Fig. 1 – Stress phase model based on Selye (1936) and amended by several authors. Shades of grey of arrows represent different genes specifically expressed during the individual stress phases. Note, the gene profiles in the various stress phases are unique, even when exposed to the same stressor at a different intensity (see Section 2).

In recent years, DNA array technology has been applied to explore changes in gene expression profiles following exposure to environmental pollutants and natural chemical stressors. This allows a better understanding of whole genome expression responses to chemical stress and, in turn, aids in the identification of ecological and toxicological modes of action. The terms 'ecogenomics' (Chapman, 2001) and 'ecotoxicogenomics' (Snape et al., 2004) in their broadest sense encompass not only transcript profiling (*transcriptomics*) but also determine protein composition (*proteomics*) and metabolic constituents (*metabolomics*). The purpose of ecotoxicogenomics is to provide an insight into the physiological status of organisms and decipher responses and interactions of organisms to the environment and to one another. More pragmatic, ecotoxicogenomics is an approach that identifies gene classes which are switched on or off upon exposure and, hence, decrypts how organisms cope with complex environmental stressors. Transcription is the initial step in gene expression, thus, a transcriptional response can give an indication of cellular mechanisms that are affected by a pollutant and in consequence provide a sensitive starting point to assess ecological and toxicological (= ecotoxicological) responses. However, each application depends on basic assumptions (Oberemm et al., 2005), including that all ecotoxicologically relevant effects are indeed accompanied by alterations in gene expression profiles. Likewise, the specific expression profiles induced by a chemical stress is expected to be similar for all chemical compounds which act in a similar toxic mode of action and thus can be used to categorize any kind of individual chemicals as well as mixtures of chemicals into different modes of action. This notion forms the basis for predictive ecotoxicology. Several studies have validated classification systems (Burczynski et al., 2000) and large amounts of gene expression data are accumulating in dedicated databases (Waters et al., 2003).

Gene expression profiles obtained by DNA microarrays are also believed to provide a more comprehensive, sensitive and characteristic insight into toxicity than typical toxicological parameters such as morphological changes, altered reproductive capacity or mortality (Hamadeh et al., 2001; Menzel et al., 2005a). In addition to these classical ecotoxicological parameters, ecotoxicogenomics is a powerful tool that unravels mechanistic processes, reveals novel modes of action, and provides the opportunity to get a dynamic picture of biological systems and the ability to comprehensively dissect different states of biological activities in cells, tissues or whole organisms. Finally, ecotoxicogenomics aims to develop new predictive models for identifying environmental or human health hazards and precise and fast molecular biomarkers of exposure to natural as well as man-made chemical stress.

This review will focus on environmental stress-mediated gene expression profiles from selected bacteria, plants, invertebrates, and fish with a strong preference on methology driven papers. Incorporating the evidence of a genetic basis of hormesis, this paper attempts to answer if (i) all responses to environmental stressors have an underlying genetic basis and (ii) functional environmental genomics is more than just a sophisticated means for biomonitoring the effect of chemicals.

2. Genetic basis of hormesis

In many instances, the dose–response of exposed organisms does not obey a linear, but biphasic, relationship. Hormesis describes this biphasic dose–response, where a low-dose stimulation or beneficial effect precedes a high-dose inhibitory or toxic effect. Numberless case studies display biphasic responses of cells or organisms to chemicals or changing environmental conditions and numerous meta-analyses have been published (e.g., Calabrese, 2005; Calabrese and Baldwin, 2001) describing hormetic dose–response curves. While not all toxic factors may induce a biphasic dose–response in cells and organisms (e.g., Weltje et al., 2005 who convincingly show this for xenoestrogens), many clearly do. The underlying mechanisms have been described as an adaptive compensatory response following an initial disruption in homeostasis. However, the term 'adaptive response' implies that low- and high-dose exposures activate more or less identical defense pathways and, furthermore, that the low-dose exposure trains the defense systems for future adverse, high-dose exposures. To date, this explanation remains descriptive, but differential gene expression analysis appears to be a promising method to substantiate this notion. Indeed, there is an increasing body of evidence that the gene expression profiles of low-dose exposures differ from higher doses.

Evidence comes from studies on radiation (Ding et al., 2005; Sokolov et al., 2006), gravity (Allen et al., 2007), or toxic chemicals. For instance, Toyoshiba et al. (2006) analyzed low- and high-dose effects of acetaminophen (paracetamol) administered to rats. The authors identified two gene interaction networks clearly segregated by the two doses: at lower doses, oxidative stress signaling pathways did not interact with the apoptosis-related genes, but did in the higher doses. In a more environmentally relevant example, Gong et al. (2007) show a hormetic increase in reproduction when the earthworm *Eisenia fetida* was exposed to trinitrotoluene. This effect was mirrored by transcriptional responses specific to the low-dose exposure.

These examples indicate that hormesis is not limited to a simple adaptive response but accompanied by a transcriptional activation of different pathways. Future studies should aim at deciphering the precise profiles and networks that drive hormesis rather than extend the existing catalog of the hormetic phenomena.

3. Stress-related gene expression profiles in bacteria

One illustrative example is *Escherichia coli* exposed to seawater. Any enteric bacterium, including *E. coli*, is challenged by a combination of hostile conditions threatening their viability, such as pH, salinity, radiation, oxidative stress, etc. Nevertheless *E. coli* and other enteric bacteria can survive in seawater for extended periods which can only be achieved via the mobilization of diverse defense mechanisms on several physiological and molecular levels (Rozen et al., 2002). Microarray experiments conducted on *E. coli* exposed to seawater demonstrated that the expression of the majority

of genes remained unchanged with less than 10% of the genes down-regulated and 25% up-regulated. In detail, Gene Ontology analysis identified that (1) Cell division and the synthesis of nucleic acid components had stopped; (2) Carbon and energy metabolism was modulated; (3) Systems needed for energy supply were induced, including both aerobic and anaerobic respiration; (4) Cells were geared towards rapid movement (whether in a chemotactic search for nutrition or in flight from the inhospitable conditions imposed upon them) (Rozen et al., 2002).

4. Stress-related gene expression profiles in plants

Most gene expression studies in plants deal with basic physiological processes, such as nutrient deficiency or excess. Since exposure to heavy metals is a severe stress to all plants studies have recently emerged on this issue, covering taxa from a 'simple' green alga to spermatophytes.

4.1. *Stress phase gene markers in the green alga, Chlamydomonas reinhardtii, under copper excess*

Copper is an essential micronutrient for plants and algae. However, in excess, Cu^{2+} can displace endogenous metal cofactors from their cellular binding sites. Cu-induced reactive oxygen species (ROS), which are able to oxidize a large variety of biological macromolecules, are known to provoke cell death either by necrosis or programmed cell death (Mittler, 2002). Plants have developed efficient defense mechanisms to prevent the formation of ROS and to repair the damaged components. Cu-induced ROS has been shown to be eliminated via the induction of several antioxidants (for more details, see Luis et al., 2006). In a study with *C. reinhardtii*, Luis et al. (2006) aimed to identify changes in gene expression that coincide with the "alarm", "resistance", and "exhaustion" phase as defined by the previously mentioned stress concept (Fig. 1). To identify gene transcript profiles as hallmark features for each of these phases, Luis et al. monitored: (1) culture growth rate and cell size changes, together with the regulation of the cyclin-dependent protein kinase (*cdk*) gene, an important gene for the cell cycle regulation (John et al., 1989); (2) expression of genes coding for photosystem proteins (e.g. *psbA* for photosystem II and *psaA* for photosystem I) and the ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (*rbcL*) concomitantly with the photosynthetic efficiency and capacity; (3) superoxide dismutase (SOD) and catalase (CAT) activities, together with the corresponding gene expression profiles; and (4) α-tocopherol contents and lipid peroxidation levels together with the transcript abundance of the gene *VTE3* coding for one of the enzymes responsible for the α-tocopherol synthesis.

The stromal photosynthetic functions were shown to be more sensitive to ROS than the membrane-located reactions. While the up-regulation of *rbcL* was able to counteract the damage induced by low levels of Cu, the transcriptional up-regulation of α-tocopherol biosynthesis led to the protection of membrane reactions. According to Larcher's (1987) stress concept, these results are synonymous of gene markers for the alarm (*rbcL*), the hardening (*FeSOD*, *VTE3*) and the exhaustion [cyclin-dependent protein kinase (*cdk*), *psbA*] phases and thus can be used to evaluate the state of oxidative stress in algae and putatively other plant cells.

4.2. *Metal hyperaccumulation in spermatophytes*

To enable survival in variable soil conditions, plants possess homeostatic mechanisms that regulate the concentration of essential heavy metal ions. Plants that are capable of inhabiting heavy metal-enriched or -contaminated soil, so-called hyperaccumulators, have great potential to provide information and strategies for phytoremediation. To provide a better understanding of the underlying mechanisms of hyperaccumulation, Chiang et al. (2006) used an *Arabidopsis* cDNA microarray to compare the gene expression profiles of the Zn/Cd hyperaccumulator *A. halleri* and the non-hyperaccumulator *A. thaliana*. Comparing the expression profiles of metal-chelators, antioxidation-related genes, and transporters, Chiang et al. (2006) revealed that certain metal transporters (e.g. metallothioneins) and genes of the ascorbate–glutathione pathway (e.g. ascorbate peroxidases) were expressed at higher levels in *A. halleri*. Furthermore, the authors were able to confirm that the enzymatic activity of ascorbate peroxidase and class III peroxidases were highly elevated in *A. halleri*, an observation that correlates well with the ability of *A. halleri* to detoxify H_2O_2 produced by oxidative stress agents. In consequence, Chiang et al. (2006) suggest that higher peroxidase activities contribute to the heavy metal tolerance in *A. halleri* by alleviating the ROS damage.

The comparison of *A. thaliana* with another hyperaccumulator, *Thlaspi caerulescens*, which particularly hyperaccumulates Zn within the roots, is presented by van de Mortel et al. (2006). Between the two species in excess of 2200 genes were significantly differentially expressed at each of the Zn exposures. While a large proportion of genes are of hitherto unknown function, many differentially expressed genes appear to be key players in metal homeostasis, abiotic stress response or lignin biosynthesis. In particular Zn homeostasis genes are evident in *T. caerulescens* exposed to high levels of Zn. Genes with a suggested function in lignin biosynthesis and genes implicated in suberin biosynthesis were up-regulated by the Zn-hyperaccumulator, a finding that correlates with differences in the deposition of lignin in the endodermis, namely two layers in *T. caerulescens* roots and only one in *A. thaliana*. In summary, although many genes were found to be equally responsive to metal stress in *A. halleri* and *A. thaliana*, which suggests an overlap in the mechanisms of metal accumulation and metal tolerance, specific transcriptional profiles were identified that were unique to the metal hyperaccumulator.

4.3. *Comparative effect evaluation of explosives in Arabidopsis thaliana*

Explosives, such as trinitrotoluene, TNT, or hexahydro-1,3,5-trinitro-1,3,5-triazine, RDX, are highly recalcitrant environmental contaminants at munitions manufacturing and disposal facilities. They are known to induce a variety of toxic

effects in humans, mammals, birds, fish, and invertebrates. Uptake of explosives by plants is of concern, firstly because of the potential exposure to the food chain, and secondly because it also opens the door to the cost-effective treatment of contaminated sites through phytoremediation (Ekman et al., 2005). Although both explosives bear some structural similarities, the transcriptomic responses differ markedly. TNT exposure resulted in an up-regulation of 52 gene and a downregulation of 47 genes (Mentewab et al., 2005) with cytochrome P450 enzymes, glutathione S-transferase as well as an ABC transporters and a nitroreductase being the genes with the strongest differential expression (Ekman et al., 2003, 2005; Mezzari et al., 2005). In contrast, RDX exposure induces genes known to respond to a variety of general stresses, including genes encoding for several molecular chaperones and transcription factors as well as vacuolar proteins and peroxidases. Strongly repressed were transcripts encoding for ribosomal proteins, a cyclophilin, a katanin, and a peroxidase. A further explosive has recently been studied, namely 2,4-dinitrololuene (2,4-DNT), a byproduct in the TNT synthesis (Yoon et al., 2006). Glutathione and genes involved in its synthesis, glutathione S-transferase and a cytochrome P450 were all induced significantly in response to 2,4-DNT. Overall this suggests that *Arabidopsis* tolerates DNT and TNT via common metabolic pathway that are distinct from the response following RDX exposure.

4.4. *Toxicity of and resistance to the herbicide 2,4-D in Arabidopsis thaliana*

Herbicides are agrochemicals that control the growth of undesired plant species, bringing about a significant overall increase in crop productivity. The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin and is one of the most successful selective organic herbicides used in agriculture. However, the widespread and intensive use of 2,4-D has led to the emergence of herbicide-resistant plants and, therefore, might give rise to several environmental problems (Teixeira et al., 2007).

Auxins are known to induce de novo synthesis of 1-aminocyclopropane-1-carboxylic acid synthase. Enhanced levels of this acid, the immediate precursor of ethylene, lead to a substantial increase in the concentration of the gaseous phytohormone involved in stress responses, plant growth and senescence. Increased concentrations of ethylene (possibly generated via the cleavage of xantophyll), triggers the biosynthesis of abscisic acid, which in turn results in growth inhibition, morphological abnormalities and senescence. Consistent with this model, recent genome-wide expression studies on the effect of herbicidal concentrations of 2,4-D in *Arabidopsis* reported the modulation of several genes known to be involved in the auxin response, ethylene signaling, and abscisic acid biosynthesis, signaling, and response. In addition, the herbicide caused a down-regulation of genes involved in cell growth and elongation, and an up-regulation of genes involved in the response to both abiotic and biotic stresses. Other stress-associated genes, such as resistance and defense genes or those involved in osmolyte biosynthesis were also modulated (Teixeira et al., 2007).

5. Stress-related gene expression profiles in invertebrates

Several invertebrates appear to be well established model or even pioneer organisms in ecotoxicogenomics. This particularly applies to the nematode, *Caenorhabditis elegans*, the crustacean genus, *Daphnia*, and earthworms.

5.1. *Studies with C. elegans*

Due to the availability of the whole genome sequence, *C. elegans* has long been subject to gene expression studies. The examples selected here follow the chronology of the publications rather than a classification of the chemical stressors.

5.1.1. *Steroid hormones*

In order to assist in the identification of possible endocrine disrupting chemicals, the global gene expression profiles were assessed in response to three natural steroid hormones, testosterone, 17β-estradiol, and pro-estrogen (Custodia et al., 2001). The study showed that the *C. elegans* genome is widely responsive to the three vertebrate steroid hormones, but the overall gene expression profiles differ among the three chemicals. 17β-estradiol and progesterone induced 1496 and 1077 genes, respectively. Moreover, 349 genes were found to be down-regulated by 17β-estradiol, and 939 by progesterone. In particular, genes involved in xenobiotic metabolism and general stress responses were up-regulated. Transcription of several vitellogenin genes was increased by 17β-estradiol, but decreased by progesterone.

5.1.2. *Humic substances*

Even naturally occurring compounds, such as humic substances, may serve as chemical stressors to organisms (Steinberg et al., 2006). In *C. elegans*, the majority of humic materials tested were found to stimulate reproduction (Höss et al., 2001) and to act as estrogen-receptor agonists (Steinberg et al., 2004; Lutz et al., 2005). Moreover, it has been shown that humic substances act as powerful chemoattractants in *C. elegans* (Menzel et al., 2005b) and cause a variety of energy-consuming chemical defense reactions (Timofeyev et al., 2006a,b). In terms of gene expression response, a synthetic humic substance had the strongest effect, marked by an up-regulation of 554 genes, compared to only 240 genes by a humic isolate from a brown-water lake. Moreover, 685 genes were found to be down-regulated by the synthetic humic substance, and only 350 by that one from the brown-water lake. Taking into account that both humic material sources were able to attract the nematode, it is remarkable that 18 genes coding for putative chemoreceptors were found to be differentially regulated. Furthermore, a limited number of enzymes involved in biotransformation were found to be differentially expressed. Humic materials are likely to produce oxidative stress caused by H_2O_2 and other reactive oxygen species (Timofeyev et al., 2006a,b), which may generate oxidative stress in the nematode. Interestingly, one of the most responsive genes induced was F32A5.2, a putative peroxidase.

A follow-up study investigating why an organism is seemingly attracted to a humic substance that represents a stressful environment hypothesized that a contributing factor may be the potential to significantly expand their life-span (Steinberg et al., 2007) — however the ecological consequence remains obscure.

5.1.3. *Polychlorinated biphenyl (PCB52)*

Polychlorinated biphenyls (PCBs) are ubiquitous organic chemicals that pose a global environmental health problem. Menzel et al. (2007) performed whole genome DNA microarray experiments using synchronized *C. elegans* populations exposed to PCB52, a non-coplanar PCB. The DNA microarray experiment identified 1158 up-regulated and 560 down-regulated genes. Following Gene Ontology clustering, the data was analyzed further by aligning all identified gene classes with each other to reveal over-represented and overlapping functional gene classes, resulting in clearly arranged Venn diagrams (Fig. 2). The up-regulated genes encompassed in particular the processes of oxidative catabolism, also transcriptional regulation was found to include numerous members, followed by the esterase/lipase/thioesterase class, lipid binding, and specific chemoreceptors. Furthermore, the presence of general toxicity was evidenced by the induction of several small *hsp-20* genes and caspases and likewise, the increased oxidoreductase activity suggested that the nematode is capable of metabolizing PCB52. The considerable up-regulation of lipid-modifying enzymes firmly implies that PCB52 disrupts the lipid homeostasis in *C. elegans*. The vast majority of down-regulated genes were found to be members of general physiological processes such as cellular metabolism, binding, organelle biogenesis, and cell integrity.

In summary, this paper provides strong evidence of the molecular mechanisms that underlie the toxicity of non-coplanar PCBs, and verifies that the activation of lipid metabolism and increase in lipid storage are likely to be major factors that drive the toxic effect of PCB52. Moreover, it is another example of how experiments can succeed in revealing toxic modes of action on the basis of DNA microarray data.

5.1.4. *Phthalate (DEHP)*

Di(2-ethylhexyl)phthalate (DEHP) is added as a softener to polyvinyl chloride (PVC), an abundant component of cables, floor tiles, garden hoses, containers, footwear and clothing. Although DEHP has been shown to be present in the environment, little is known about its ecotoxicological properties. Roh et al. (2007) investigated the toxicity of DEHP to *C. elegans* using multiple toxic parameters, including expression profiling of stress-related genes. Although true statistical evaluation of the data set could have improved the message of the paper, the authors were able to demonstrate that exposure

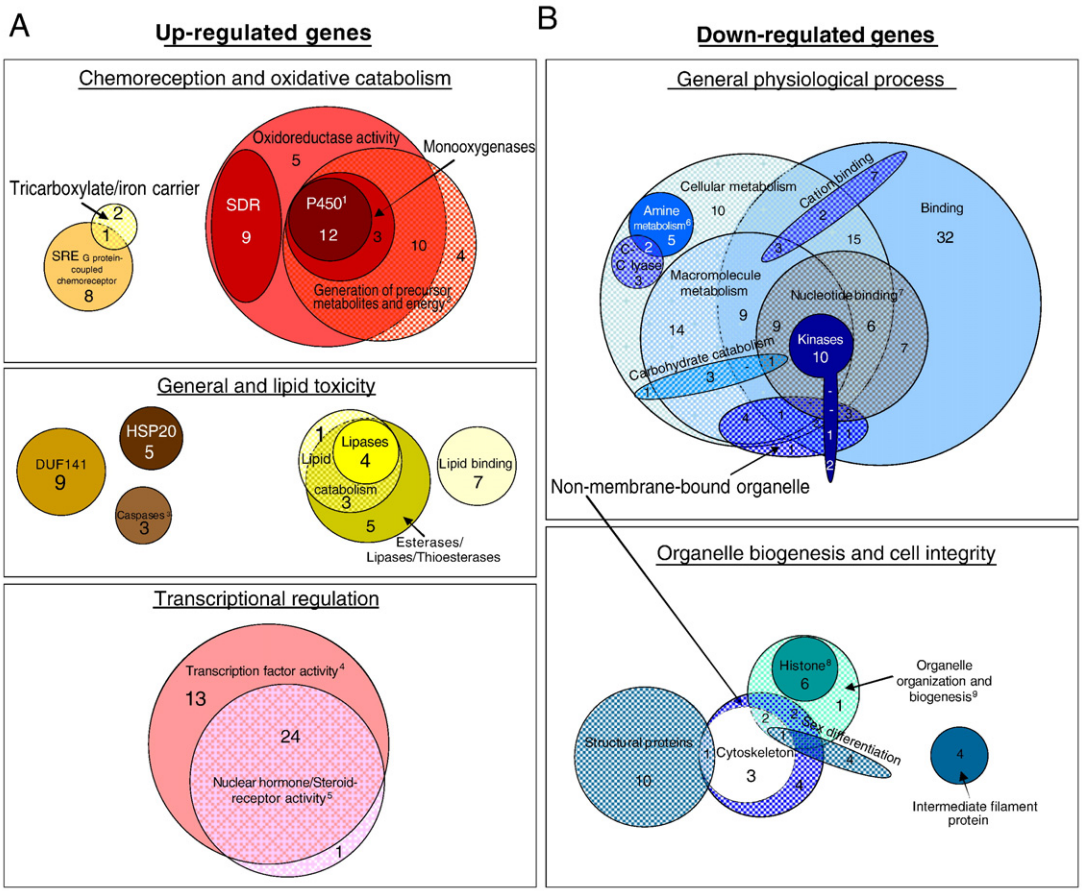

Fig. 2 – Venn diagrams illustrating the overlap between over-represented gene classes of significantly PCB52 induced (A) and repressed (B) genes, respectively. The digits refer to gene numbers (from Menzel et al., 2007).

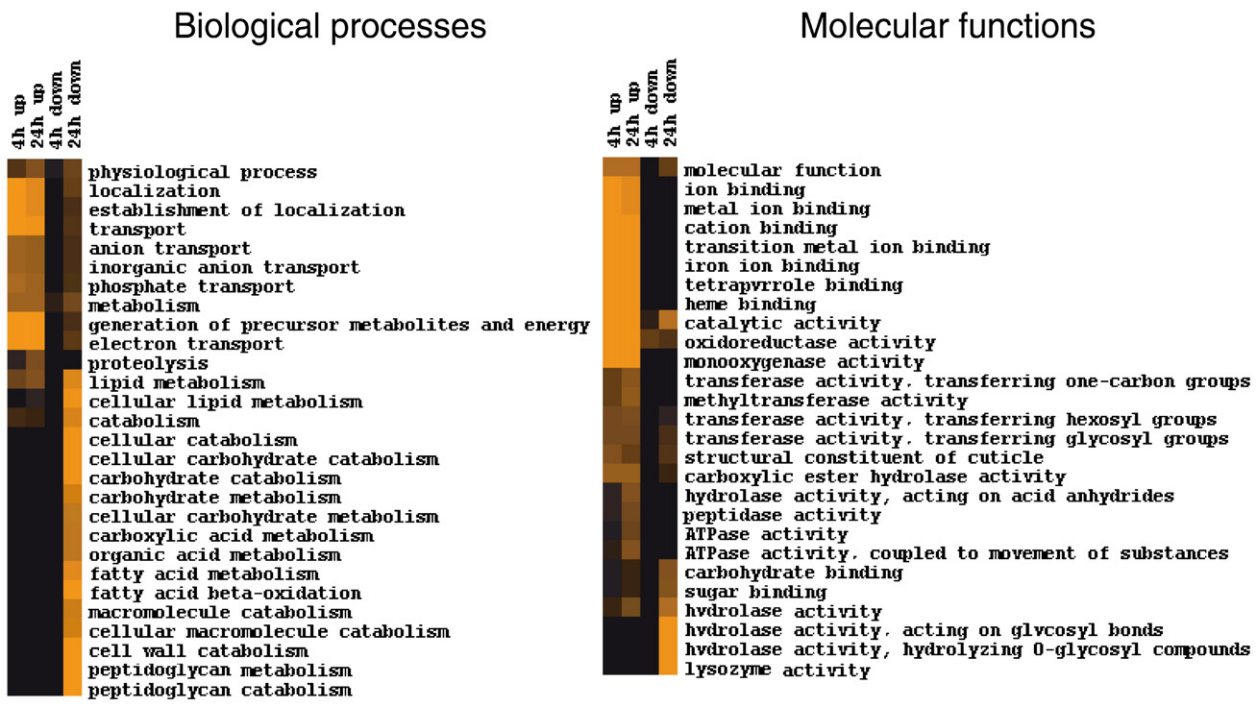

Fig. 3 – Biological processes and molecular functions enriched with Cd-responsive genes in *C. elegans*. Brightness (orange) translates into significance and enrichment of the pathway (from Cui et al., 2007).

to DEHP induces a strong and differential induction/repression of 40 genes. In detail, the expression of heat shock proteins *hsp-16.1* and *hsp-16.2* was decreased by DEHP exposure, a trend however that could not be confirmed in transgenic *hsp-16::GFP* strains following the exposure to DEHP. Cytochrome P450 *cyp-35A2*, the UDP-glucuronosyltransferase *ugt-21*, the ABC transporters *pgp-1*, and the vitellogenin *vit-1* were all strongly induced. This occurred concomitantly with the deterioration of the physiological state, which suggests an increase in expression of those genes is likely to be reaction to toxicity, rather than a homeostatic response.

5.1.5. *Cadmium*

Cd is a persistent environmental toxicant that is associated with a variety of diseases. The DNA microarray experiments of Cui et al. (2007) identified 237 up-regulated and 53 down-regulated genes that significantly changed following either 4h or 24h exposure to Cd. These genes were clustered into early and late response genes. The former encompasses pathways, which regulate the localization and transportation of different chemical species (in particular metal ions) (Fig. 3). This suggests that the first response to Cd intoxication is a transcriptional adjustment to maintain ion homeostasis and readjustment of perturbed energy supply. During the 24h exposure period, metabolic and localization pathways were enriched within up- and down-regulated gene lists (Fig. 3). Cd exposure resulted in the over-expression of 25 biotransformation genes. In addition, the expression of four ABC transporters, *pgp-1*, *pgp-8*, *pgp-9* and *mrp-3*, was induced by Cd, and *pmp-5* was down-regulated. Moreover, proteolysis was significantly enriched, possibly leading to an accumulation of damaged proteins. Pathways that were over-represented within down-regulated genes included fatty acid metabolism, cellular lipid metabolism and cell wall catabolism, indicating that Cd disrupts multiple cellular functions. The discovery of novel genes provided valuable information aiding our understanding of the toxicogenomic response of Cd in a fashion that goes far beyond classical biomarker studies.

5.2. *Studies with Daphnia magna and Daphnia pulex*

D. magna, a small freshwater crustacean, is a keystone species in ecological food webs and a model species for toxicant exposure.

5.2.1. *Heavy metals*

The objective of a study by Poynton et al. (2007) was to compare distinct expression profiles in *D. magna* subjected to sublethal Cu, Cd, and Zn exposures. The results support known mechanisms of metal toxicity (e.g. via two probable metallothioneins, or the iron-response element containing ferritin) as well as reveal novel modes of action such as the Zn induced inhibition of chitinase activity. Most interesting was the finding that, although the three metals belong to the same class of chemicals, the expression profiles were distinct and unique (Fig. 4) (Poynton et al., 2007).

Soetaert et al. (2007a) go further by applying three concentrations of Cd over two exposure periods to show that dynamic time and dose responsive expression profiles can be obtained. Cd affected molecular pathways associated with processes such as digestion, oxygen transport, cuticular metabolism and embryo genesis and development. In particular, the induction of the yolk protein vitellogenin, was down-regulated by the long-term exposure. Furthermore, Cd

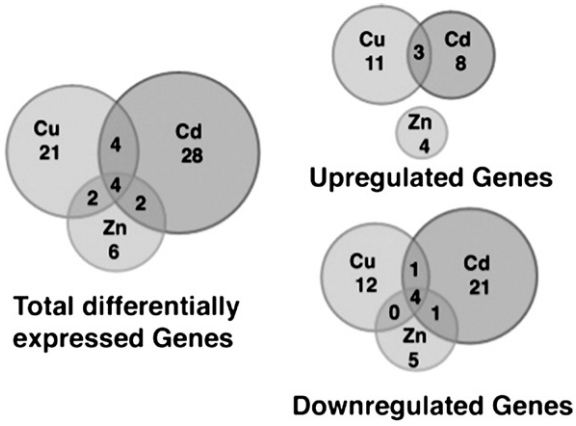

Fig. 4 – Venn diagrams showing Cu, Cd, and Zn causing the differential expression of a unique set of cDNAs in *Daphnia magna*. The number of differentially expressed genes determined by microarray analysis for each metal is shown including the genes that overlap in expression by more than one metal (Poynton et al., 2007).

affected the expression of genes coding for proteins involved in molecular pathways associated with the immune response, stress response, cell adhesion, visual perception and signal transduction.

Finally, a Cd exposed *D. pulex* cDNA microarray yielded three novel metallothioneins (Shaw et al., 2007), thereby demonstrating elegantly that details of the underlying molecular genetics of how an organism interacts with its environment can be unraveled — a process that is more complex than indicated by unidimensional biochemical biomarkers alone.

5.2.2. *Fungicides*

Analogous to the Cd-experiment with *D. magna*, embryogenesis has been shown to be impaired in neonates exposed to the fungicide propiconazole. As before, vitellogenin was down-regulated resulting in the impaired maturation of oocytes (Soetaert et al., 2006). These findings were mirrored at the organismal level, where the highest concentration of propiconazole adult significantly impaired growth and induced developmental effects in the offspring.

A slightly more complex picture emerges when *D. magna* is exposed to another fungicide, fenarimol, which acts as molting hormone in arthropods, including *D. magna* (Soetaert et al., 2007b). As expected, genes belonging to molting specific pathways were differentially expressed upon exposure to fenarimol. At the highest exposure concentration, a set of proteolytic enzymes were induced whereas different cuticular proteins were down-regulated. Moreover, effects on embryo development were apparent at the gene expression level (including vitellogenin) as well as at the organismal level (resulting in a significant increase in embryo abnormalities in the offspring).

5.3. *Studies with earthworms*

Earthworms (Phylum Annelida, Class Oligochaeta) have been renowned as 'ecosystem engineers' in recognition of the direct and indirect effects they have on water, nutrient and carbon cycling in temperate and tropical soils. Natural contamination and anthropogenic pollution of soils are likely to be major determinants of functioning and survival of such keystone invertebrates. Earthworms will have both evolutionary adaptation and genetically programmed responses to these toxic chemicals, but mechanistic understanding of such is sparse. Research frequently focuses only on easily measurable endpoints, typically mortality, although more sensitive tests on effect endpoints such as reproduction and growth with certain test species (Eisenia fetida, E. andrei, and *Lumbricus rubellus*) are also widely used. These bioassays can produce sensitive estimates of population effects, but are not suited for the elucidation of mechanisms of action, and thus may be difficult to generalize from. Thus, a major challenge for ecologists and ecotoxicologists is to gain an understanding of the toxic mechanisms at a molecular level, and demonstrate how these molecular changes relate to functional changes at the organism and population level. Very recently, several complex studies have been published.

Owen et al. (2008) and Svendsen et al. (2008) have generated an 8000-element transcriptome microarray for *L. rubellus*. Strikingly, less than half the putative genes (41%) were assigned annotations from the Gene Ontology system. This reflects the phylogenetic uniqueness of earthworms compared to well-annotated model animals. The microarray identified substantive differences in the transcript profiles of juvenile and adult animals. Annotation of genes indicated that transcripts associated with macromolecular biosynthesis, energy production, and connective tissue synthesis (all processes associated with rapid growth rate) were over-expressed in juveniles. The microarray was further used to determine dose–response transcription profiles following exposure to three xenobiotics from different chemical classes: inorganic (the heavy metal cadmium), organic (the polycyclic aromatic hydrocarbon fluoranthene), and agrochemical (the herbicide atrazine). Analysis of these profiles revealed compound-specific fingerprints which identify the molecular responses of the annelid to each contaminant. The majority of genes that show statistically significant relationships between xenobiotic dose and transcript levels do not have informative similarity to known proteins and were thus annotated as genes 'regulated by xenobiotic exposure'.

A clearer insight is gained with copper, Cu, and *L. rubellus*. Being an essential element that is also highly toxic to

earthworms in high concentration it can be expected that not only general toxic-response pathways are induced/perturbed, but also specific biological mechanisms that are essential for copper handling and homeostasis. Therefore, copper is an excellent model toxin for demonstrating an integrative ecotoxicogenomics approach. Bundy et al. (2008) exposed worms to sub-lethal levels of copper in a semi-field situation using buried mesocosms, and monitored the dose–response by transcriptomics and metabolomics. The data provided evidence that the copper exposure led to a disruption of energy metabolism: transcripts of enzymes from oxidative phosphorylation were significantly over-represented, and increases in transcripts of carbohydrate metabolizing enzymes (maltase-glucoamylase, mannosidase) had corresponding decreases in small-molecule metabolites (glucose, mannose). Treating both enzymes and metabolites as functional cohorts led to clear inferences about changes in energetic metabolism (carbohydrate use and oxidative phosphorylation), which would not have been possible by taking a 'biomarker' approach to data analysis.

When exposing the redworm, *E. fetida*, to an explosive (TNT), Gong et al. (2007) discovered that the expression of genes involved in multiple biological processes was altered, including muscle contraction, neuronal signaling and growth, ubiquitination, fibrinolysis and coagulation, iron and calcium homeostasis, oxygen transport, and immunity. Sublethal doses of TNT affected the nervous system, causing blood disorders similar to methemoglobinemia, and weakened immunity in *E. fetida*. The findings provide new insights into the toxicological mechanisms of TNT at the global gene expression level. This information furthered our understanding how TNT causes toxic effects in a soil organism and allowed the comparison with higher organisms.

A synopsis of gene expression profiles of selected bacteria, plant, and invertebrate species exposed to various environmental chemical stresses is presented in Tables 1a and b. It is apparent that, upon exposure to different chemical stressors, one of the main common strategies is the reduction of cellular metabolism, such as nucleotide biosynthesis, fatty acid metabolism, nutrient absorption, oxygen transport, and cell division.

6. Stress-related gene expression profiles in fish

Fish have attracted the most attention regarding gene expression profiling under chemical stress, and most studies are highly advanced. Furthermore, gene expression profiling seems to be the tool of choice in particular with chemicals/pharmaceuticals which do not show clear or even no effects in classical ecotoxicological tests.

6.1. *Metals and metalloids*

Gene profiling studies that investigate the effect of metals have only recently been forthcoming. For instance, Klaper et al. (2006) studied the pathways of methylmercury (MeHg) toxicity in fish. Hg in its methylated form is neurotoxic, particularly to the developing nervous system. However, the mechanistic effects of MeHg on other physiological processes such as reproduction are at large unknown, and there are comparatively few studies that examine risks of MeHg exposure. Klaper et al. (2006) found that the expression of genes commonly associated with endocrine disruption was altered due to the exposure to Hg. Vitellogenin gene expression, for example, significantly declined in female fish exposed to increasing concentrations of Hg. Other genes included those associated with egg fertilization and development, sugar metabolism, apoptosis and electron transport. Klaper et al. also observed differences in expression profiles in male and female fish (including genes not specifically associated with reproduction), suggesting that the differences in physiology and MeHg toxicity are potentially interlinked.

Cu is an essential micronutrient and fish assimilate it either through the gills from the surrounding water, or the diet via the digestive tract. Though essential, elevated levels of Cu can cause a range of negative effects, including reduced growth, interference with ion-regulation, and endocrine disruption. Many of these responses are, in part, due to the reactivity of Cu with H_2O_2 and its potential to undergo (Fenton-like) redox reactions to form ROS. The resulting cellular damage can be membrane peroxidation, DNA damage, and protein carbonyl production. Like other organisms, fish combat elevated levels of ROS with protective ROS-scavenging enzymes, such as SOD and CAT. However, once these enzymes are saturated, irreversible cellular damage and death can occur (Craig et al., 2007 with further references). In two experiments Craig et al. (2007) examined the impacts of Cu on gene expression, oxidative damage, and cell oxidative capacity in the liver and gills of zebrafish. Soft water-acclimated zebrafish exposed to environmentally relevant Cu concentrations resulted in significant increases of cytochrome *c* oxidase subunit 17 and catalase, associated with both increased Cu load and protein carbonyl concentrations in the gill and liver. Furthermore, there were indications that Cu alters normal mitochondrial biogenic processes, possibly though cytochrome *c* oxidase subunit 17. However, unlike MeHg, Cu did not modulate endocrine disruption.

Arsenic, As, is a prominent environmental toxicant and carcinogen; nevertheless, its molecular mechanism of toxicity and carcinogenicity remains poorly understood. Recently, Lam et al. (2006) performed microarray-based expression profiling the liver of zebrafish exposed to As(V). The authors found that there was an increase in transcriptional activity associated with metabolism, especially for biosyntheses, membrane transporter activities, the cytoplasm, and the endoplasmic reticulum. Many differentially expressed genes encoding heat shock proteins and genes involved in DNA damage/repair, antioxidant activity, hypoxia induction, iron homeostasis, as well as ubiquitin-dependent protein degradation were identified, suggesting strongly that DNA and protein damage causes major cellular injury as a result of As metabolism and oxidative stress (Lam et al., 2006).

6.2. *Nanoparticles*

Bioactive nanoparticles may cause harm to human health and the ecosystem. Carbon nanoparticles, i.e. fullerenes (C_{60}), are already widespread (see Henry et al., 2007). Several studies

Table 1a – Synopsis of gene expression profiles of selected bacteria and plant species exposed to various environmental chemical stresses

Species	Target genes	Condition	Marker genes/gene clusters		Reference
			Up-regulated	Down-regulated	
Bacteria					
Escherichia coli	Whole genome	Exposure to Mediterranean seawater	Degradation of fatty acids, amino acids and other carbon compounds; aerobic and anaerobic respiration; chemotaxis and mobility	Cell division, nucleotide biosynthesis	Rozen et al. (2002)
Plants					
Chlamydomonas reinhardtii	8 stress genes	Copper access	*rbcL* (alarm phase), *VTE3* and *SOD* (hardening phase), *CDK* (adjustment phase), *PsaA* and *PsbA* (exhaustion phase)	*CDK* (exhaustion phase)	Luis et al. (2006)
Arabidopsis thaliana	Whole genome	Zink exposure	sHSPs, Cu/Zn superoxide dismutase, metal homeostasis (various metal transporters other than ZIP)	Metal homeostasis (ZIP metal transporters), Fe superoxide dismutase	van de Mortel et al. (2006)
Thlaspi caerulescens	Whole genome (A. thaliana)	Zink exposure	Lignin and suberin biosynthesis, metal homeostasis	Fe superoxide dismutase	van de Mortel et al. (2006)
Arabidopsis thaliana	Whole genome	Herbicide 2, 4-D	Auxin response, ethylene signaling, and abscisic acid biosynthesis, signaling and response;	Cell growth and elongation	Teixeira et al. (2007)
Arabidopsis thaliana	Serial analysis of gene expression	TNT	Cytochrome P450 enzymes, glutathione S-transferase, ABC transporters, nitroreductase	Protease inhibitors, a dehydrogenase, elongation factor 1 B α-subunit	Ekman et al. (2003)
Arabidopsis thaliana	5 stress genes	TNT, RDX	Glutathione S-transferase, nitroreductase	Not analyzed	Mezzari et al. (2005)
Arabidopsis thaliana	Serial analysis of gene expression	RDX	Molecular chaperones, transcription factors, vacuolar proteins, peroxidase	Ribosomal proteins, a cyclophilin, a katanin, a peroxidase	Ekman et al. (2005)
Arabidopsis thaliana	14,000 unique clones	TNT	52 genes, ABC transporters, peroxidases, resistance proteins, transferases	47 genes, glucose transporter, defense related proteins, amino acid and protein metabolisms	Mentewab et al. (2005)

have shown that fullerenes can interact with lipids in membranes (see Oberdörster et al., 2006). In *Daphnia* long-term exposures resulted in a delay in molting and reduced offspring production at environmentally realistic concentrations. However in fish, neither the mRNA nor protein-expression levels of biotransformation enzymes changed. The peroxisomal lipid transport protein PMP70 was significantly reduced in fathead minnow (*Pimephales promelas*), but not medaka (*Oryzias latipes*). In a study with the zebrafish (*Danio rerio*) Henry et al. (2007) investigated whether nanoparticles or the vehicle (tetrahydrofuran, THF, which is used to generate aqueous aggregates of C_{60}) is the toxic agent. The authors found that the survival of larval zebrafish was reduced in THF–C_{60} and THF–water, but not in C_{60}–water. The greatest differences in gene expression were observed in fish exposed to THF–C_{60} and most of these genes were similarly expressed in fish exposed to THF–water. Significant up-regulation of genes involved in controlling oxidative damage was observed after exposure to THF–C_{60} and THF–water. Overall these findings suggest that THF and/or the degradation products are the causative agent for toxicity, and not C_{60}.

6.3. *Single organic compounds*

By exposing juvenile rainbow trout to carbon tetrachloride, CCl_4, or pyrene, Krasnov et al. (2005) identified opposite transcriptional responses/trends for several genes. Pyrene affected mainly transcripts implicated in the maintenance of the genetic apparatus, immune response, glycolysis, and iron homeostasis, whereas CCl_4 affected structural proteins and genes involved in cellular stress, protein folding, and steroid metabolism. Overall, pyrene suppressed a range of protective or acclimative reactions, many of which were stimulated by CCl_4. In addition, gene profiling indicated adaptive and potentially maladaptive reactions to toxicity. For instance, stimulation of mitochondrial proteins coincided with suppression of catalase, whereas CCl4 down-regulated fatty acid metabolism and peroxisomal proteins. Despite these differences, common responses to chemical toxicity were observed for metallothioneins, HSP90 and mitochondrial proteins of oxidative phosphorylation. Most of the effects are implicated in hematopoiesis and immune response.

6.4. *Endocrine disrupting compounds*

Gene expression profiling has been exploited to evaluate chemicals where evaluation by means of classical toxicity tests has been problematic or inconclusive. In fish, these are typically pharmaceuticals (van der Ven et al., 2005, 2006) and hormone-like compounds (Kishi et al., 2006; Larkin et al., 2002a, 2003; Brown et al., 2004a,b; Iguchi et al., 2006; Tilton et al., 2006; Williams et al., 2007). Since the presence

Table 1b – Synopsis of gene expression profiles of selected invertebrate species exposed to various environmental chemical stresses

Species	Target genes	Condition	Marker genes/gene clusters		Reference
			Up-regulated	Down-regulated	
Caenorhabditis elegans	Whole genome	Steroid hormones	Glutathione S-transferases, cytochromes P450, HSP70 proteins, metallothionein, vitellogenin	Glutathione S-transferases, cytochromes P450	Custodia et al. (2001)
Caenorhabditis elegans	Whole genome	Humic substances	Chemoreceptors, peroxidase, biotransformation	Chemoreceptors, transporter	Menzel et al. (2005b)
Caenorhabditis elegans	Whole genome	PCB52	Chemoreceptors, oxidative catabolism, transcriptional regulation, esterase/lipase class, lipid binding, HSP16	Cellular metabolism, binding, organelle biogenesis, cell integrity	Menzel et al. (2007)
Caenorhabditis elegans	Whole genome	Di(2-ethylhexyl) phthalate (DEHP)	UDP-glucuronosyltransferases, cytochromes P450, ABC transporter, vitellogenin	HSP16	Roh et al. (2007)
Caenorhabditis elegans	Whole genome	Cadmium access	Glutathione S-transferases, cytochromes P450, UDP-glucuronosyltransferases, ABC transporters, Proteolysis	Fatty acid metabolism, cellular lipid metabolism, and cell wall catabolism	Cui et al. (2007)
Daphnia magna	5000 cDNA clones	Sublethal Cu, Cd, and Zn exposures	Metallothioneins, ferritins, monooxygenases, and cell signaling (Cd,Cu), oxidative stress response (Cd), proteases and sulfotransferases (Cu)	Digestion and nutrition absorption, Chitinase (Cd,Zn), development (Cd,Cu), proteases (Zn)	Poynton et al. (2007)
Daphnia magna	2455 cDNA clones	Cadmium access	Carbohydrate and lipid digestion, cuticula metabolism (50μg/L), immune response, acid–base balance response	Oxygen transport, vitellogenins, cuticula metabolism (100μg/L)	Soetaert et al. (2007a)
Daphnia pulex	3602 cDNA clones	Cadmium access	Cuticula metabolism, hydroxylase activity, ion binding	Oxygen transport	Shaw et al. (2007)
Daphnia magna	2455 cDNA clones	Propiconazole	Transcriptional/translational regulation, energy metabolism, HSP90	Vitellogenins, larval-specific genes and chaperonins	Soetaert et al. (2006)
Daphnia magna		Fenarimol	Proteolysis, alpha-amylase	Cuticula proteins, vitellogenin	Soetaert et al. (2007b)
Lumbricus rubellus	~ 8000 cDNA clones	Cadmium, fluoranthene, atrazine	Glutathione S-transferases (Cd, Fla, Atz), cytochromes P450 (Cd, Atz,) DNA repair (Fla, Atz); metallothioneins (Cd); transcriptional regulation (Fla), mitochondrial electron transport, protein degradation (Atz)	Respiratory chain (Cd, Fla), ubiquitin specific protease, DNA repair, ferritin (Cd); proteasome, FKBP (Fla); Oxidative phosphorylation (Atz)	Owen et al. (2008), Svendsen et al. (2008)
Lumbricus rubellus	~ 8000 cDNA clones	Sublethal Cu exposure	Carbohydrate metabolism, oxidative phosphorylation metallothionein, HSP70, glutathione S-transferases, DNA repair, cell cycle control,	Gluconeogenese, oxidative phosphorylation, apoptosis, DNA, repair, cell cycle control	Bundy et al. (2008)
Eisenia fetida	4032 cDNA clones	2,4,6-trinitrotoluene (TNT)	Oxygen transport, muscle contraction, metallothionein, ubiquitinylation, Ca^{2+}-signaling, neurological function	Neurological function, immune response — wound healing, ferritin, glutathione S-transferase, fibrinogen	Gong et al. (2007)

of pharmaceuticals in the aquatic environment was first reported some 25years ago, many studies have demonstrated a great variety of pharmaceutical substances in waste-, surface- and drinking water, including neuropharmaceutical agents, hormones, anti-inflammatory, antibiotics, analgesics, lipid regulators and cytotoxic drugs (van der Ven et al., 2005 with further references). Although concentrations remain relatively low, pharmaceuticals are by definition biologically very active, and therefore should be assessed appropriately. However, the environmental fate and ecotoxicological effects of many pharmaceuticals remain, to date, poorly understood. This shortfall is slowly starting to be redressed, van der Ven et al. (2005), for example, developed a zebrafish brain-specific microarray. Following exposure to the antipsychotic drug Chlorpromazine, 56 genes were shown to be differentially expressed in brains of male and/or female zebrafish, of which most genes were down-regulated. This study is one of the first reports describing the molecular effects of a human neuro-active pharmaceutical in freshwater non-target organisms.

Zebrafish exposed to the antidepressant Mianserin were subjected to whole genome microarray analysis to pinpoint mechanistic drivers that modulate Mianseri specific gene

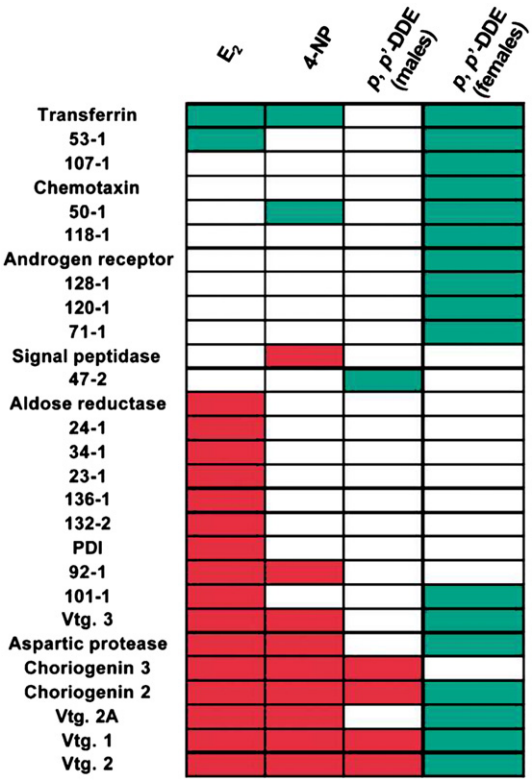

Fig. 5 – Gene expression profiles of largemouth bass livers exposed to 17-β-estradiol (E_2) and two chemicals that behave as estrogens. Red indicates up-regulated genes and green indicates down-regulated genes (from Larkin et al., 2002b). Despite the common expression of various vitellogenin genes by all exposures, the differences between the hormone, 17-β-estradiol, and the two endocrine disruptors, 4-NP and *p,p′*-DDE, are evident.

expression profiles (van der Ven et al., 2006). Analysis of brain and gonad tissue clearly demonstrated the estrogenic activity of Mianserin and its potency to disrupt endocrine signaling, based on induction of molecular biomarkers of estrogenicity (e.g. vitellogenin and zona pellucida proteins). The possible mechanism underlying the estrogenic activity of Mianserin may be caused by the disturbance of the Hypothalamo-Pituitary-Gonadal axis via serotonergic and adrenergic systems in the brain of zebrafish.

The European flounder is another favorite candidate for assessing the effects of endocrine disruptors, particularly in European estuaries. Williams et al. (2007) showed that known biomarkers of estrogen exposure, choriogenin L and vitellogenins, were induced over a time-course. Among the 175 identified genes showing significant induction or repression, those associated with the Gene Ontology terms mitochondria, amino acid synthesis, ubiquitination, and apoptosis were over-represented while those associated with immune function, electron transport, cell signaling and protein phosphorylation were under-represented.

A consensus response is now beginning to emerge with the ever increasing number of gene expression studies on the effects of estrogens in fish liver. It is evident that the vitellogenins and choriogenins are most responsive upon estradiol treatment and thus represent effective biomarkers of estrogen or xenoestrogen exposure. However, being members of multigene families, they require more attention in order to fully characterize their expression responses. Several other studies highlight the counter-intuitive effects on apoptotic as well as proliferative pathways, and demonstrate the impact of estrogen on mitochondria and steroid transporters. Overall, the studies provide valuable insights into the mode of action of 17-β estradiol in liver and show that its gene expression profile differs from chemicals that are estrogen analogs, such as DDE or nonylphenol (Fig. 5) (Larkin, 2002b, 2003).

6.5. *Mixture toxicity*

6.5.1. *Broad range of environmental toxicants*

Most of the examples so far presented have focused on the effect of a single compound. The following investigation (Yang et al., 2007) determined the parallel toxicogenomic profiles of 11 well known environmental toxicants in *D. rerio* larvae, namely methylmercury chloride (MeHg), $CdCl_2$ (Cd), $PbCl_2$ (Pb), As_2O_3 (As), Aroclor 1254 (PCB), acrylamide (AA), *tert*-butylhydroquinone (tBHQ), 4-chloroaniline (4CA), 1,1-bis-(4-chlorophenyl)2,2,2-trichloroethane (DDT), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and valproic acid (VA). The gene expression profiles induced by the toxicants were found to be related but sufficiently different to identify toxicant-specific profiles (Fig. 6). Moreover, Yang et al. (2007) were able to detect gene expression changes at concentrations inducing no phenotypic effect. The differences in gene responses were particularly striking at early developmental stages and when a mixture of compounds (Cd, Pb, MeHg, and As) was applied at low doses, synergistic effects were detected. This

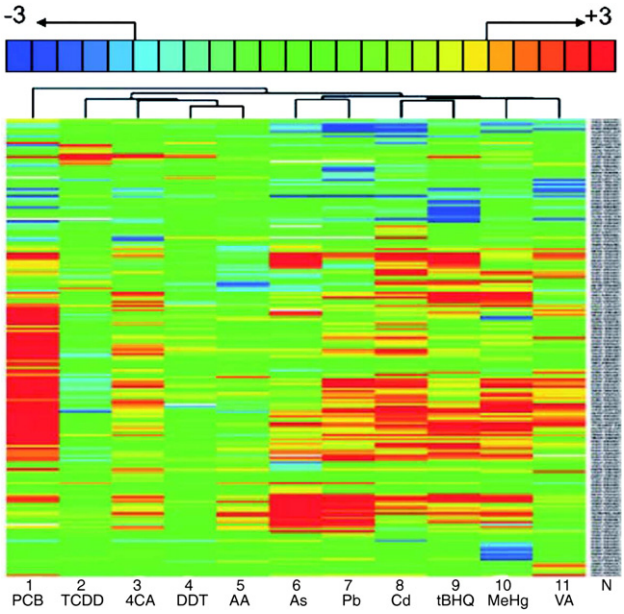

Fig. 6 – Toxicants can induce highly specific toxicogenomic profiles. Hierarchical clustering of gene responses in zebrafish embryos treated with the environmental toxicants as indicated. For each toxicant exposure, vehicle controls were carried out in parallel. The gene names are indicated (N) and are legible upon magnification of the original PDF version of this figure (Yang et al., 2007). The key at the top indicates the color code for fold changes, changes greater than three are not indicated explicitly but are included.

work provides proof of principle that the fish embryos can serve as a specific and highly sensitive whole-animal model to monitor the ecotoxicogenomic impact of chemicals.

6.6. *Effluent toxicity*

Wastewater effluents, sediments or abandoned industrial sites are typically contaminated by a suite of toxic compounds. Single compound laboratory exposures are therefore highly simplistic and somewhat artificial. Although effluents are major sources of direct pollution, few papers have addressed the issue of complex toxicity.

When exposing juvenile brown trout to subacute concentrations of a mixture of resin acids (a characteristic effluent of the pulp and paper industry), Meriläinen et al. (2007) found dose-dependent changes in a large number of genes. Resin acids interfered with iron metabolism (as evidenced by the decrease in transcripts for iron transporters and heme-containing proteins) and down-regulated the expression of genes encoding for enzymes degrading reactive oxygen species.

Moens et al. (2007) unraveled the modes of action of whole wastewater effluents on the common carp. Microarray analysis revealed that the effluent mainly affected molecular pathways associated with the energy balance of the fish, including the down-regulation of carbohydrate and lipid metabolism, as well as digestive enzymes. However, this particular effluent did not cause direct endocrine disruption in carp, nor did the effluent interfere with any of the molecular pathways associated with an immune response. The composition within and between effluents changes over time, and therefore this study demonstrates the power of gene profiling to provide a snap shot of the toxicity that arises from the exposure to effluents.

6.7. *Sediment toxicity*

Sediments from waterways with high vessel traffic and (purified) waste water loadings can comprise a complex mixture of potentially toxic compounds. Gene expression profiling of zebrafish eggs and embryos exposed to this very complex toxicity, provides an excellent and realistic means of assessment. In a recent comprehensive study, Kosmehl (2007) compared the gene expression profiles obtained from zebrafish exposed to two sediments of the River Rhine (Reckingen and Iffezheim). Genes associated with fatty acid transport (e.g. high density lipoprotein), fatty acid metabolism and beta-oxidation of fatty acids were found to be down-regulated in response to extract exposure. For instance apolipoprotein D, a protein-component of high density lipoproteins, was significantly down-regulated. Cytochrome P450 1A1 and 1C1 were up-regulated by a factor of 100 and 30, respectively. Classical inducers of P450 are polycyclic aromatic hydrocarbons, PCBs, dibenzo-*p*-dioxins like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polychlorinated naphthalenes, and furans.

Another group of up-regulated genes was the heat-inducted chaperones HSP (heat shock proteins). Likewise, the Natural Killer Cell Enhancing Factor was up-regulated in exposed zebrafish embryos. In addition to cytotoxicity, this factor acts as an antioxidant by increasing cellular resistance to oxidative damage by hydrogen peroxide and by protecting cells from alkyl hydroperoxide and heavy metals such as methyl mercury (Kosmehl, 2007, with further references). A group of down-regulated genes was structural related proteins, in particular serin proteases (elastase 2 and A) and α-amylase.

Kosmehl (2007) concluded that the observed results in the embryos may suggest that the regular heterotrophic

Table 2a – Synopsis of gene expression profiles of selected fish species exposed to various environmental chemical stresses exposed as single compounds

Species	Target genes	Condition	Marker genes/gene clusters		Reference
			Up-regulated	Down-regulated	
Metals and metalloids					
Pimephales promelas (liver)	200 genes	Methyl mercury (MeHg)	Vitellogenin (male), egg fertilization and development, sugar metabolism, apoptosis, electron transport	Vitellogenin (female)	Klaper et al. (2006)
Danio rerio (liver and gill)	4 genes	Copper	Cytochrome c oxidase subunit 17 (COX-17) and catalase (CAT)		Craig et al. (2007)
Danio rerio (liver)	~ 14,900 genes	Arsenic	Transporter activities (localizing in membrane, cytoplasm, and ER), glutathione transferases, S-adenosylmethionine-dependent methyltransferases, thioredoxins, ArsA arsenite transporter, HSP genes,	Blood factors, hormone activity, lipid metabolism, growth factors, cytoskeleton, transcription factors	Lam et al. (2006)
Nanoparticles					
Danio rerio (larvae)	~ 14,900 genes	C_{60} nano-aggregates + tetrahydrofuran	Metalloproteinase ; oxidative stress-inducible genes (including glutathione S-transferases) only THF)		Henry et al. (2007)
Single organic compounds					
Oncorhynchus mykiss (kidney, liver)	1273 cDNA clones	Carbon tetrachloride and pyrene	Metallothioneins, HSP90, mitochondrial proteins of oxidative phosphorylation	Hematopoiesis and immune response, peroxidase activity	Krasnov et al. (2005)
Danio rerio (brain)	682 cDNA clones	Chlorpromazine		Cell structure and cytoskeletal organization, intermediary and energy metabolism, cell signaling and neurotransmitter metabolism	van der Ven et al. (2005)
Danio rerio (brain and gonad tissue)	Whole genome	Mianserin	Vitellogenin and zona pellucida glycoprotein, intermediary and energy metabolism (brain)	Intermediary and energy metabolism (gonad)	van der Ven et al. (2006)
Platichthys flesus (liver)	13,824 clones (inc. controls)	17-β estradiol	Mitochondria, amino acid synthesis, ubiquitination, and apoptosis	Immune function, electron transport, cell signaling and protein phosphorylation	Williams et al. (2007)

metabolism of the zebrafish embryos is reduced in order to combat the contamination and for ongoing transformation processes, indicating an oncogenic potential of the sediments. This finding is further supported by an up-regulation of the anticancer gene p53 (one of the major tumor suppressors) and p53-associated activating transcription factor 3 during extract exposure. Unfortunately, it was difficult to distinguish between the different sample locations, which may reflect the complexity of pollutants present in the sediment extracts.

A synopsis of gene expression profiles of selected fish species exposed to various environmental chemical stresses is presented in Tables 2a and b.

6.8. *Population genetics*

Another giant step towards realistic environmental situations is done when gene profiling utilizes a whole population or even different populations of one species. Nacci et al. (1999), for example, describe that aquatic species, such as the estuarine fish *Fundulus heteroclitus* (mummichog), can adapt to local environmental pollution conditions.

The mummichog populations, studied by Nacci et al. (1999), are indigenous to an urban estuary in Massachusetts, USA, contaminated with persistent and bioaccumulative toxicants, namely dioxin-like compounds, that are particularly toxic to developing fish. While there was variation in the responsiveness to dioxin-like compounds within each group, fish from the contaminated site were profoundly less sensitive to the toxicants than reference fish. Specifically, concentrations of dioxin-like compounds similar to those measured in mummichog eggs from the contaminated site were lethal to reference embryos. Furthermore, responsiveness to dioxin-like compounds was inherited at least to the F2 generation and independent of maternal contaminant contributions.

In contrast to the Massachusetts mummichogs, it was found that resistance is not highly heritable in a Virginia population originating from contaminated sites of the Elizabeth River (Meyer and Di Giulio, 2002). However, the authors show that offspring of this population of Elizabeth River killifish are also resistant to the teratogenicity and cytochrome P4501A-inducing activity of PCB congener 126, a prototypical coplanar polyhalogenated aromatic hydrocarbon.

The genetic evaluation at the population level solved this contradiction. Fisher and Oleksiak (2007) used *Fundulus* cDNA arrays to compare metabolic gene expression profiles of the brains from individuals of nine populations including the aforementioned populations. The authors found that up to 17% of metabolic genes had evolved adaptive changes in gene expression in the polluted populations. Two genes in

Table 2b – Synopsis of gene expression profiles of selected fish species exposed to various environmental chemical stresses exposed as mixtures

Species	Target genes	Condition	Marker genes/gene clusters		Reference
			Up-regulated	Down-regulated	
Danio rerio (larvae)	16,399 genes	11 model compounds: MeHg, Cd, Pb, As, Aroclor 1254, acrylamide, *tert*-butylhydroquinone, 4-chloroaniline, DDT, TCDD, valproic acid	Specific expression profiles for each of the chemicals, the identity of the toxicant and could be predicted from the expression profiles with high probability, for details please see original paper.		Yang et al. (2007)
Salmo trutta m. *lacustris* (liver)	1273 genes	Resin acids (RA)	Cell proliferation and reparation of tissues	Iron transporters and heme-containing proteins, oxidative stress response, protein biosynthesis, defense and immune responses, energy metabolism	Meriläinen et al. (2007)
Cyprinus carpio (liver)	960 cDNA clones	Industrial effluent	Carbohydrate/ protein digestion, detoxification and stress response	Glucose/glycogen metabolism, steroid metabolism, apolipoproteins	Moens et al. (2007)
Danio rerio (larvae)	~14,900 genes	Two differently contaminated river sediments	Detoxification and stress response (CYP, HSP, GST), metalloproteinases	Fatty acid transport and metabolism, structural related proteins	Kosmehl (2007)

particular showed a conserved response among three polluted populations, suggesting common, independently evolved mechanisms for adaptation to environmental pollution in these natural populations.

7. Does it really all lie in the genes?

Recent research suggests that epigenetic processes are likely to play a significant role in acclimation to environmental stresses. Epigenetics comprises non-Mendelian inheritance and metastable genetic characters (Bird, 2007; Bossdorf et al., 2008) and includes all heritable changes in gene expression and function that cannot be explained by changes in DNA sequences (Richards, 2006). Hence, epigenetics refers to any change in gene expression that are stable between cell divisions, and sometimes between generations, but do not involve changes in the underlying DNA sequence of the organism. Consequently, these changes cannot be traced by microarrays and may thus contribute to the variability of microarray studies.

The underlying mechanism includes methylation of cytosine residues in the DNA, remodeling of chromatin structure through chemical modification, regulatory processes mediated by small RNA molecules as well as any other kind of gene silencing, e.g., by chaperones. With relevance to environmental impact, there are two well documented epigenetic pathways: methylation of DNA or RNA and gene silencing by HSP90.

In a pioneering study, Fieldes and Amyot (1999) altered the DNA methylation in flax by applying 5-azacytidine (a drug that removes methyl groups from DNA) to show that the resultant experimental phenotype lasts for four subsequent generations. To date, ecologically important genes with methylated epialleles have been found to affect several traits, including pathogen resistance in plants (Kalisz and Purugganan, 2004). In mice, environmental contaminants, such as endocrine disrupters (vinclozolin, Crews et al., 2007) and dietary supplements with relatively high doses of methyl-donors (Cropley et al., 2006) induce changes in DNA methylation that are inherited over several generations. Although these examples clearly elucidate the mechanisms, the quantity of chemicals required to induce the observed effects is far from being environmentally realistic.

Gene silencing by chaperones appears to be the best documented pathway of the non-Mendelian interrelationship between the environment and the individual phenotypes and their responses. This is due to the fact that even mild environmental stressors may disturb the silencing function of the heat shock protein 90, HSP90. To identify the precise mechanisms, Queitsch and Sangster (2002) applied anti-tumor antibiotics to *A. thaliana* to inhibit the molecular chaperone HSP90 and cause multiple inheritable phenotypes. Persistent organic xenobiotics which are usually subject of ecotoxicological studies have, so far, not been applied to *A. thaliana*. Nevertheless, the authors show that even other, much milder environmental stressors than the antibiotic application, such as the nature of the growth substratum, also had the capacity to uncover HSP90-buffered traits. In consequence, one may expect that environmental chemicals have the potential to activate the epigenetic machinery.

The role of HSP90 in buffering the expression of genetic variation is not restricted to plants, but is conserved across the animal kingdom. Queitsch and Sangster (2002) show that, under stressful conditions, HSP90 is induced. When HSP90 buffering is compromised, for example by exposure to chemicals, cryptic variants are expressed and selection can lead to expression of these traits, even after HSP90 function is restored (Rutherford and Lindquist, 1998; Sollars et al., 2003).

From an environmentalist's perspective, there are profound reasons why epigenetics should be considered in assessing and evaluating effects of chemicals and other environmental stresses on organisms: epigenetics may

explain some (or even most) of the heritable variation observed in natural population that cannot be explained by differences in the DNA sequence. Furthermore, taking epigenetics into account will provide a broad insight into the response mechanisms of organisms exposed to the variety of environmental stresses.

8. Potential future development

DNA microarrays are currently the most widely used methodology for gene expression profiling, although some limitations persist. In the past, the quality of data sets has been rather variable. Some, but not all, experiments provided replicates, some entries included raw array data and others only processed data. In this context, the Microarray and Gene Expression Data (MGED) society has developed guidelines and standards for the user community. Now widely accepted, MIAME[1] (*Minimum Information About a Microarray Experiment*) defines the minimum quantity and quality of information that is required to interpret and verify results (Brazma et al., 2001). Indeed, many journals now require that MIAME compliant microarray data is uploaded to public databases as a prerequisite for publication in an effort to ensure unhindered public access to primary, good quality, data (Ball et al., 2004). Further limitations of DNA microarrays include hybridization and cross-hybridization artifacts as well as dye-based detection issues (Okoniewski and Miller, 2006; Eklund et al., 2006; Casneuf et al., 2007), which, to date, have not been completely solved.

A potentially more comprehensive method of measuring transcriptome composition (and as a by-product aids in the confirmation/discovery of intron–exon boundaries) is by direct ultra-high-throughput sequencing of cDNA (Ng et al., 2006; Mortazavi et al., 2008; Nagalakshmi et al., 2008; Marioni et al., 2008). The sequencing approach has clear advantages over DNA microarrays by elucidating the exact nucleotide content of target DNA sequences. However, so far a major constraint has been the inhibitive cost and low/slow capacity in generating data. New ultra-high-throughput massively parallel sequencing technology may overcome this limitation, by being able to generate a vast number of sequence reads (~ 200,000 single reads per 5-hour run), thus facilitating the identification of genome-wide transcripts. If sufficient reads are collected from a sample, it should theoretically be possible to detect transcripts from all biologically relevant classes. The resulting sequence reads can then be individually mapped to the source genome and quantified to obtain the number and density of reads corresponding to RNA from each known exon, splice event or even new candidate gene. In short, ultra-high-throughput sequencing technology provides a comprehensive method to analyze gene expression patterns and may well supersede DNA microarrays in the near future. This will enhance current gene profiling techniques and ultimately aid unravel issues of environmental and regulative importance.

[1] Please refer http://www.mged.org/.

9. Conclusions

The application of DNA microarrays in ecotoxicogenomics is still at an early stage and complex challenges remain. Nevertheless, the case studies presented here underline the value of a DNA microarray approach. Its use links ecotoxicological effects that result from an exposure with specific expression profiles of several thousand genes. In this respect, DNA microarray approach facilitates the identification and classification of gene responses to drugs and environmental pollutants, and potentially may contribute toward aiding in risk assessment and biomarker research. However, it must be emphasized that, to date, most studies lack appropriate bioinformatic and statistical support and thus fail to exploit the true power of global transcript screening. Moreover, a major stumbling block remains, namely the challenge of defining the link between molecular genetic data and phenotypic effects of ecotoxicological importance. Indeed, once reliable differential expression profiles have been determined the following question arises: 'What do these differences mean in an ecotoxicological context?' Due to the huge number of regulated genes, it is still difficult to distinguish between specific responses to a single substance and more general detoxification of the organism. Moreover, the multitude of significant gene regulations may correlate with the huge number of potential contaminants that are potentially present in the environment. The challenge is to discriminate those alterations associated with toxicity from changes that are not related or occur as a normal adaptive response which is not associated with injury to the individual cell or organ. By further selecting the appropriate set of genes for investigation, one single DNA microarray experiment could possibly incorporate numerous biologically relevant endpoints within one assay. Ideally, the integration of genomic (and also proteomic and metabolomic) data with traditional ecotoxicological parameters will identify mechanistically based agglomerative biomarkers and elucidate mechanistic networks that can be used to develop predictive quantitative models. Then this approach can be used to establish thresholds of toxicity and predict exposure levels of a contaminant or complex mixtures required to elicit a particular biomarker or adverse response (Boverhof and Zacharewski, 2006). As mentioned before, adverse effects, however, can rarely be attributed to an individual event. Most responses to a pollutant will involve complex interactions between genes, proteins, and metabolites. At present, only ecotoxicogenomics provide the key to determine (simultaneously) the broad molecular status of an organism experiencing a toxicological challenge. Ecotoxicogenomics, in its present form, typically identifies genes that are differentially expressed in a largely descriptive manner and devoid of hypotheses and assumptions. Another promising approach is the inter-species comparison of gene expression profiles, which will reveal evolutionary conserved molecular events in response to toxicosis. Ecotoxicogenomics is therefore not limited to identifying novel candidate genes as biomarkers but also aims to pinpoint stressor specific expression signatures and reveal stress modulated molecular pathways, finally translating DNA microarray from a laboratory bench exercise to the future environmental safety assessments — undoubtedly one of the holy grails.

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Glossary

ABC transporters: ATP-binding cassette transporters, are transmembrane proteins that function in the transport of a wide variety of substrates across extra- and intracellular membranes
Apoptosis: main type of programmed cell death in multicellular organisms, involves a series of biochemical events leading to a characteristic cell morphology and death
cDNA: a single-stranded DNA complementary to an RNA, synthesized from it by in vitro reverse transcription.
Chaperonins: protein complexes that assist the folding of these nascent, non-native polypeptides into their native, functional state, belong to a large class of molecules that assist protein folding, called molecular chaperones
Cuticle: or cuticula is a multi-layered structure outside the epidermis of many invertebrates, notably roundworms and arthropods, in which it forms an exoskeleton
Cyclophilin: proteins that bind to ciclosporin (cyclosporine A), an immunosuppressant
DNA array: collection of DNA spots, commonly representing single genes, arrayed on a solid surface by covalent attachment to a chemical matrix, qualitative or quantitative measurements utilize the selective nature of nucleic acid hybridization under high-stringency conditions and fluorophore-based detection.
Ecotoxicogenomics: integration of genomics into ecotoxicology, application to organisms that are representative of ecosystems and used to study the hazardous effects of chemicals on ecosystems as well as individuals on the level of genomics
Epialleles: alleles that differ from each other in the patterns of methylation of DNA nucleotides of the gene, rather than stable nucleotide mutations
Eukaryotes: organisms whose cells are organized into complex structures by internal membranes: animals, plants, fungi, and protists.
Gene expression: process in which the inheritable information in a gene is made into a functional gene product, such as RNA and/or protein
Genomics: methods to analyze a given genome (the entire genetic material of a cell)
Hematopoiesis: formation of blood cellular components. All of the cellular components of the blood are derived from hematopoietic stem cells.
Homeostasis: the property of a living organism, that regulates its internal environment so as to maintain a stable, constant condition.
Katanin: a microtubule-severing AAA protein (= **A**TPase **A**ssociated with diverse cellular **A**ctivities)
Metabolomics: study of unique chemical fingerprints (especially small-molecule metabolite profiles) that specify cellular processes leaving behind, can vary with external environmental conditions
Methemoglobinemia: disorder characterized by the presence of a higher than normal level of methemoglobin in the blood. Methemoglobin is a form of hemoglobin that does not bind oxygen. When its concentration is elevated in red blood cells, anemia and tissue hypoxia can occur
Necrosis: accidental death of cells and living tissue; in contrast to apoptosis, does not send cell signals which tell nearby phagocytes to engulf the dying cell.
p53: also known as protein 53 (TP53), is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. It is important in multicellular organisms as it helps to suppress cancer.
Prokaryotes: group of organisms that lack a cell nucleus (= karyon), or any other membrane-bound organelles.

Proteomics: research into the creation, function and effect of the entire protein material in cells and/or organisms
ROS: reactive oxygen species, include oxygen ions, free radicals, and peroxides, both inorganic and organic. During environmental stress, ROS levels can increase dramatically, which can result in significant damage to cell structures.
Transcriptomics: study of the set of all messenger RNA (mRNA) molecules or 'transcripts', produced in one or a population of cells or even a whole organism; can vary with external environmental conditions
Ubiquitination: or ubiquitylation refers to the post-translational modification of a protein by the covalent attachment of one or more ubiquitin monomers. Ubiquitin is a highly-conserved small regulatory protein that is *ubiquitous* in eukaryotes. The most prominent function of ubiquitin is labeling proteins for degradation. Besides this function, ubiquitination also controls the stability, function, and intracellular localization of a wide variety of proteins.
Vitellogenin: egg yolk precursor protein expressed predominantly in female organisms, classified as a glyco-lipoprotein, having properties of a sugar, fat, and protein.
Zona pellucida: glycoprotein membrane surrounding the plasma membrane of an oocyte