Meeting the Challenges of Aquatic Vertebrate Ecotoxicology

MICHAEL J. CARVAN III, JOHN P. INCARDONA, AND MATTHEW L. RISE

The field of ecotoxicology uses biomarkers to assess the health of populations of sentinel organisms and to determine risk associated with environmental chemicals. The tools of modern biology are being used to develop promising new suites of biomarkers that must be rigorously tested and validated within a comprehensive mechanistic understanding of how toxic chemicals in the environment influence basic physiology and behavior. The zebrafish is a well-established laboratory model organism with a well-equipped molecular toolbox for basic biology and biomedicine with logical applications in ecotoxicology. As a model organism for ecotoxicology, the zebrafish can be used to develop mechanistic models of gene-environment interactions that will provide a foundation for the development of genomic resources in other fish species. Integration of mechanistic molecular data from multiple fish species will lead to the development of integrated dynamic models that will enable better diagnosis and treatment of environmental disease and improved ecological risk assessments.

Keywords: ecotoxicology, zebrafish, biomarkers, comparative transcriptomics

An understanding of the impacts of harmful chemicals at the population, community, and ecosystem levels is the primary aim of ecotoxicology. The ability to diagnose the health effects of toxic chemicals at these scales, primarily in nonhuman organisms, often hinges on biomarkers, characteristics that can be objectively assessed as an indicator of a pathophysiological state. Because of the increasing use of gene products as biomarkers, ecotoxicology is one of the many biologically based scientific fields that will advance dramatically in the next few decades, thanks to technological innovations in the detection of biomolecules and continued rapid developments in computing. The grand challenge in ecotoxicology is to develop integrated dynamic models of the relationships among organismal-, population-, and ecosystem-scale changes that incorporate fundamental physical, chemical, and biological processes along varied temporal, organizational, and spatial scales, from high-frequency molecular dynamics to decadal regional and global changes (figure 1). Biologically based systems are inherently nonlinear and stochastic, making the mathematical, statistical, and computational challenges of such an endeavor quite formidable (Hastings et al. 2005). Many subsets of this modeling task have commenced in separate fields but need to be integrated for the purposes of ecotoxicology to make predictions of ecological change on the basis of efficiently acquired environmental and biomarker data.

Ecotoxicology and environmental public health are very closely related—there is a strong natural linkage between the health of ecosystems and the health of people that are part of them. Despite considerable overlap, however, the primary focus of these fields is quite different (see box 1). Ecotoxicology is inherently concerned with risks to the health of species, populations, communities, and ecosystems, whereas environmental public health is concerned with risks to individuals or populations of humans from stressors other than toxic chemicals. The high degree of evolutionary conservation among vertebrates often blurs the distinction between human health and the health of other organisms. At the same time, mechanistic studies in environmental toxicology are crucial for the development of biomarkers. Throughout this article, we will focus primarily on ecotoxicology in aquatic and marine systems; however, many of the concepts are applicable across all areas of ecotoxicology.

Michael J. Carvan III (e-mail: carvanmj@uwm.edu) is a Shaw associate scientist at the Great Lakes WATER Institute, University of Wisconsin–Milwaukee. His research focuses on the effects of developmental toxicants on adult phenotypes and the role of genetic variability in susceptibility and resistance to environmental pollutants. John P. Incardona is a research toxicologist at the Environmental Conservation Division of National Oceanic and Atmospheric Administration's Northwest Fisheries Science Center in Seattle, Washington. His research focuses on contaminant impacts on fish health and on conservation medicine. Matthew L. Rise is an assistant professor and Canada Research Chair (Tier 2) in Marine Biotechnology at the Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Canada. His research focuses on the development and use of genomic tools for studies related to fish diseases, environmental toxicology, and aquaculture. *© 2008 American Institute of Biological Sciences.*

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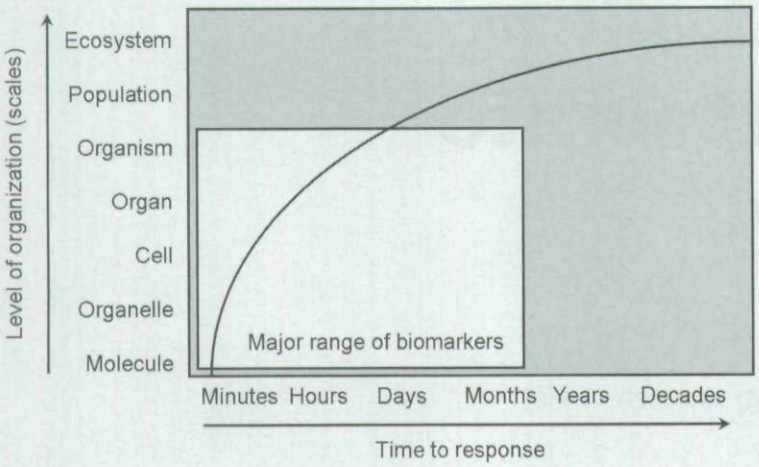

Figure 1. Biological organization and responses to stress. The line represents the relationship between the levels of biological organization and the time period until manifestation of a biological response. Biomarkers can reflect responses from the molecular to the organismal scale; however, responses at the higher end of the scale are the most likely ones to have ecological relevance. Responses at the cellular level are the basis for transcriptomics, proteomics, and metabonomics. Source: Based on a figure in Braunbeck (1998).

Biomarkers

Biomarkers reflect an integrated response to all the toxicological and pharmacological interactions of the mixture of compounds to which a sentinel organism is exposed and any other components of the environment in which it resides. Biomarkers have been used in ecotoxicology for decades, and lately advances in the fields of modern molecular biology and analytical chemistry have created a great deal of enthusiasm for the use of molecular biomarkers in ecotoxicology. The term "biomarker" is used in many different ways in the fields of biology and medicine. At its most basic level, a biomarker is a biologically derived quantifiable parameter that is used as an indicator of the biological state of an organism or cell. Some of the early biomarkers used in medicine are familiar and are still in use today—for example, oral body temperature, blood pressure, and hematocrit (packed red blood cell volume). Molecular biomarkers are widely used in the field of toxicology to assess responses to xenobiotic chemicals, and in cancer biology for preventative screening and tumor-typing. One of the earliest biomarkers in ecotoxicology was thinning eggshells in predatory birds, which was determined to be caused by DDE (dichlorodiphenyldichloroethylene), a metabolite of the then widely used pesticide dichlorodiphenyltrichloroethane, or DDT (Heath et al. 1969).

Ecotoxicological biomarkers can generally be considered indicators of health status, exposure, effect, or susceptibility. A number of biomarkers that are currently used in the field of ecotoxicology and a few examples are listed in table 1. These biomarkers were developed in the laboratory and later validated in field studies as predictive of chemically induced pathogenesis. Although each of the these biomarkers is considered a specific indicator of exposure or effect for a specific chemical class or classes, this is not necessarily true because a number of factors can modulate their responsiveness (Tilton et al. 2006, Hansen et al. 2007, Hwang and Bowen 2007). The "universal" nature of these biomarkers for a given individual may be affected by gender, nutritional status, genetic differences, seasonal cycles, life stage, acclimation, organ or tissue measured, and possibly other factors. In a recent review by Forbes and colleagues (2006), an example of the relationship between tissue polychlorinated biphenyl (PCB) levels and CYP1A1 induction (Smith et al. 2003) is used to illustrate that unknown variables influence biomarker expression. The data show that tissue PCB levels explain 35% to 40% of the variation in CYP1A1 induction. This may not be a very good correlation for each individual, but on a population scale for ecological risk assessment, this single biomarker is a decent predictor of risk.

This example also illustrates the primary difference between the use of biomarkers in humans and in wildlife. Biomarker levels in individual humans are used to assess risk in that particular individual, whereas biomarker levels in samples from wildlife

Box 1. Glossary of terms.

Ecotoxicology is the science relating to the harmful effects of chemicals and chemical aggregates (e.g., pollutants, pharmaceuticals, food additives, nanoparticles) on species, populations, and the natural environment. Ecotoxicology is dependent on the fields of ecology, toxicology, chemistry, mathematics, statistics, and environmental science.

Environmental public health is the science relating to the effects of environmental chemicals, chemical aggregates, radiation, noise, and other stressors on human disease. Many aspects of environmental health are also included within the fields of pharmacology and occupational health.

Genomics is the study of the structure and function of genomes. A genome is the complete gene complement of an organism.

Metabonomics is the study of response in the metabolome to changes in physiological state. The metabolome is the complete complement of the small-molecule metabolites of an organism.

Proteomics is the study of the proteome. A proteome is the complete protein complement of an organism.

Transcriptomics is the study of transcriptional initiation, processing, and degradation of the transcriptome. A transcriptome is the complete messenger-RNA complement of an organism.

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Table 1. Selected molecular biomarkers commonly used in ecotoxicology and the biological state for which it is used as an indicator.

Biomarker (reference)	Biological state
CYP1A induction—enzyme activity, messenger RNA (mRNA), protein (Sarkar et al. 2006)	Exposure to aromatic hydrocarbons
Vitellogenin induction—mRNA, protein (Thorpe et al. 2007)	Exposure to estrogen receptor agonist in adult male fish
Metallothionein induction (Sarkar et al. 2006)	Exposure to metals
Increased DNA damage—adducts, strand breaks (Shugart and Theodorakis 1998)	Exposure to (and effects of) promutagens and mutagens
Acetylcholinesterase inhibition—activity (Sarkar et al. 2006)	Exposure to (and effects of) carbamate and organophosphate pesticides
Heat shock protein induction—mRNA, protein (Mukhopadhyay et al. 2003)	General stress indicator

populations are used to assess risk to the entire population. In many cases, sentinel species are used to assess risk to a number of populations (or species) that may be evolutionarily closely related or may occupy a similar niche within an ecosystem. The incorporation of additional biomarkers may improve the predictive ability of any assessment, but the biomarkers must be integrated into a mechanistic model and anchored either to exposure or to a toxicologically relevant phenotype.

Development of new biomarkers

Ideally, the development of new biomarkers—actually, suites of biomarkers—will follow from mechanistic, dynamic, multiscale models of organism-environment interactions with a strong connection to pathogenesis and fitness. These new suites of biomarkers would be subjected to stringent laboratory validation procedures followed by rigorous validation in the field. The models should provide evidence for the strengths and limitations for each individual biomarker and support for the linkages within the entire suite of biomarkers. Unfortunately, such models do not yet exist.

Fortunately, there are a number of high-throughput technologies available for the development of new biomarkers from the complete assemblage of messenger RNA (mRNA) molecules, proteins, or small molecules in a sample, using techniques based in transcriptomics, proteomics, and metabonomics, respectively (see box 1). Most species of direct ecological relevance do not have the genomic resources (either a fully sequenced genome or a very large collection of expressed complementary DNA [cDNA] sequences) necessary to efficiently utilize transcriptomic or proteomic techniques. The latest generation of ultra-high-throughput DNA sequencers (e.g., Roche's Genome Sequencer FLX System, based on 454 pyrosequencing technology) will produce many tens of thousands of expressed cDNA sequences longer than 200 base pairs in a single run, at very low cost per base. A single run from an ecologically relevant species with little or no genomic resources can produce enough sequence data for the fabrication of a custom oligonucleotide microarray that can be used to identify promising new biomarkers.

Custom arrays will be vital for testing the suitability of mechanistic models for new organisms but the construction of new mechanistic models will require analysis of the full complement of gene products and small molecules. A model organism with the ability to genetically manipulate the expression of key biomolecules is critical for testing these models and for validating the utility of biomarkers identified using these dynamic, mechanistic models. For aquatic ecotoxicology, the zebrafish (*Danio rerio*) is very likely the best model organism for the vast majority of aquatic vertebrates.

The zebrafish model system

The development of zebrafish as a model system for genetic analysis is largely due to the pioneering efforts of the late George Streisinger (Grunwald and Eisen 2002). Originally a bacteriophage geneticist, Streisinger recognized that zebrafish had many of the qualities that made invertebrates such as the fruit fly *Drosophila* so amenable to genetic studies. These include high fecundity, short generation time, and ease of husbandry; moreover, hundreds of zebrafish embryos can be reared in Petri dishes, and, importantly, rapid searches for interesting phenotypes can be done by a simple visual screen. Also, as discussed in detail below, the zebrafish embryo was particularly accessible for traditional embryological study and manipulation.

However, major technical challenges had to be surmounted to bring the zebrafish model to the high level of efficiency and sophistication that we know today. By the early 1980s, Streisinger and colleagues had developed methods to mutagenize females with gamma irradiation, screen for mutants in homozygous embryos generated by parthenogenesis (production of diploid embryos from eggs only), and map relative locations of genes on chromosomes (Grunwald and Eisen 2002). After Streisinger's death, his efforts were carried on by his students and colleagues (particularly Charles Kimmel), who used these tools to isolate and characterize a small number of informative developmental mutants. This groundwork demonstrated the tractability of vertebrate developmental genetics in the zebrafish.

Several advancements in the 1990s, built on Streisinger's foundation, established the zebrafish as a high-throughput "forward" genetic system. First were the techniques for efficient chemical mutagenesis of the male germ line (using ethylnitrosourea, or ENU) and for maintaining large numbers of fish in individual families while maximizing their health and fecundity. Methods for insertional mutagenesis, which greatly facilitates cloning target genes, were also developed (Amsterdam et al. 1999). Second is the ability to locate newly isolated mutations on a conceptual genetic map based on recombination frequencies. A linkage map of

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generally anonymous DNA markers (e.g., microsatellites) has been generated using random amplified polymorphic DNAs, simple sequence length polymorphisms, single-nucleotide polymorphisms (SNPs), and other microsatellite methods. Using these markers, mutations are most often mapped by polymerase chain reaction (PCR)-based bulked segregant analysis or centromere-linkage analysis (Johnson et al. 1996, Postlethwait and Talbot 1997). A radiation hybrid map has been developed with zebrafish or mouse hybrid cell lines containing random zebrafish chromosome fragments produced by irradiation, on which expressed sequence tags are mapped relative to chromosome breakpoints by PCR (Geisler et al. 1999). A final requirement for the zebrafish forward genetic system is a physical DNA map, ideally integrated with the linkage map through cytogenetics, and the ability to clone the gene of interest. Most genes are isolated by positional cloning, whereby a molecular marker that is tightly linked to the mutated gene is used to identify a cloned genomic DNA fragment from a region near the gene (Talbot and Schier 1999). The genome database and the expanding database of gene expression patterns often implicate candidate genes. Although greatly facilitated by the zebrafish genome project, mapping and cloning of mutated genes remain the most challenging aspects of zebrafish genetics.

Zebrafish mutagenesis screens

As with *Drosophila*, zebrafish embryos produced from mutagenized adults can be rapidly scanned for lethal effects or even subtle changes in embryonic structures. This is because the zebrafish chorion (or eggshell) is clear, allowing easy viewing of embryogenesis as it rapidly progresses from fertilization to hatching in two to three days. The chorion is also delicate and there is a large perivitelline space, allowing easy dechorionation and microinjection, which is important for labeling cells in the embryo, transplanting cells between embryos, and numerous other manipulations. The early embryo itself is transparent and relatively large, and the yolk is pigment free, conditions that allow the direct observation of virtually every tissue and organ system developing in real time with very simple optics. These features allow a detailed description of key developmental landmarks and standardization of a method for staging embryos (Kimmel et al. 1995), which is crucial for monitoring the development of specific tissues in mutants.

The ability to follow dye-labeled cells in the live embryo, and to transplant labeled cells between mutant and wild-type embryos, allow two important processes: (1) the identification of regions in the early gastrula embryo that gave rise to distinct tissue types, and (2) the creation of genetically mosaic embryos. The latter permits the determination of cell autonomy, that is, whether or not a gene's function is required within the cells that show the mutant phenotype. This is a key strength of the zebrafish system, and a wide range of approaches for generating specific types of mosaics have been developed (Carmany-Rampey and Moens 2006). The intersection of genetics and development in the zebrafish model illuminates the broadly conserved nature of developmental mechanisms and signaling pathways among all vertebrates, and paved the way for the large-scale screen initiated in 1993.

The details of the tour de force screens carried out simultaneously in Boston and Tübingen have been reviewed elsewhere (Eisen 1996, Grunwald 1996). Briefly, two groups comprising several labs and a few score students and postdocs screened more than a million F3 progeny of mutagenized fish (figure 2). Using simple dissecting microscopes, embryos were examined for structural changes and assayed for some simple locomotor behaviors. The combined results were 1858 mutations in about 500 different genes affecting nearly all aspects of embryogenesis and early larval development, as well as some affecting the neural circuitry underlying simple behaviors. While the F3 screen is still the most commonly used and simple approach, there are a variety of screening strategies that can be tailored for specific needs (Patton and Zon 2001), and new screens are limited only by the ingenuity of individual investigators. For example, efficient methods for producing transgenic fish have even further expanded the utility of zebrafish genetic screens. There is an growing collection of transgenic lines that express reporters such as green fluorescent protein in specific tissues. With confocal microscopy, this allows real-time *in vivo* imaging of cells and developing structures in three dimensions. These lines provide an essential link between cell biology and development, and they also provide the basis for mutagenesis screens precisely targeting specific tissues or processes.

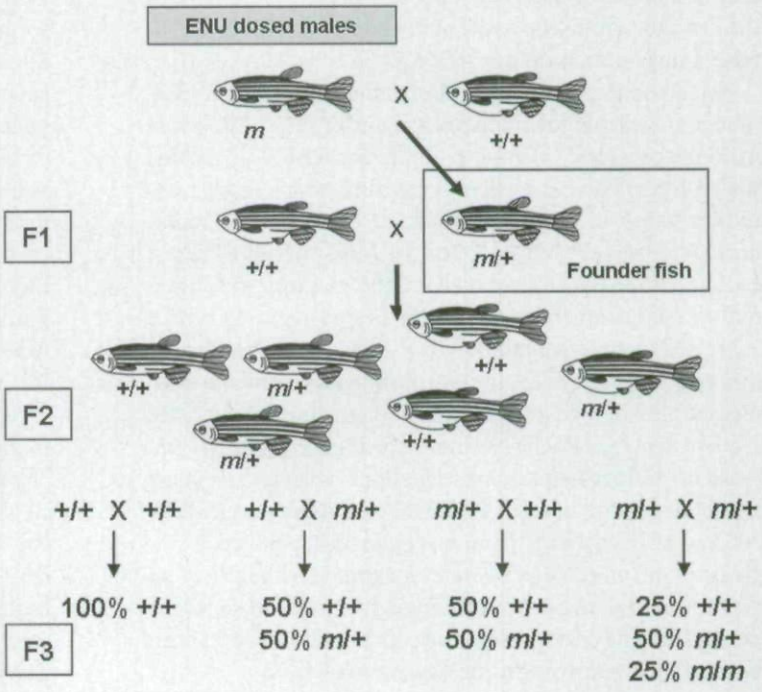

Figure 2. Schematic of an ethylnitrosourea-mutagenized F3 screen.

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The ongoing sequencing and annotation of the zebrafish genome continues to identify novel genes for which mutants are not yet isolated. The functions of these genes can be studied through "reverse" genetics, that is, starting with a known gene product and working backward to genetically alter its function. The powerful gene targeting (or "knockout") methods used in the mouse are currently impossible in zebrafish because of a lack of embryonic stem-cell lines. One feasible but very resource-intensive approach is "resquencing," or TILLING (target-induced local lesion in genomes; Wienholds et al. 2003).

A now common and widely used technique in zebrafish uses antisense morpholino oligonucleotides to block production of a gene product (Nasevicius and Ekker 2000). Morpholinos are single-stranded oligonucleotides containing a morpholine backbone instead of ribose, which renders them resistant to ribonucleases and hence long-lived when injected into cells or embryos. Designed using a sequence that complements either the ribosome binding site in mRNAs or intron-exon splice-site junctions in pre-mRNAs, morpholinos bind the target site and block access of the proteins necessary for translation or intron splicing, respectively. When microinjected into early cleavage–stage embryos, morpholinos are distributed to all the cells of the embryo. The result is a marked reduction in the normal protein product that can mimic, or phenocopy, loss-of-function mutations in the same gene (Nasevicius and Ekker 2000). The disadvantage of morpholinos is that their utility is restricted to the embryo and early larva, with the effect decaying thereafter. Nevertheless, reverse genetic techniques are an area of intensive investigation in zebrafish, and improved methods are not far off the horizon.

Genomic tools in zebrafish

Because of the zebrafish's utility as a model system for developmental genetics, a number of microarray-based tools have become commercially available. Affymetrix (Santa Clara, CA), Agilent (Santa Clara, CA), and Nimblegen (Madison, WI) market products for transcriptomic analysis. These tools have been used to explore the molecular mechanisms involved in biological processes such as endocrine disruption (King Heiden et al. 2008), caudal fin regeneration (Schebesta et al. 2006), and eye development (Leung et al. 2007). Additional tools are commercially available for analysis of gene-protein interactions (ChIP-on-chip), comparative genome hybridization, and micro-RNA expression, while individual labs have developed custom tools for gene mapping and limited SNP analysis (Stickney et al. 2002). A wide variety of tools are available for genomic and transcriptomic analysis of zebrafish responses to ecotoxicologically relevant stressors.

Zebrafish as a model in toxicology

The use of zebrafish in toxicology and, more specifically, ecotoxicology has been fairly limited, and has not strayed very far from the roots of traditional toxicology. For obvious reasons, zebrafish have figured most prominently in developmental toxicology and teratology. Among the most intensively studied environmental contaminants are the halogenated dioxins, which are thought to have contributed strongly to the decline of lake trout (*Salvelinus namaycush*) in the Great Lakes through impacts on early life-history stages (Cook et al. 2003). Exposure of fish embryos to halogenated dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes a characteristic syndrome of cardiovascular defects and edema, which are initiated by activation of the aryl hydrocarbon receptor (AHR; Peterson et al. 1993). The AHR is a ligand-activated transcription factor that controls the expression of a battery of genes encoding enzymes that convert many hydrophobic xenobiotics to water-soluble derivates that are excreted. These enzymes include mixed-function oxygenases such as members of the CYP1A gene family. Halogenated dioxins and many polychlorinated biphenyls are potent AHR ligands, but as poor substrates for CYP1A and other enzymes, they are not metabolized and therefore bioaccumulate.

A combination of descriptive developmental toxicity, molecular biology, and morpholino knockdown studies have led to a model in which TCDD developmental toxicity in fish results from persistent activation of the AHR in the developing heart, leading to a failure of cardiomyocyte proliferation and concomitant heart malformation. These effects are apparently independent of CYP1A induction, implicating a mechanism involving other AHR target genes. Gene expression profiling of TCDD-exposed zebrafish larvae has been performed using DNA microarrays, with one study using a transgenic line with fluorescent cardiomyocytes to aid in isolation of cardiac-specific RNA. Particular changes in cardiac-specific gene expression were identified, and a cluster of cell-cycle progression genes are also dramatically downregulated (Chen et al. 2008). There is still no clear link between downstream targets of the AHR and the biological effects of TCDD.

Generally, the small-molecule screening approach has proved fruitful in zebrafish developmental toxicology, although it has been used on a limited scale. Novel pathways of toxicity have been identified for several pesticides (Stehr et al. 2006), metals (Blechinger et al. 2007), and polycyclic aromatic hydrocarbons (PAHs; Incardona et al. 2004, 2005). While all these studies were aided by many of the conveniences of the zebrafish system, some illustrate that one of the most powerful tools is simply the continually growing collection of zebrafish mutants. Comparison of chemical-induced effects and mutant phenotypes is an efficient way to identify target tissues or pathways (Incardona et al. 2004, Stehr et al. 2006). The PAHs are a particularly illuminating case.

PAHs are a large and diverse family of compounds derived from fossil fuels and their combustion. Because PAH levels in the environment are directly proportional to human population density, these ubiquitous compounds are increasing in aquatic habitats as a result of atmospheric deposition. Studies following the 1989 *Exxon Valdez* oil spill in Prince William Sound, Alaska, identified a syndrome that occurred in fish embryos and larvae exposed to PAHs in weathered crude oil; the syndrome's features overlapped considerably with the effects

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of halogenated dioxins. The family of PAHs includes hundreds of compounds that always occur in complex mixtures, providing an ideal case for application of the zebrafish model. A small-scale but systematic comparison of the biological effects of crude oil to nine individual PAHs (representing abundant classes) has identified novel modes of action (Incardona et al. 2004, 2005). Because some PAHs are known to be AHR ligands, morpholino knockdown of AHR/CYP1A pathway components was used to determine the role of this pathway in the effects of different PAHs. In this case, several three-ringed PAHs were found to have novel impacts on embryonic cardiac function that were independent of the AHR (Incardona et al. 2005), while comparison with zebrafish cardiac mutants suggested the compounds directly target proteins involved in the conduction of electrical impulses during cardiac contraction, such as ion channels (Incardona et al. 2004).

Despite the increasing use of the zebrafish model in ecotoxicology, its strongest aspect, forward genetics, has not yet been used at all. In the same manner that forward genetics has been used in zebrafish to identify developmental signaling pathways, it could be applied to the identification of pathways that are disrupted by chemical exposure. A powerful and proven technique in *Drosophila* genetics is the identification of mutations that enhance or suppress a particular phenotype (St Johnston 2002). Similarly, mutagenesis screens for modifiers of toxicity could help characterize involved genes or pathways. For example, a visual screen for embryos that do not develop edema when exposed to TCDD could identify mutants with suppressed TCDD toxicity, and ultimately AHR target genes. The prevention of TCDD toxicity by an AHR morpholino indicates the feasibility of such a screen; presumably, AHR loss-of-function mutations would be included among resistant mutants. Screens identifying mutants with altered sensitivity to cocaine (Darland and Dowling 2001) and resistance to drug-induced seizures (Baraban et al. 2007) also attest to the feasibility of this approach. At the same time, it is remarkable that early vertebrate embryos (e.g., cleavage or gastrula stage) exhibit a high degree of resistance to many xenobiotics, possibly because of the presence of multidrug transport proteins (Hamdoun et al. 2004). Mutagenesis screens for enhanced susceptibility to chemicals in zebrafish could potentially identify unknown detoxification pathways, as well as provide insight into pathways disrupted by chemical exposure. Although insertional mutagenesis screens are still fairly resource intensive, ENU mutagenesis screens focusing on robust phenotypes can be carried out with a few people and a modest fish colony, and require far fewer resources than do most transcriptomic approaches.

Other capabilities and modern applications of the zebrafish model remain relatively underutilized in ecotoxicology. There is increasing use of transgenic lines for studies of toxic mechanisms and contaminant effects (Blechinger et al. 2007). The same features that make zebrafish a good forward-genetics system are excellent for other high-throughput techniques. Libraries containing thousands of small molecules can be screened for a range of biological activities using zebrafish embryos arrayed singly or in triplicate in 96-well plates. Although investigators generally are still required to visually inspect embryos, the process is facilitated by the use of robotics to distribute test compounds, and in some cases imaging techniques for quantifying biological effects. Such "small-molecule screens" on zebrafish embryos are now common in the area of drug discovery (Murphey and Zon 2006). However, these methods are especially applicable to evaluating the joint toxicity of chemical mixtures, a major challenge in both human and aquatic environmental toxicology. The difficulty is due in part to the sheer numbers of chemicals that enter the environment. To incorporate ecological realism, laboratory studies of mixture toxicity must contend with the expanding complexity and expense of a factorial experimental design. This goal is feasible with zebrafish embryos in multiwell plates and robotics to generate factorial combinations of contaminants, for example, different PAHs and metals.

Virtually all of the methods developed for zebrafish genetic screens can be applied to ecotoxicological questions. However, there are some limitations. The greatest strengths of the zebrafish system are the embryo and its manipulability, the extensive genomic tools and resources, and the collection of developmental mutants. The tools and capabilities are much more limited for assessing effects in late larval, juvenile, and adult stages. Their small size makes biochemical and some physiological studies more challenging. Nevertheless, the zebrafish model is in many ways still under construction, and many of these limitations will undoubtedly be overcome.

Comparative transcriptomics in ecotoxicology

Toxicogenomics is a relatively new field that uses genomic research tools and techniques, such as suppression subtractive hybridization (SSH) cDNA library construction and characterization, DNA microarrays, and quantitative reverse transcription–PCR to determine how exposure to chemicals and chemical aggregates affect molecular pathways and biological processes in individuals and populations. Ecotoxicological research aims to shed light on the mechanisms by which chemicals and chemical aggregates exert their effects on wildlife species, and the nascent field of ecotoxicogenomics involves the use of genomic resources and methods in ecotoxicological research. Teleost fish play important roles in toxicogenomics and ecotoxicology research, just as teleost models have played and continue to play key roles in research conducted in many fundamental areas of vertebrate biology (e.g., development, physiology, evolution).

Actinopterygii (ray-finned fishes) and Sarcopterygii (lobe-finned fishes and terrestrial vertebrates) diverged approximately 450 million years ago (Christoffels et al. 2004). More than 99% of Actinopterygii species (approximately 24,000) are teleosts (i.e., in the division Teleostei), making teleost fishes the most diverse and successful group of vertebrates (Christoffels et al. 2004). Since teleosts are found in most aquatic habitats on Earth, they serve as valuable sentinels of accelerated environmental change caused by human

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activities. Furthermore, research on how environmental degradation influences the gene expression and health of teleost fish will improve our understanding of how anthropogenic stressors may affect other vertebrates, including those in the Sarcopterygiian lineage (e.g., humans).

There is growing evidence that genomic tools and techniques are effective for identifying fish genes involved in responses to individual toxicants and to toxicant mixtures such as industrial effluents (table 2). Targeted cDNA library construction and characterization (Diatchenko et al. 1996) and DNA microarray hybridization have been popular transcriptomic techniques for identifying fish genes that respond to environmental toxicants (biomarkers of exposure).

For researchers working on nonmodel fish species, with little available DNA sequence information and no species-specific microarrays for global gene expression profiling, SSH libraries present the advantage of not requiring prior characterization of the transcriptome. SSH libraries are targeted gene discovery tools that are enriched for genes induced in "tester" samples (e.g., methylmercury-exposed liver of juvenile rainbow trout) relative to "driver" samples (i.e., control, nonexposed liver of juvenile rainbow trout). Reciprocal SSH libraries allow identification of genes that are induced or suppressed by a given toxicant (i.e., candidate biomarkers genes). As examples, SSH libraries have been used to identify carp genes potentially responsive to en-

Table 2. Examples of toxicogenomics studies involving fish species other than zebrafish.

Species	Reference	Microarray platform	Application
Carp (*Cyprinus carpio*)	Moens et al. 2006	1K *C. carpio* cDNA	Liver response to endocrine disrupting compounds
	Reynders et al. 2006	0.6K *C. carpio* cDNA	Liver gene expression response (GER) to cadmium
	Moens et al. 2007	1K *C. carpio* cDNA	Liver GER to 21-day exposure to industrial effluent
European flounder (*Platichthys flesus*)	Williams et al. 2003	0.2K *P. flesus* cDNA	Liver biomarkers of exposure to polluted water
	Sheader et al. 2006	0.5K *P. flesus* cDNA	Liver GER to cadmium injection
	Williams et al. 2006	13K *P. flesus* cDNA	Liver transcriptome changes after cadmium injection
	Williams et al. 2007	13K *P. flesus* cDNA	Liver transcriptome changes after estradiol injection
Fathead minnow (*Pimephales promelas*)	Klaper et al. 2006	0.2K *P. promelas* cDNA	Liver GER to dietary methylmercury exposure
	Wintz et al. 2006	5K *P. promelas* cDNA	Liver GER to waterborne exposure to explosive 2,4-DNT
	Larkin et al. 2007	2K *P. promelas* oligo.	Liver GER to waterborne exposure to 17ß-estradiol (E2)
	Mager et al. 2008	5K *P. promelas* cDNA	Whole fish GER to chronic lead exposure
Goldfish (*Carrasius auratus*)	Martyniuk et al. 2006	1.2K *C. auratus* brain on goldfish-carp microarray[a]	Brain GER to waterborne exposure to pharmaceutical estrogen (17α-ethinylestradiol)
	Marlatt et al. 2008	1.2K *C. auratus* brain[a]	Hypothalamus GER to E2 implantation
Medaka (*Oryzias latipes*)	Kishi et al. 2006	22.5K *O. latipes* oligo.	Whole fish transcriptome comparisons of males versus females, and adult male GER to E2
Rainbow trout (*Oncorhynchus mykiss*)	Koskinen et al. 2004	1.4K *O. mykiss* cDNA	Whole fish GER to sublethal toxicant exposures
	Krasnov et al. 2005	1.3K *O. mykiss* cDNA	Kidney and liver GER to sublethal toxicant doses
	Tilton et al. 2005	1.6K *O. mykiss* oligo.	Biomarkers of aflatoxin B_1 (AFB_1)-induced liver cancer
	Tilton et al. 2006	GRASP 3.5K cDNA	Liver GER to three different carcinogenic compounds
	Skillman et al. 2006	2 different platforms[b]	Male liver GER to 17α-ethynylestradiol exposure
	Tilton et al. 2007	1.6K *O. mykiss* oligo.	Impacts of DIM[c] and E2 on AFB_1-induced liver cancer
	Finne et al. 2007	GRASP 16K cDNA	Impacts of four individual toxicants and a synthetic mixture on the transcriptome of primary hepatocytes
	Aluru and Vijayan 2008	147 gene targeted *O. mykiss* cDNA1	Brain GER to AhR agonist or antagonist exposure

K, approximately 1000 genes.

Note: The species affiliations of probes (immobilized DNA on glass microarrays, also called "features" or "spots") are given under "microarray platform." The 1.6K rainbow trout microarray is composed of 70-base (70-mer) single-stranded DNA fragments called oligonucleotides ("oligos"), and the 22.5K medaka microarray is composed of 60-mer oligos. The GRASP (Genomic Research on Atlantic Salmon Project) 3.5K microarray includes 3119 unique Atlantic salmon (*Salmo salar*) cDNA probes and 438 unique rainbow trout cDNA probes (Rise et al. 2004), and the GRASP 16K microarray includes 13,421 unique Atlantic salmon cDNA probes and 2576 unique rainbow trout cDNA probes (von Schalburg et al. 2005). Information on fabrication and additional applications of the salmonid microarrays may be found in Rise and colleagues (2007). The 147-gene-targeted rainbow trout cDNA microarray includes fragments of genes with a variety of molecular functions, including metabolism, stress, and immune response (Wiseman et al. 2007). The 0.2K fathead minnow array (*www.ecoarray.com/Product/nylon_sub.htm*) is printed on nylon by EcoArray, Inc. (Alachua, FL). The 5K fathead minnow platform was an anonymous microarray (i.e., cDNA samples printed on slides were sequenced and identified only if they were informative experimentally).

a. This mixed-species cDNA microarray contains 1.2K goldfish genes from a subtracted brain cDNA library and 8K carp genes (Gracey et al. 2004). For closely related species, such as goldfish and carp (diverged less than 10 million years ago; Martyniuk et al. 2006) or rainbow trout and Atlantic salmon (diverged 8 million to 20 million years ago; Rise et al. 2004), cross-species (heterologous) hybridizations have been shown to provide high-quality gene expression data.

b. This study used both the GRASP 3.5K microarray platform (Rise et al. 2004) and a 32-gene microarray primarily containing estrogen-responsive genes.

c. DIM = 3,3'-diindolylmethane (found in cruciferous vegetables, and sold as a chemoprotective dietary supplement). In this study, DIM was found to promote tumorigenesis (Tilton et al. 2007).

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docrine disruptors (Moens et al. 2006), and European flounder genes potentially responsive to cadmium, benzo(a)pyrene, or a mixture of environmental contaminants from a polluted estuary (Sheader et al. 2004).

DNA microarrays are currently the most popular research tools for global gene expression studies, and microarray-based tools and techniques are becoming integral in many areas of research, including agriculture and medicine. Because of their importance as research models (e.g., biomedical, developmental biology, toxicology) and in global aquaculture, genomic resources such as expressed sequence tag (EST) databases and DNA microarrays have been developed for several teleost species. For example, large-scale genomic projects exist for salmonids (e.g., Genome Canada–funded Genomic Research on Atlantic Salmon Project, or GRASP; *http://web.uvic.ca/cbr/grasp*), Atlantic cod (e.g., Genome Canada–funded Atlantic Cod Genomics Project; *http://codgene.ca/index.php*), catfish (e.g., Auburn University's Catfish Genome Project; *www.ag.auburn.edu/fish/peaks-of-excellence/aquaculture/catfishgenome.php*), and other species of interest to the global aquaculture industry. Large-scale genomic research projects on fish generally begin with gene discovery efforts involving cDNA library construction, DNA sequencing, and EST database creation (e.g., Rise et al. 2004, 2007, 2008). After the gene discovery phase, these projects often focus on building resources for functional genomics research (e.g., cDNA or oligonucleotide microarrays).

Resources arising from large-scale genomics projects, such as EST databases and DNA microarrays, are valuable tools for toxicogenomic research. As examples, GRASP microarrays (Rise et al. 2004, von Schalburg et al. 2005) have been used to identify rainbow trout liver genes that are responsive to carcinogenic compounds (Tilton et al. 2006) and other toxicants such as 17α-ethinylestradiol (EE2) and TCDD (table 2; Finne et al. 2007). In addition, DNA microarrays have been used to study carp, European flounder, fathead minnow, goldfish, and medaka, and gene expression responses to toxicants (table 2 and references therein). In addition to their use in research related to toxicology (table 2), fish DNA microarrays have been used to identify genes and molecular pathways involved in fish responses to pathogens, vaccines, stress, and growth hormone transgenesis. Reviews of fish microarray platforms and their applications have been published recently (e.g., Douglas 2006, Rise et al. 2007).

All of these previously mentioned studies involved single fish species, although research approaches involving two or even three species should provide more reliable mechanistic information and more informative biomarkers. Comparative toxicogenomic research aimed at identifying toxicant-responsive gene expression signatures in evolutionarily divergent fish species will allow the identification of molecular biomarkers, and the mechanisms influencing toxicity, that are likely to be conserved among all fish. This type of multispecies approach (figure 3) uses the rapidly growing genomic resources for fish species such as zebrafish, Atlantic salmon, rainbow trout, medaka, and fathead minnow (table 3), as well as other publicly available resources such as the Comparative Toxicogenomics Database (Mattingly et al. 2006). Furthermore, cross-species

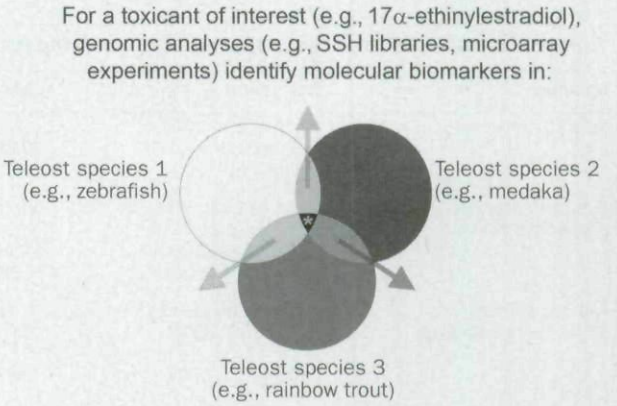

Figure 3. Example of a comparative toxicogenomics approach.

Table 3. Expressed sequence tag collections of selected fish species.

Selected orders of teleosts (bony fish)	Examples of species with genomic resources	Number of expressed sequence tags in GenBank
Superorder Protacanthopterygii		
Salmoniformes	Atlantic salmon (*Salmo salar*)	433,337
	Rainbow trout (*Oncorhynchus mykiss*)	260,887
Superorder Paracanthopterygii		
Gadiformes	Atlantic cod (*Gadus morhua*)	181,350
Superorder Ostariophysi		
Siluriformes	Channel catfish (*Ictalurus punctatus*)	44,767
Cypriniformes	Zebrafish (*Danio rerio*)	1,379,829
	Fathead minnow (*Pimephales promelas*)	249,941
	Common carp (*Cyprinus carpio*)	32,046
	Goldfish (*Carassius auratus*)	2924
Superorder Acanthopterygii		
Cyprinodontiformes	Killifish (*Fundulus heteroclitus*)	74,755
Pleuronectiformes	European flounder (*Platichthys flesus*)	7977
Beloniformes	Japanese medaka (*Oryzias latipes*)	609,699
Tetraodontiformes	Torafugu (*Takifugu rubripes*)	26,069
Gasterosteiformes	Three-spined stickleback (*Gasterosteus aculeatus*)	276,992

Note: Numbers of sequences were acquired from NCBI Entrez Taxonomy (nucleotide EST database; *www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy*).

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toxicogenomics research takes advantage of the strengths of different fish models. As an example, we will consider a two-species comparative toxicogenomics approach utilizing zebrafish (a warm-water fish) and rainbow trout (a cold-water fish). Zebrafish is a well-known genetic model for vertebrate developmental biology and genetics (Carvan et al. 2005), and rainbow trout is a prominent model for studies involving carcinogenesis, comparative immunology, and physiology (Thorgaard et al. 2002). Both zebrafish and rainbow trout have served as important model organisms for toxicology research (Thorgaard et al. 2002, Carvan et al. 2005). There are large EST sets for zebrafish and salmonids (table 3), and excellent microarray resources for these fish. Since naturally reproducing populations of rainbow trout appear on several continents, this species serves as an ecotoxicogenomic model, bridging the gap between the laboratory and natural aquatic environments. For ecotoxicology research, such multispecies toxicogenomic models will allow identification of molecular biomarkers that are conserved (e.g., shown using SSH or microarray experiments to be toxicant responsive in multiple fish species, and therefore potentially useful for studying the impact of pollutants on any teleost species of interest), as well as those biomarker genes that are toxicant responsive in some species (or lineages) but not in others (figure 3). Similarly, biomedical research models that utilize multiple teleost species and their respective genomic resources will potentially be more powerful and applicable to human health issues than single-species models, since genes and molecular pathways found to be involved in a biological process (e.g., response to pathogens or pollutants) in evolutionarily divergent fish species (figure 3) will potentially be conserved among all vertebrates, including humans.

Conclusions

Traditional toxicology—the study of poisons—connotes processes such as tissue and organ damage, serious illness, and even death. Not coincidentally, the most widespread tool in toxicology is the LD_{50} (the dose that is lethal to 50% of the organisms), and the biological pathways most often studied are those involved in metabolism and detoxification. Most of the problems facing modern ecotoxicology are much more subtle, however. The broader and more widespread problems are the exposure of animals to low levels of pollutants chronically, and exposure to complex mixtures of pollutants (Eggen et al. 2004). Our greatest challenges relate to our understanding of how non–point source pollution associated with rapid worldwide urbanization and coastal development have affected—and will continue to affect—marine and aquatic resources and the conservation and recovery of threatened or endangered species. Integrated dynamic models are needed that can link changes at the organismal, population, and ecosystem scales to a detailed mechanistic understanding of chemical impacts at the cellular, organ, and organismal levels. Suites of biomarkers are required to accurately assess the health of individuals and populations and to provide data for predictive models.

The zebrafish, which provides the foundation for mechanistic models and the development of new biomarkers in aquatic ecotoxicology, can make particularly strong contributions when used in this novel context. Contributions from other fish species with extensive genomic resources will strengthen the models and provide field validation for new biomarkers. The same high degree of conservation among vertebrates that led the National Institutes of Health to invest in developing resources for the zebrafish as a model for human diseases indicates that studies of chemical effects in zebrafish will be applicable to threatened or endangered fish species and to those fish that are difficult to rear in a laboratory setting.

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