Pure Appl. Chem., Vol. 75, Nos. 11–12, pp. 2467–2475, 2003.

Workshop 3.5

Closing the gap between exposure and effects in monitoring studies*

Donald E. Tillitt‡ and Diana M. Papoulias

Columbia Environmental Research Center, U.S. Department of Interior, Geological Survey, 4200 New Haven Road, Columbia, MO 65201, USA

Abstract: A major challenge to contaminant monitoring programs is the selection of an appropriate suite of measurements for assessing exposure and effects. Early monitoring programs relied solely on residue analysis to detect the organochlorine compounds that were in use at that time. A shift to the use of more transient, less persistent chemicals required that a new set of tools be developed to determine if an organism had been exposed. This led to the development of cellular and biochemical assays that could indicate the presence of these types of chemicals in biota and the environment. However, it was recognized that measures of contaminant presence alone were insufficient to assess the health of biota. As a result, considerable research began to be directed toward development of diagnostic tools for measuring chemical effects in fish and wildlife. Today, contaminant monitoring programs follow a paradigm for study design that emphasizes not only the use of measures of exposure, but also measures of effect.

Using data from our monitoring and research studies for hormonally active substances, we discuss a variety of metrics of exposure and effects and their application to specific chemicals, and the current information gaps. We conclude that although several bioindicators of exposure and effect have been promoted and used, to date there continues to be a poor association between cause and effect for endocrine active substances. In part, this is due to the limited number of diagnostic tools that are available and to a lack of basic toxicological information concerning toxicokinetics and mechanisms of action of hormonally active chemicals in fish and wildlife species. In the foreseeable future, both tissue and environmental residue data, despite the many limitations, will continue to be an important component of monitoring programs for hormonally active chemicals as we continue to develop and validate more specific bioindicators of exposure and effects.

INTRODUCTION

Monitoring for effects of chemicals in the environment has become more important over the past decade. This is due to changes that have occurred in the types and patterns of chemicals used in agriculture, industry, and household products. The shift in chemicals used in commerce and industry has been from persistent, hydrophobic chemicals such as polychlorinated biphenyls (PCBs) and 2,2-bis(*p*-chlorphenyl)-1,1,1-trichloroethane (DDT) to less persistent chemicals, such as organophosphate pesticides. More stringent regulations that control the use and release of chemicals into the envi-

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* **75**, 1617–2615 (2003).

‡Corresponding author

ronment are responsible for these changes in use patterns from persistent, bioaccumulative chemicals to more water-soluble, less persistent chemicals. These "new generation" chemicals are not detected in tissues, and therefore exposure must be measured in the environment. This creates a gap between the exposure metric, environmental (e.g., water) concentration, and the potential for effects in fish and wildlife. Exposure can no longer be measured directly in the organism, as it could for the organochlorine compounds, and other persistent, bioaccumulative chemicals.

Compounding this difficulty in measuring the exposure to fish and wildlife for these "new generation" chemicals is the fact that complete models for exposure and toxicity are lacking. Exposure models that estimate the dose an aquatic organism receives at a given water concentration are inadequate for many of these chemicals. Moreover, the toxicity dose–response relationships are unknown for a vast number of these new chemicals as well as many of the not-so-new chemicals. The necessary toxicity testing in standard laboratory organisms has simply not been conducted for many of these chemicals. Additionally, the relative sensitivity of many fish and wildlife species of concern to natural resource managers are not known. As such, even when dose–response relationships have been evaluated in surrogate species, the evaluation of environmental effects remains a challenge. Last, there is little understanding of the effects of multiple chemical exposures on aquatic organisms. We simply do not understand how chemicals interact once they reach the target organ or tissue.

As a result of this lack of knowledge and the associated uncertainty, it is nearly impossible to predict toxicity or potential toxicity of the less persistent chemicals alone or in combination with the older bioaccumulated compounds. This is particularly true for that group of chemicals reported to cause endocrine-related effects in fish and wildlife species. Biological monitoring of health effects in fish and wildlife species is required to begin to evaluate any potential effects that may be caused by these chemicals. Therefore, the objectives of this presentation were to address two issues in monitoring for effects of endocrine active compounds (EACs):

Do we still need to measure the "old" organochlorine pesticides and industrial chemicals in large-scale monitoring programs?

How do we close the gap between metrics of exposure and estimates of effects in a time of more chemicals with less persistence?

IS THERE A NEED FOR CONTINUED MONITORING OF THE ENVIRONMENT FOR ORGANOCHLORINE PESTICIDES AND INDUSTRIAL COMPOUNDS?

Chemical monitoring efforts in the United States, Europe, and elsewhere in the world have been designed to measure persistent chemicals in biotic and abiotic matricies. The concentrations of chemicals measured in this fashion are compared with known threshold toxicity values to estimate or determine relative risk. The results of most of these monitoring efforts have been consistent with one another over the past three decades. Most environmental monitoring of chemicals has demonstrated the same temporal trends in concentrations of the persistent bioaccumulative compounds. There have been declines, often exponential decreases, in the concentrations of organochlorine chemicals in most of the more contaminated environments. Examples of these declines include concentrations of PCBs in Great Lakes fish and wildlife [1–3], DDT and PCB declines in the biota of the Baltic Sea [4,5]; and fish in the National Contaminants Biomonitoring Program (NCBP) [6,7]. Regulations were instituted in the late 1970s that banned or restricted the use of persistent organochlorine chemicals. The declines in concentrations of these chemicals in biota and the environment have been dramatic, more than originally estimated in some cases, and underscore the importance of regulatory actions. Globally, monitoring of the environment for organochlorine pesticides and industrial chemicals has demonstrated those declines with the exception of remote areas, such as the Arctic [8,9]. Evidence is controvertible as to whether concentrations of organochlorine pesticides and industrial compounds are on the decline or increase in these areas.

Even with the almost universal declines in concentrations of the persistent, bioaccumulative chemicals in the environment, there are still strong reasons and rationale for resource management agencies to continue to monitor the environment for these chemicals. First, areas of concern still exist. While declines have been the rule, there are still areas of environmental contamination that are well above a threshold for toxicity in fish and wildlife species. Second, environments that have experienced declines in concentrations of many persistent, bioaccumulative chemicals are in some cases still experiencing untoward effects, which may be linked to these chemical contaminants [10,11]. There are populations of fish and wildlife that continue to have symptoms of toxicity consistent with some of the organochlorine pesticide or industrial compounds. Although concentrations of these compounds have declined, these populations may be indicating that thresholds for effects need further refinement. Additionally, there may be other stressors impinging on these populations, or there may be joint effects of these chemicals in combinations that are not predicted by our laboratory-based assessments with single compounds. Until we understand the true causes for the observed population effects in some of these contaminated environments, we must continue to monitor the potential causes, which include organochlorine pesticides and industrial chemicals. Third, there remains the continued threat of new releases of some of the banned or restricted organochlorine pesticides and industrial compounds. In some cases, large amounts of these chemicals continue to be found either in landstocks or still in use. For example, even though PCBs have not been manufactured in the United States for a quarter of a century, the majority of the PCBs produced are still found in use in electrical devices [12].

The efforts to monitor persistent organochlorine pesticides and industrial compounds need to be redirected and evaluated, but not eliminated, at this point in time. There are a number of steps that could be taken to reduce the costs associated with organochlorine chemical monitoring. Steps to reduce the cost associated with persistent organochlorine pesticides and industrial compounds should include: reduced replication of analyses at a given site; composite of samples; increased temporal periods between sampling events; and increased use of new technologies. Alternative forms of analysis, such as immunoassays or bioassay screening techniques, can provide the exposure information at substantially reduced costs as compared with routine analytical chemistry analysis. Immunoassay procedures for PCBs in biota [13–15] have proven to be quantitative, and are about 20 % of the costs of the most simple PCB analysis. Similarly, the H4IIE bioassay to screen for dioxin-like chemicals in fish, wildlife, and other environmental samples is now routinely and cost-effectively used [16].

CLOSING THE GAP BETWEEN EXPOSURE AND EFFECTS IN CHEMICAL MONITORING

The veterinary products, human antibiotics, pharmaceutical drugs, among countless other chemicals that are found in our sewage treatment plant wastewater effluents present new challenges to environmental scientists and resource management agencies. The number of such chemicals that are released into the environment increases each year, yet the sources and amounts of these releases are generally not well understood. However, some specific efforts have been directed toward detection of these chemicals in streams [17]. In the United States for example, the National Water-Quality Assessment (NAWQA) program has developed methods for many of these less persistent compounds [17]. The challenge that then arises is to bridge the gap between the environmental concentrations that predict exposure to fish and wildlife species and the evaluation of the effects that may occur as a result of those exposures. Threshold values for effects of many of these chemicals are simply not known. This gap is further widened by the fact that the responses of organisms to endocrine active agents, such as steroid hormones, can have nonmonotonic responses to increases in exposure. These nontypical dose–response curves may have an "inverted U" or "J-shape", as opposed to the more classic sigmoid curve [18,19]. A consequence of these more complex dose–response relationships is that our ability to interpret measures of chemical exposure is diminished. Limitations in our ability to estimate individual chemical exposures and interpret those exposures with respect to potential effects are further exacerbated by the

complex interactions of multiple chemicals. The effects of multiple chemicals with similar modes of action or the action of chemicals with dissimilar modes of action on the same target organs and tissues are far from being understood. These complex interactions may follow simple, recognized relationships, but in most cases it is not readily apparent what type of interaction occurs.

Biological monitoring is the most direct and prudent action to address the uncertainties of chemical exposure in the environment. The U.S. Geological Survey Biomonitoring of Environmental Status and Trends (BEST) program is an example of a biological monitoring approach that is designed to develop multiple lines of evidence of contaminant exposure and effects in the aquatic environment [20]. The approach includes four major categories of methods: (1) biomarkers, (2) toxicity tests and bioassays, (3) community health, and (4) residue analysis. The use of these methods is intended to provide results that constitute a "weight-of-evidence" approach for identifying effects from a wide variety of contaminants including hormonally active chemicals. The BEST program is unique among national monitoring programs with its emphasis on characterizing the effects of environmental contaminants on the health of the biota and their supporting habitat. BEST accomplishes this through the application of both chemical analysis and biological response measurements. In addition to applying measures of biochemical, physiological, morphological, and histopathological responses, BEST assesses and synthesizes existing information from other monitoring efforts to incorporate multiple lines of evidence into an ecological risk assessment approach. An important component of the BEST program is its emphasis on integrating established monitoring tools with research to develop new methods for addressing emerging issues and problems.

BIOMONITORING AS AN INDICATOR OF ECOSYSTEM HEALTH

The BEST program's study of environmental contaminant effects on fish in the Mississippi River Basin (MRB) was a demonstration project for the BEST program [20]. The MRB is the largest basin in North America. More than 72 million people live in the basin where agriculture, mining, and industry are the main activities generating contaminants of concern. The BEST MRB project served to illustrate how well a selected set of fish health biomarkers performs, the limits on interpretation of the results from fish health biomarkers, and how monitoring studies can serve as a platform for focused investigations.

Among the objectives of the MRB study were documentation and comparison of reproductive biomarker response, and identification of sites where fish reproductive health may be affected by chemical exposure [20]. Forty-eight stations were sampled for this study at which 10 males and 10 females of carp (*Cyprinus carpio*) or bass (*Micropterus sp*.) were collected and evaluated. The bioindicators of endocrine disruptors included: gonad pathologies, sex steroid and vitellogenin levels, and GSI. Tissue residue data were also obtained for organochlorines and elemental contaminants.

An analysis of the tissue data and biomarker responses at a station and overall comparison among stations provides some idea of the association between biomarker response and exposure to potential endocrine disruptors. Of the 48 stations evaluated, 31 stations were identified as having contaminant levels of concern based on either tissue concentrations or biomarker response. Twenty-three stations were identified as having tissue residue levels sufficiently high to potentially cause endocrine disruption. Likewise, 13 of the stations were identified as having contaminant levels of concern based on biomarker response. However, biomarker and tissue data coincided for only 8 of the 31 stations. These data suggest that (1) contaminants not analyzed for in tissues caused the biomarker responses in at least 5 of the 31 stations; and (2) this suite of biomarkers did not reveal an endocrine effect of the chemicals found at 15 of the 31 stations, although those same chemicals have been shown to cause endocrine-related effects in laboratory tests.

The finding that biomarker and tissue residue data corresponded at just 8 of 31 stations with possible endocrine-disrupting chemical contamination suggests that there may be only a weak relationship between biomarkers measured and the chemicals detected. Correlation analysis on the full MRB data set was used to evaluate the relationship between the fish biomarkers and the chemicals found in their

tissues and showed reasonable correspondence between the organochlorines (cyclodiene pesticides and PCBs) and biomarkers based on extant toxicological information. *p′p′*-DDE, predominantly an anti-estrogen, correlated negatively with steroid and vitellogenin levels and positively with atresia. The results for mercury were ambiguous as it correlated positively with atresia and estradiol levels although laboratory studies indicate this may only be true for atresia; estradiol levels have been shown to correlate negatively with mercury.

Despite a general concordance between biomarkers and tissue residue data, the relationships between contaminants measured and biomarker responses at specific stations were difficult to reconcile. Station 111 was one of the eight stations at which results from the biomarker and tissue data indicated exposure to possible endocrine disruptors. Bass captured at this station had PCB concentrations averaging 1 μg/g and a high incidence of ovotestes. However, the existing toxicological data does not indicate that intersex results from exposure to PCBs. Furthermore, sex steroid levels in males were >2-fold higher than those in their counterparts from a reference location. That PCBs tend to have a depressive effect on sex steroid levels further suggests that there is no link between the high levels of PCBs and the biomarker responses at this station. The presence of ovotestes has been attributed to estrogen agonists such as those found in sewage effluents, yet exposure to estrogens typically depresses sex steroid levels in fish. The data are perhaps better explained by the presence of an aromatase enhancer, such as atrazine, which would increase endogenous estradiol levels and which in turn could also induce the ovotestes.

Monitoring data alone cannot provide define cause and effect relationships between environmental exposures and effects in fish and wildlife. Biological monitoring programs are typically designed to provide generalized information on spatial and temporal trends. Large-scale monitoring programs are usually designed to provide a large amount of information, but the depth of the studies does not allow determination of etiologies for any observed effects. The BEST program uses fish health indicators to identify areas of concern based on organismal responses and exposures. While the combined biological and chemical indicators of exposure to endocrine disruptors used in the BEST program may suggest cause and effect linkages, their greatest value is in identifying sites of potential concern. Once effects are observed in biological monitoring efforts, such as the BEST program, focused investigations are required to understand the origin of these effects. Quite often these focused investigations take the form of field-laboratory combined investigations that afford some of the complexities of the field, yet some of the control of laboratory investigations. Combined these are the studies that further our understanding of the effects of complex mixtures on endocrine systems.

FOCUSED INVESTIGATIONS CAN ORIGINATE FROM BIOLOGICAL EFFECTS MONITORING

Results from monitoring studies, such as BEST, also serve as a platform on which focused research questions can be formulated. For example, the results from Station 111, described above, led to an integrated lab–field study to understand whether fish with clinical signs of endocrine disruption (i.e., hormone imbalances, ovotestes) and tissue concentrations of known endocrine disrupting chemicals exhibit reproductive impairment. The establishment of a connection between the indicators of endocrine disruption and reproductive performance is necessary to better understand the potential significance of this condition to fish populations. The goal of this focused investigation was to capture wild fish in spawning condition to determine whether fish with signs of endocrine disruption can successfully spawn and produce healthy progeny. Fish from the contaminated site (Station 111) and a reference site were transported to laboratory ponds and maintained throughout spawning. Various observations and measurements were made on adults and offspring during the spawning, hatching, and rearing phases. Results from this study indicated that relative to the reference fish, the overall health of the adult bass from Station 111 was poor. Conclusions regarding reproductive success were confounded however, due to the

unexpected appearance of clinical signs of endocrine disruption in the reference fish and the fact that the spawning behavior of fish from Station 111 differed unpredictably from that of the reference fish.

The exposure of model organisms to extracts of environmental mixtures can be a powerful tool for interpreting the reproductive significance of chemical and biomarker results, when evaluated in conjunction with controlled reproduction studies [21,22]. These combined studies with standard test chemicals and mixtures of chemicals from the environment can be an important set of tools for elucidation of cause and effect linkages [23]. The d-rR strain of medaka is a useful teleost model because this strain bears a sex-linked color marker allowing easy determination of genetic sex even when phenotypic sex has been altered. Medaka are amenable to several exposure methods. Egg injection is one method that has been perfected for use with this species. Egg injection mimics the maternally derived exposure to the egg of the persistent bioaccumulative compounds [24]. It has also been useful in studying the effects of stage specific early embryonic exposure to the more water soluble chemicals. The eggs, hatchlings, and older stages are easily exposed in traditional water baths as well. In some instances, a combination of injection and water-borne exposures may be useful as it has been shown that while an early exposure may not have obvious immediate effects, it may potentiate the effects of exposures later in life.

Laboratory-based studies using medaka have helped us characterize a number of indicators of endocrine disruption and assess the cause and effects links. For example, because we cannot easily determine genetic sex of the monitored organisms, generally sex reversal cannot be determined from field collections. Although indirect indicators such as skewed sex ratios from field collections may suggest sex reversal or sex-specific mortality. The d-rR medaka model can elucidate the effects of environmental conditions on phenotypic sex.

Secondary sexual characteristics are quite sensitive to estrogens and androgens and many fish are sexually dimorphic. Medaka have distinct sex-specific fin shapes, and the males develop breeding tubercles on the anal fin that have been shown to be very sensitive to androgens. Also, the urogenital duct systems are distinct between the genders. Established early in development, the ducts can be altered depending on exposure to estrogens or androgens during the period of sexual differentiation. Additionally, because the secondary sex characteristics are hormone-defined, they are not seen in normal immature juveniles, but can be induced through exposure. Therefore, precocious appearance of secondary sex characteristics or quantifiable changes in the characters can be an indicator of exposure.

Gonad and gamete pathologies are important indicators of exposure to and effects of endocrine disruptors, but are difficult to interpret because of the many variables in addition to hormonally active substances that can alter normal endogenous hormonal homeostasis. The medaka model is useful for understanding this complexity through characterizing the effects of multiple biotic and abiotic variables in part because much of the normal reproductive and endocrine biology is already understood. For example, the pattern of ovotestes observed in the MRB study has also been observed in both juvenile and adult male medaka exposed to aqueous solutions of natural and synthetic estrogens. However, it is also known that abrupt temperature and pH changes among other factors can induce ovotestes. Using the medaka model, we can examine the development of ovotestes in vivo or use ex vivo cultures of germ cells to look at effects of chemicals on development of such abnormalities.

Vitellogenin has been a keystone biomarker of estrogen exposure. But there remain difficulties in interpreting field results. Often, as in the MRB study, the vitellogenin levels don't coincide with endogenous estradiol levels or stage of development and individual variation seems to be great. Replacing or complimenting this measurement with measurement of the zona radiata protein, choriogenin, which has been shown to be more sensitive to estrogen than vitellogenin in medaka and other species, may be warranted. And for both measures, using juveniles that should have normally very low levels of endogenous sex hormones would be preferable.

Studies with medaka and other species have shown that estrogen and androgen levels are affected by exposure to certain chemicals including natural and synthetic sex steroids. The underlying mechanisms however are still unknown and this contributes to the difficulty in interpreting their significance in monitoring studies. In the MRB example, males tended to have higher than normal levels of 11-keto-

testosterone and estradiol for their stage of development, time of year, and geographic location. Sensitive steroid test kits designed to use small volumes of plasma facilitate the use of models such as the medaka in designing studies to interpret such monitoring results.

SUMMARY

A shift from the manufacture and usage of persistent bioaccumulative chemicals to chemicals that are water soluble or are readily metabolized and eliminated from an organism has necessitated a concomitant paradigm shift in our approach to monitoring chemicals in the environment. No longer can we assess exposure and predict effects based solely on tissue residue information. Contemporary monitoring programs must be modified so as to maintain some aspects of traditional residue-based evaluations, yet also incorporate biological measures of exposure and effect consistent with the toxicological properties of the new generation of chemicals. Using data from our monitoring and research studies for hormonally active substances, we have discussed a variety of metrics of exposure and effects, their application to specific chemicals, and the current information gaps. We conclude that although several bioindicators of exposure and effect have been promoted and used, to date there continues to be a less robust association between cause and effect than could formerly be made based on tissue residue information. In part, this is due to the limited number of diagnostic tools that are currently available, to a lack of basic toxicological information concerning toxicokinetics and mechanisms of action of chemicals (e.g., endocrine disruptors) in fish and wildlife species, and to a lack of understanding of the effects of environmental mixtures. In the foreseeable future, measurement of chemical exposure in tissues and in the appropriate environmental compartment (i.e., water), will continue to be an important component of monitoring programs despite the many limitations. We must continue to develop and validate more specific bioindicators that will help close the gap between exposure and effects of endocrine-disrupting chemicals.

Research needs for development and implementation of successful monitoring programs for endocrine active compounds

1. **Understand basic endocrine function in representative fish and wildlife species.** The species important to resource managers often do not have some of the basic information on endocrine function. Basic endocrine and life history information such as seasonal steroid hormone cycles, gonad development patterns, signals and patterns of sexual maturation, and cues for spawning and seasonal migrations. This information is need for species of fish and wildlife that are important to resource managers.
2. **Develop dose–response models for EACs.** The complexity of endocrine function and the vast numbers of new chemicals introduced into commerce requires that model dose–response relationships continue to be developed for EACs.
3. **Develop new technologies for monitoring EACs (ELISA, bioassay).** Screening systems for both the quantification of EACs in the environment and evaluation of effects (biomarkers) in fish and wildlife need to be developed or validated for environmental use. As a better understanding of basic endocrine function and models of response to EACs are developed, our knowledge of key control points of reproductive processes will aid in the development of assays, biomarkers, and other tools for environmental monitoring for EACs.
4. **Develop estrogen/androgen responsive genetic markers for screening with microarrays.** Toxicogenomic responses of fish and wildlife species toward EACs have the potential to provide a better understanding of initial events in responses to EACs. Abnormal or altered changes in gene expression are often some of the primary events in an organism's response to EACs. The tools being developed in genomics, such as microarrays, have the potential to allow screening of hundreds or thousands of genes simultaneously and nonlethally. However, the utility of these tools is

dependent on extensive characterization of genes and the dose and temporal response patterns of those genes to EACs.

5. **Target indicators that are significant relative to higher-order functional effects need to be validated for EACs.** Metrics of population- and community-level effects of EACs in fish and wildlife with linkages to behavioral, physiological, and biochemical measures need to be developed and validated for environmental monitoring. Apical endpoints, those that integrate the combined responses to multiple chemicals or multiple stressors, need continued evaluation for incorporation into environmental monitoring for EACs. Most measures used in contaminant monitoring are organismal level of biological organization. These are important and tend to be the best defined, yet we need to continue to strive towards measures of population-level indicators of reproductive health.

ACKNOWLEDGMENTS

The authors would like to acknowledge the BEST program, directed by Timothy Bartish, and the collaborating scientists: C. J. Schmitt, J. E. Hinck, J. J. Whyte, V. Blazer, T. Gross, J. Coyle, and P. Anderson. The results of the BEST MRB study used as the example in this presentation are a result of the combined efforts of these scientists and their support staff. Funding for this work and the BEST program was provided by the U.S. Geological Survey, Department of Interior.

REFERENCES

1. P. C. Baumann and D. M. Whittle. *Aquat. Toxicol*. **11**, 241–257 (1988).
2. D. V. Weseloh, T. W. Custer, B. M. Braune. *Environ. Pollut.* **59**, 141–160 (1989).
3. D. E. Tillitt, C. J. Schmitt, D. V. Weseloh, C. M. Bunck. In *Ecotoxicological Risk Assessment of the Chlorinated Organic Chemicals,* J. Carey, P. Cook, J. Giesy, P. Hodson, D. Muir, W. Owens, K. Solomon (Eds.), pp. 259–288, SETAC Press, Pensacola, FL (1998).
4. M. Olsson. In *PCBs and the Environment*. *Vol. III*, J. S. Waid (Ed.), pp. 181–208, CRC Press, Boca Raton, FL (1986).
5. M. Olsson and L. Reutergårdh. *Ambio* **15**, 103–109 (1986).
6. C. J. Schmitt and W. G. Brumbaugh. *Arch. Environ. Contam. Toxicol.* **19**, 731–747 (1990).
7. C. J. Schmitt, J. L. Zajicek, P. H. Peterman. *Arch. Environ. Contam. Toxicol.* **19**, 748–782 (1990).
8. R. J. Norstrom and D. C. G. Muir. *Sci. Total Environ.* **154**, 107–128 (1994).
9. D. J. Gregor, A. J. Peters, C. Teixeira, N. Jones, C. Spencer. *Sci. Tot. Environ.* **160/161**, 117–126 (1995).
10. A. Fairbrother, G. T. Ankley, L. S. Birnbaum, S. P. Bradbury, B. Francis, L. E. Gray, D. Hinton, L. L. Johnosn, R. E. Peterson, G. van der Kraak. In *Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates,* R. T. Di Giulio and D. E. Tillitt (Eds.), pp. 283–362, SETAC Press, Pensacola, FL (1999).
11. D. E. Tillitt, G. T. Ankley, J. P. Giesy, J. P. Ludwig, H. Kurita, D. V. Weseloh, P. S. Ross, C. A. Bishop, L. Sileo, K. L. Stromborg, J. Larson and T. J. Kubiak. *Environ. Toxicol. Chem.* **11**, 1281–1288 (1992).
12. S. Tanabe. *Environ. Pollut.* **50**, 5–28 (1988).
13. J. L. Zajicek, D. E. Tillitt, J. N. Huckins, J. D. Petty, M. E. Potts, D. A. Nardone. In *Environmental Immunochemical Methods*, J. Van Emon, C. Johnson, Gerlach (Eds.), Chap. 26, pp. 307–325, ACS Symposium Series 646, American Chemical Society, Washington, DC (1996).
14. J. L. Zajicek, D. E. Tillitt, T. R. Schwartz, C. J. Schmitt. *Chemosphere* **40**, 539–548 (2000).
15. J. A. Lasrado, C. R. Santere, J. L. Zajicek, J. R. Stahl, D. E. Tillitt, D. Deardorff. *J. Food Sci.* **68** (1), 133–7 (2003).
16. J. J. Whyte, C. J. Schmitt, D. E. Tillitt. *Crit. Rev. Toxicol.* **34** (1), (2004).

17. D. W. Kolpin, E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, H. T. Buxton. *Environ. Sci. Technol.* **36** (6), 1202–12 (2002).
18. E. J. Calebrese and L. A. Baldwin. *Hum. Experiment. Toxicol.* **19**, 2–31 (2000).
19. H. A. Tilson. *Environ. Health Perspect.* **106** (Suppl. 3), 807–811 (1998).
20. C. J. Schmitt (Ed.). *Biomonitoring of Environmental Status and Trends (BEST) Program: Environmental Contaminants and their Effects on Fish in the Mississippi River Basin*, p. 241, U.S. Geological Survey, Biological Science Report 2002-0004 (2002).
21. M. K. Walker, P. M. Cook, B. C. Butterworth, E. W. Zabel, R. E. Peterson. *Fundam. Appl. Toxicol.* **30**, 178–186 (1996).
22. P. M. Cook, E. W. Zabel, R. E. Peterson. In *Chemically-Induced Alterations in the Functional Development and Reproduction of Fishes*, R. Rolland, M. Gilbertson, R. Peterson (Eds.), Chap. 2, pp. 9–27, SETAC Press, Pensacola, FL (1997).
23. P. J. Wright and D. E. Tillitt. *Aquat. Toxicol.* **47** (2), 77–92 (1999).
24. M. K. Walker, E. W. Zabel, G. Åkerman, L. Balk, P. J. Wright, D. E. Tillitt. In *Techniques in Aquatic Toxicology*, G. Ostrander (Ed.), Chap. 4, pp. 41–72, CRC Press/Lewis Publishers, Boca Raton, FL (1996).