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Mechanistic Research in Aquatic Toxicology: Perspectives and Future Directions

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Abstract

On the thirtieth anniversary of the journal, I provide a perspective on some of the questions and opportunities for new understanding that will interest aquatic toxicologists during the next thirty years. I focus on mechanisms of toxicity involving transcription factors, signalling pathways, and gene networks involved in toxic and adaptive responses in aquatic animals. Prominent questions address the value of a toxicity pathways approach in aquatic systems, issues involving extrapolation among species, identification of susceptibility genes and useful biomarkers of adverse effect, new emerging contaminants, the importance of epigenetic mechanisms, effects of multiple stressors, evolutionary toxicology, and the relative roles of technical and conceptual limitations to our understanding of chemical effects on aquatic systems.

Introduction

The thirtieth anniversary of the journal *Aquatic Toxicology* provides an opportunity to look back and reflect on what has happened in the field since the founding of this journal and to look forward to the promise and challenges of the next thirty years. After briefly commenting on the state of aquatic toxicology in 1981 and what we have learned since then, I consider some of the important questions that may occupy aquatic toxicologists for the next thirty years. Aquatic toxicology is a broad field; my comments focus primarily on efforts to understand mechanisms of toxicity and especially the role of transcription factors, signalling pathways, and gene networks in mediating toxic and adaptive responses to chemical exposure.

Aquatic toxicology in 1981

What were the topics being addressed in the field of aquatic toxicology in 1981? What were the most pressing problems? What were the methodological limitations? What advances have enabled progress to be made since then? What didn't we know then, but have learned since, changing the way we think about aquatic toxicology?

A glance at the founding editorial (Malins and Jensen, 1981) reveals that the overall goals of the field have remained largely unchanged: "…identifying…potentially toxic substances and…relating their presence in environments and organisms to alterations in life processes."

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Similarly, some of the major challenges of the field in 1981 still resonate today: “Methods for identifying biological alterations in aquatic organisms” and “the ability to link… chemical exposure to biological change.”

Papers in the first volume of the journal addressed these challenges by applying toxicological, physiological, and biochemical approaches to research in a handful of taxa, including fish (mostly trout and flounder) and molluscs. The major compounds of interest in this first issue were metals and PAHs; subsequent issues dealt with pesticides, PCBs, and a few other agents. The analytical methods were rather non-specific. For example, chemical analysis measured total PCBs rather than congeners; assays for cytochrome P450 induction were limited to measurement of total P450 and of hybrid activities such as AHH (aryl hydrocarbon hydroxylase) rather than individual CYP forms. Despite the limitations, much was learned about the exposure of aquatic organisms to chemicals and about the responses of organisms to those exposures. The broad outlines of important response pathways such as that mediated by the aryl hydrocarbon receptor (AHR) were established even without an understanding of the molecular details.

Aquatic Toxicology in 2011

If the goals and challenges faced today are similar to those faced by aquatic toxicologists in 1981, how is the field different now? What has been accomplished? What are the pressing problems today? What kinds of problems are anticipated in the next decade and beyond?

One obvious change is in the approaches and methods that have been developed—in many cases borrowed from biomedical researchers—to measure chemicals and their effects with ever increasing sensitivity, specificity, and sophistication. For example, the “unresolved complex mixture” of petroleum has been resolved (Frysinger et al., 2003) and the application of molecular and genomic techniques has enabled important advances in our understanding of how chemical exposure alters gene expression (Wang et al., 2010; Yang et al., 2007). There also has been substantial effort devoted to, and much progress made in, identifying and characterizing the genes and proteins involved in mechanisms of toxicity—the genetic tool kit. For example, numerous studies have addressed the comparative biology of transcription factors, biotransformation enzymes, and transporters that influence chemical effects (the “chemical defensome”), and how they vary among taxa (Goldstone et al., 2006).

Despite this new knowledge, there remain major gaps in our understanding of chemical impacts in aquatic systems—indeed, in all systems (Novak et al., 2011). What are some of these knowledge gaps, and what will be required to fill them?

Going Forward: Research Questions in Mechanistic Aquatic Toxicology

• *How do chemicals perturb biochemical pathways and cellular gene networks in aquatic organisms?*

In 1981, aquatic toxicologists were studying enzyme activities as indicators of altered gene expression in exposed animals. Subsequently, changes in the expression of single genes could be measured at the level of their encoded proteins (western blots) and mRNAs (various methods from *in vitro* translation to real-time RT-PCR). More recently, we have developed the ability to measure changes in the expression of thousands of transcripts (microarrays or deep sequencing), proteins (proteomics), or the resulting products of enzymatic reactions (metabolomics). How are all of these changes connected? Pathway and interactome analyses (e.g. Alexeyenko et al., 2010) are beginning to reveal a more integrated view of how these changes lead to altered cellular function, but our understanding in this area remains rudimentary. Fundamental research in model systems such as the sea urchin is

elucidating the nature of gene regulatory networks and how they function during biological processes, including development (Davidson, 2010; Peter and Davidson, 2011). Research to investigate how chemicals and other stressors perturb the function of those networks (e.g. Amit et al., 2009) will illuminate both mechanisms of toxicity and the resilience of the networks in the face of environmental change.

• *How do toxicological pathways and networks vary among species and higher taxa? Can we develop an evolutionary (and therefore predictive) view of how chemicals impact these networks?*

A recent report from the U.S. National Research Council (National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents, 2007) provided a vision of toxicology and toxicity testing in the 21st century, focusing exclusively on toxicology in relation to human health. That report and a series of papers exploring its implications (Andersen and Krewski, 2009, 2010; Collins et al., 2008) discussed a shift from whole-animal testing of chemicals to a system in which *in vitro* tests (using human cells) are used to identify and elucidate *toxicity pathways*—cellular response pathways that are perturbed by chemicals, disrupting cellular function. Data from dose-response modelling of how these pathways are affected by chemicals *in vitro* would be extrapolated using pharmacokinetic and exposure models to provide predictions of risk to human individuals and populations.

Such an approach involving the identification of toxicity pathways could have value in aquatic toxicology, but the challenges of applying this model to aquatic systems are immense. For example, instead of focusing on one species (humans), aquatic toxicologists are concerned with thousands of species. Establishing cell lines from each of those species, or even a representative few, is not practical. *In vivo* test systems such as zebrafish embryos or small invertebrates might be suitable (i.e. amenable to automation; inexpensive) for identifying toxicity pathways of relevance to aquatic toxicity in representative taxa, but we are left with the substantial challenge of extrapolating across species, and in some cases across families and orders, to make predictions about effects in exposed populations.

What features of pathways and networks elucidated in one species can be extrapolated to others? The evolutionary conservation of many important pathways, presumably including many of those impacted by toxicants (toxicity pathways), means that the task is not hopeless. However, it will require an evolutionary framework, because the degree of uncertainty involved in extrapolation across species will be related to the evolutionary distance separating the species. In addition, we must develop efficient ways to identify taxon-specific variations that may have evolved in some pathways, making them more or less sensitive to perturbation by chemicals. Identifying such variations will be facilitated by the ability to sequence—rapidly and relatively inexpensively—the genome of any target species. In this case, we will need to develop ways to extract the relevant information about pathway function from the component gene sequences and other information, such as expression analysis and molecular modelling of encoded proteins. Developing this capability will require the identification of better genetic markers of species differences in susceptibility to chemicals as well as early markers of adverse effects.

• *Can we identify and apply sensitive, specific, and truly useful biomarkers of adverse effect so that we can detect incipient damage to populations or ecosystems?*

Thirty years of biomarker research has led to valuable discoveries about how gene expression can be altered by chemical exposure (an example of one type of biomarker) with potential use of such changes as indicators of exposure or effect (but not necessarily *adverse* effect). Much effort has gone into developing such biomarkers, but practical application of

these tools has lagged. Aquatic toxicologists can now measure chemically altered gene expression across the genome with great sensitivity. To maximize the utility of the wealth of functional genomic data that are being generated, it will be increasingly important to tie those changes in gene expression to specific toxic endpoints ("phenotypic anchoring"). Thus, the most useful gene expression studies will incorporate multiple doses and assessment of relevant toxicological endpoints (e.g. Whitehead et al., 2010).

• *What are the key genes that influence susceptibility? Can we identify differences in gene sequences that are predictive of differences in chemical sensitivity among populations and species?*

Identifying susceptibility genes or "biomarkers of susceptibility" is a longstanding goal in both biomedical and environmental toxicology. In the biomedical arena, pharmacogenetic differences that predict the response to drugs are well known and individualized medicine based on such information is advancing rapidly. Progress has been made in aquatic toxicology as well, for example in defining the role of AHR variants and species differences in controlling the sensitivity of populations (Wirgin et al., 2011) or species (Head et al., 2008; Karchner et al., 2006) to dioxin-like compounds. However, not all differences in sensitivity to chemicals will be able to be assigned as unambiguously to a single locus or small number of key amino acid residues.

• *What are tomorrow's "emerging contaminants"?*

Over the past decade, increasing attention has been paid to groups of compounds considered together under the general heading of "emerging contaminants" or "contaminants of emerging concern". It certainly is appropriate that understudied or recently introduced chemicals such as brominated flame retardants, phthalates, nanoparticles, pharmaceuticals and personal care products have been targeted recently for enhanced scrutiny. In 2011, however, these chemicals are no longer "emerging" contaminants; they have emerged—even if we still do not fully understand their impacts. What chemicals are in the environment now but are not yet receiving sufficient research attention? What new chemicals will be introduced into products or processes and subsequently released into the environment over the next 30 years? Even if we cannot predict what some of those chemicals will be, does our basic understanding of toxicological mechanisms and our development of test systems and procedures ensure that adverse impacts can be identified in time to prevent long-term damage to aquatic systems?

• *How important are epigenetic mechanisms in aquatic toxicology?*

Epigenetics is all the rage in human health research, with emerging epidemiological and experimental evidence for non-genetic transgenerational inheritance of traits, imprinted genes, paramutation, and adult disease resulting from environmental factors acting in the fetal period ("fetal programming"). Reports suggesting the involvement of epigenetic mechanisms in chemical effects in rodents have stimulated additional research on the role of epigenetics in biomedical toxicology (Jirtle and Skinner, 2007). Nevertheless, the general importance of epigenetic mechanisms in toxicology remains unclear (with additional confusion caused by controversy over exactly which types of mechanisms are considered "epigenetic"). It seems likely that within the next few years, research in mammalian models will establish the mechanisms and relevance of epigenetics in toxicology. That understanding will facilitate research on the role of epigenetics in aquatic toxicology by allowing more focused questions to be asked. On the other hand, research in some aquatic model systems (e.g. fish embryos) could help establish fundamental features of epigenetic mechanisms of toxicity.

• *How does simultaneous exposure to multiple stressors influence the effects of environmental contaminants?*

Understanding effects of chemical mixtures has been a goal of toxicology for many years, and there are many examples of how chemicals interact, sometimes in unpredictable ways, to cause toxicity. While chemical mixtures continue to be of great interest, the study of interactions has expanded to include non-chemical stressors, which may affect the ability of a species or population to deal with chemical exposures. Well-known stressors of relevance to aquatic environments include hypoxia, thermal stress, and ocean acidification (Diaz and Rosenberg, 2008; Hofmann and Todgham, 2010). All of these have taken on increasing importance as a result of climate change, nutrient-fed "dead zones", and other human impacts on the aquatic environment. Although the questions are clear, it will be challenging to design experimental and field studies that provide rigorous tests of hypotheses about effects of multiple stressors and co-exposures.

• *How does chemical exposure drive evolutionary changes in populations and species? How do evolutionary adaptations to chemically contaminated environments affect the ability of the population or species to deal with other stressors (including other chemicals)?*

The relatively young subfield of "evolutionary toxicology" seeks to understand the effects of chemicals on genetic diversity and allele frequencies in populations of exposed organisms (Bickham, 2011). Beyond simply characterizing allelic variation and genes subject to selection in exposed populations, it will become increasingly important to determine the functional properties of the variants (Dalziel et al., 2009; Storz and Wheat, 2010). One example is the evolved resistance to PCBs that occurs in some populations of fish, recently linked to an allelic variant of AHR2 that encodes an AHR protein with reduced binding affinity for dioxin-like compounds (Wirgin et al., 2011).

An important set of questions in evolutionary toxicology concern the unanticipated consequences of chemically driven adaptation. In most cases, little is known about whether or how changes at specific loci (those linked to evolved resistance), or an overall loss of genetic diversity after chemical exposure, affect the sensitivity of the populations to other, coincident stressors. A deeper understanding of toxicity pathways and networks (see above) may permit more accurate predictions of potential "costs" associated with chemically induced genetic change.

• *What are the current limitations to understanding effects of chemicals on aquatic systems, and how might they be overcome?*

Methodological limitations: When one thinks about limitations in science, what often comes to mind first are the technical limitations on our ability to make critical measurements with sufficient sensitivity, specificity, and spatial and temporal resolution to answer the most pressing questions. While there remain important limitations in our ability to collect the appropriate types of data, the major advances in analytical capabilities, especially analytical chemistry and molecular biology, have revealed a new limitation: our ability to process and integrate the troves of data so that the valuable biological information can be extracted. In the decades to come, collaborations between computational biologists and experimental biologists, already important, will become increasingly crucial to advances in aquatic toxicology.

Understanding the role of specific genes and proteins in toxicological mechanisms will be aided by new developments in the ability to perform loss-of-function and gain-of-function experiments both in aquatic models and—importantly—directly in species of environmental concern. Until recently, the ability to knock out genes in order to determine their functional

roles was limited to a few model organisms such as the mouse or fruit fly. Gene knock-down (partial loss of function) technologies developed in established models such as the zebrafish have only slowly been transferred to environmental models (Matson et al., 2008) and are imperfect. Recently, more powerful techniques such as specific gene targeting by zinc finger nucleases (ZFNs) show great promise for application to a variety of aquatic species, for the first time allowing knock-out technology to be applied broadly to address questions in aquatic toxicology (Sander et al., 2011). Undoubtedly, new tools for gene targeting will emerge in the next decade, providing new opportunities to probe gene and protein function in relation to chemical effects (e.g. Clark et al., 2011).

Aquatic toxicology is both a laboratory-based and field-based science. While the laboratory side often acquires technology from the biomedical sciences, field toxicologists are part of a broader environmental sciences research community. Challenges shared by the environmental sciences include adverse sampling conditions, the difficulty of making measurements with sufficient temporal and spatial resolution to capture environmental variability at the relevant scales, and the inability to observe environmental systems in real time. New technologies originated in other areas of environmental science such as oceanography may prove useful to toxicologists. For example, *in situ* biological and chemical sensors (e.g. Campbell et al., 2010) will permit early warnings of contamination events and allow long-term monitoring of specific locations in real time. “Ecogenomic sensors” that sample, process, identify, and measure gene expression in microorganisms, all *in situ*, have recently been deployed (Scholin, 2010) and will only become more sophisticated. How can aquatic toxicologists use such emerging technologies to assess the condition of the environment and animal responses to it?

Limitations in biological understanding: Although we often think of limitations as technical in nature, such as those described above, aquatic toxicologists also are limited by gaps in our fundamental understanding of biological systems and how they function. What are the concepts in basic biology that we don’t yet understand (and may not even be aware of) but that, once we know them, will change the way we think about how chemicals disrupt biological processes?

Predicting these “new concepts” is difficult, but we can gain insight by looking at recent examples of new findings in biology that have potential to alter our view of mechanisms of toxicity. One obvious example is the discovery of small non-coding RNAs such as microRNAs and their widespread and important roles in the post-transcriptional regulation of gene expression. Discovered only 18 years ago (and largely ignored for almost a decade after that), microRNAs are now known to be diverse, abundant, and evolutionarily conserved molecules with regulatory roles in multiple life stages of animals and plants. The role and importance of altered microRNA expression or function in the effects of chemicals is still not well understood, but emerging evidence suggests the potential involvement of small RNAs in the effects of at least some chemicals (Hudder and Novak, 2008).

There are other examples of fundamentally new concepts in biology that have clear or potential relevance for mechanisms of toxicity. Epigenetic inheritance was discussed above. Whole genome duplications, now known to have occurred in certain lineages such as teleost fish, have led to gene family diversifications that must be considered in extrapolating across taxa (Postlethwait et al., 2004). In population genetics, a recent finding is that so-called silent, synonymous changes in DNA sequence (single-nucleotide polymorphisms that do not change the encoded amino acid) may not be silent after all, because they can affect the kinetics of mRNA translation and thus the co-translational folding of the resulting protein (Komar, 2007). A very recent and surprising result, not yet fully explained, is that the sequence of mRNA in a cell does not always reflect the sequence of DNA that encodes it (Li

et al., 2011). The transcriptional or post-transcriptional RNA editing that leads to such differences provides an additional source of variation that could affect protein function and thus susceptibility to chemicals.

Final thoughts

Aquatic toxicology is a trans-disciplinary science, requiring expertise in environmental chemistry, oceanography, molecular biology, genomics, mathematics, evolutionary biology, zoology, and many other fields. Of course, no one person can be expert in all of these areas, so progress in aquatic toxicology will require collaborations across disciplinary boundaries. Such collaborative efforts will certainly become more prominent as we move forward. In addition, we must remain aware of new developments in other fields and open to thinking about how they can be applied to provide new insight into longstanding questions about the impacts of chemicals on aquatic life.

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