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Hazard/Risk Assessment

ADVERSE OUTCOME PATHWAYS: A CONCEPTUAL FRAMEWORK TO SUPPORT ECOTOXICOLOGY RESEARCH AND RISK ASSESSMENT

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Abstract—Ecological risk assessors face increasing demands to assess more chemicals, with greater speed and accuracy, and to do so using fewer resources and experimental animals. New approaches in biological and computational sciences may be able to generate mechanistic information that could help in meeting these challenges. However, to use mechanistic data to support chemical assessments, there is a need for effective translation of this information into endpoints meaningful to ecological risk—effects on survival, development, and reproduction in individual organisms and, by extension, impacts on populations. Here we discuss a framework designed for this purpose, the adverse outcome pathway (AOP). An AOP is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment. The practical utility of AOPs for ecological risk assessment of chemicals is illustrated using five case examples. The examples demonstrate how the AOP concept can focus toxicity testing in terms of species and endpoint selection, enhance across-chemical extrapolation, and support prediction of mixture effects. The examples also show how AOPs facilitate use of molecular or biochemical endpoints (sometimes referred to as biomarkers) for forecasting chemical impacts on individuals and populations. In the concluding sections of the paper, we discuss how AOPs can help to guide research that supports chemical risk assessments and advocate for the incorporation of this approach into a broader systems biology framework. Environ. Toxicol. Chem. 2010;29:730–741. © 2009 SETAC

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INTRODUCTION

Ecological risk assessors face increasing demands to assess more chemicals, with greater speed and accuracy, and to do so using fewer resources and experimental animals. Legislation such as the Food Quality Protection Act (FQPA) and Safe Drinking Water Act (SDWA) in the United States and the Registration, Evaluation and Authorisation of Chemicals (REACH) program in the European Union (EU) creates mandates to assess potential risks from an expanding number of chemicals or to consider a broader suite of effects than has commonly been considered in previous assessment efforts. Regulatory programs are also faced with the need to assess emerging contaminants of concern, such as pharmaceuticals and nanomaterials, for which existing assessment procedures may be inadequate [1].

At the same time, the fields of biology and toxicology have seen a number of important developments. Advances in computational capabilities and bioinformatics, measurement technologies (e.g., genomics), and fundamental toxicological understanding at the molecular level have increased the amount and types of information available and potentially useful to risk assessors. However, for most regulatory assessments, broad suites of in vivo toxicity tests continue to provide the basic information underlying the decision-making process. The time and resources necessary to support this approach run counter to the demands being faced. As argued by Bradbury et al. [2], circumstances require that we move away from an overdependence on in vivo testing and make greater use of computational, molecular, and in vitro tools.

Similar challenges are faced by scientists involved in human health risk assessment. In 2007, the National Academies of Science released an expert panel report, *Toxicity Testing in the 21st Century* [3], which described a vision for the future of toxicity testing to support human health risk assessments. That report acknowledged many of the issues identified above for ecological risk assessment and emphasized the need to develop a focused assessment approach that maximizes use of existing knowledge and the efficient and targeted search for critical new knowledge, while minimizing reliance on resource-intensive testing approaches. Strategies proposed by Bradbury et al. [2] and the National Research Council (NRC) [3] have as a common recommendation the need to collect basic information about biological systems and how chemicals perturb them, in order to improve the ability to predict which chemicals are likely to cause adverse effects or, for retrospective assessments, deduce which chemicals are most likely to be causing observed effects.

Bringing the full range of emerging tools and understanding to bear on ecological risk assessment requires the development of a framework within which data and knowledge collected at many levels of biological organization can be synthesized in a

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way that is useful to risk assessors and the ecotoxicologists who support this activity. In the present paper, we propose the adverse outcome pathway (AOP) as this framework. As discussed below, we believe AOPs provide a useful structure within which existing knowledge can be organized, from which key uncertainties and research priorities can be identified, and through which we can improve predictive approaches needed to advance regulatory ecotoxicology [4]. Several case examples are presented to illustrate the nature and merits of the approach. In the concluding sections, we discuss the development and use of AOPs, relate this approach to existing assessment tools, and explore the relationship between AOPs and emerging biological systems models.

DEFINITION OF ADVERSE OUTCOME PATHWAYS

An AOP is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment (Fig. 1). As such, AOPs are generally a sequential series of events that, by definition, span multiple levels of biological organization. The content connecting the initiating event to the outcome can take various forms, depending on the types and extent of biological information from which the AOP is derived or the risk context that it is being used to address. Relationships among levels of biological organization may be causal, mechanistic, inferential, or correlation based, and the information on which they are based may derive from in vitro, in vivo, or computational systems. Such linkages provide the critical foundation for greater use of predictive approaches in ecotoxicology and ecological risk assessment.

The term *adverse outcome pathway* was developed in part to draw a distinction from the term toxicity pathway as defined and used by NRC [3]. A toxicity pathway was defined by NRC as a ''cellular response pathway that, when sufficiently perturbed, is expected to result in adverse health effect'' [3]. Although connection to an adverse outcome is implicit in this definition, the NRC focus is almost exclusively on initiating events and proximal cellular responses that can be measured and modeled in vitro. Thus, the NRC report [3] and associated forum articles describe toxicity pathway assays as in vitro assays that measure ''critical mechanistic end points involved in the induction of overt toxic effects rather than the effects themselves'' [3,5]. Within this framework, the linkage of pathway disruption to adverse outcomes is regarded as part of the science base required to implement the vision [3], but the pathway itself is at the cellular level.

Adverse outcome pathways and toxicity pathways will overlap considerably and derive from the same scientific research. By definition, however, AOPs represent a set of plausible connections that leads all the way from the molecular initiating event to an adverse effect considered relevant in risk assessment. In the case of ecological risk assessment, this generally means well-quantified endpoints of demographic significance that can be used to predict or infer potential population impacts. Thus, AOPs can be viewed as encompassing and extending beyond the NRC [3] definition of toxicity pathway (Fig. 1).

The AOP concept was also developed in response to uncertainties in the field regarding usage of the terms *mechanism of action* and *mode of action*. Mechanism of action has been defined as ''a complete and detailed understanding of each and every step in the sequence of events that leads to a toxic outcome'' [6], which includes detailed knowledge of the causal and temporal relationships among all the steps leading to a specific effect [7]. In contrast, mode of action has been defined as ''a common set of biochemical, physiological, or behavioral responses that characterize an adverse biological response where major, but not necessarily all, linkages between a direct initiating event and an adverse outcome are understood'' [6,7]. In practice, however, the term mechanism of action is often used to describe just a portion of the biological response lying

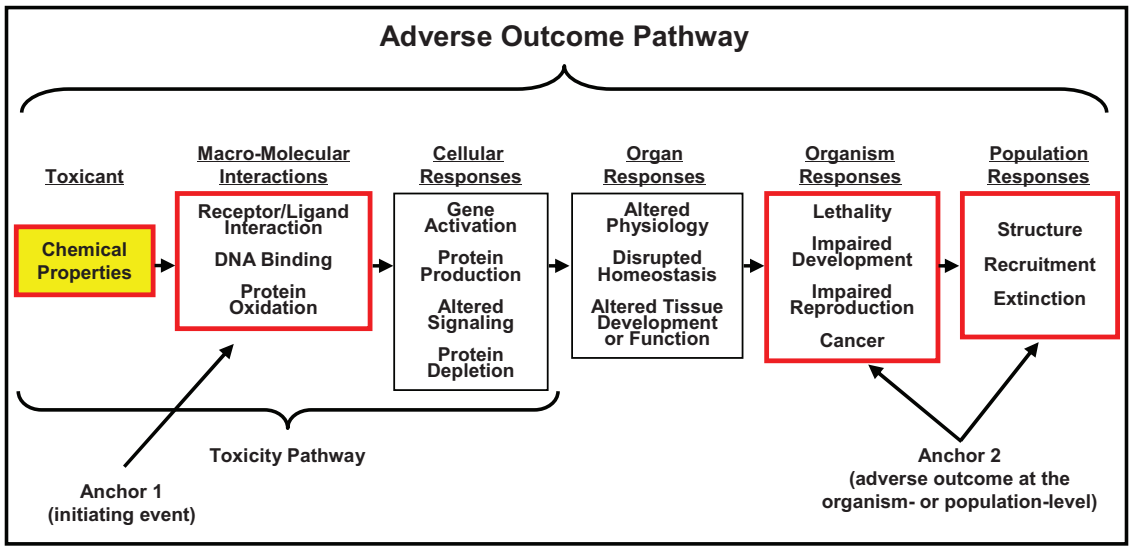

Fig. 1. Conceptual diagram of key features of an adverse outcome pathway (AOP). Each AOP begins with a molecular initiating event in which a chemical interacts with a biological target (anchor 1) leading to a sequential series of higher order effects to produce an adverse outcome with direct relevance to a given risk assessment context (e.g., survival, development, reproduction, etc.; anchor 2). The first three boxes are the parameters that define a toxicity pathway, as described by the National Research Council [3].

between an initiating event and adverse outcome, whereas the term mode of action is commonly defined by an outcome or initiating event but is rarely anchored to both. The AOP concept addresses such terminology problems by requiring an anchor to both a molecular initiating event and an adverse outcome with significance to risk assessment. In addition, AOPs can accommodate whatever linkages are appropriate to a particular assessment, based on current knowledge. A fully developed, mechanistically based AOP would fulfill the definition of mechanism of action provided above. In the case of AOPs that have gaps and black boxes in which mechanistic details are either unknown or not needed, AOPs could be regarded as similar to mode of action. In either case, the same terminology is applied to whatever level of detail is appropriate.

With mechanism and mode of action as predecessors, the AOP concept is not revolutionary, but rather represents an evolution of a framework more useful for risk assessments. Adverse outcome pathways provide a consistent structure and terminology for organizing our ecotoxicological understanding across levels of biological organization and thus facilitate more effective application and integration of diverse information and identification of key uncertainties and research needs. A parallel may be drawn to advances in ecological risk assessment (ERA) science achieved during the 1980s and 1990s. Many elements of the proposed ERA frameworks were not new to the field; they had roots in a number of existing scientific and regulatory practices. What was most significant was the development of a consistent and comprehensive structure and terminology that supported more effective risk assessments. A key element of ERA is a conceptual model that provides the linkages of various types of information to assessment objectives. An AOP adds more structure to this conceptual model with regard to the use of a variety of ecotoxicological information, maintaining a connection to the individual organism- and population-level risks that we wish to manage, even as our science and regulatory practice focus more on the development and use of computational and in vitro tools.

ADVERSE OUTCOME PATHWAYS: CASE EXAMPLES

Several case examples are presented below to help illustrate the components of AOPs and highlight their utility for ecological risk assessment and ecotoxicology research. These particular examples were selected to include a variety of pathway types as well as variation in the detail available for the pathways. The case examples also were selected to exemplify various ways in which AOPs can contribute to addressing significant risk assessment needs and uncertainties.

AOP case example 1: Narcosis

Adverse outcome pathways may have gaps where details on the exact chain of events leading to adverse outcome are unknown, but enough information is available about the pathway to improve risk assessment decisions. Although narcosis is the least detailed of the AOPs that we will discuss, it is particularly important in ecotoxicology because approximately 60% of all industrial organic chemicals are thought to act via this AOP [8]. Narcosis is described by Overton [9] as nonspecific toxicity resulting from weak and reversible hydrophobic interactions and is referred to in ecotoxicology as *baseline toxicity*, meaning that, if a chemical does not produce toxicity by some more specific mechanism, it will act by narcosis, providing it is sufficiently soluble in water at high enough concentrations to achieve the required chemical activity [10]. In fish, narcosis is characterized by decreased respiratory rate, slowed metabolic rate, low blood oxygen, loss of equilibrium, and eventual plasma ion imbalance prior to death [11].

Contrasting theories of narcosis exist (e.g., Yamakura et al. [12]), but all involve hydrophobic interactions between chemicals and cellular membranes as the molecular initiating event of this AOP. Narcosis occurs across a diverse set of chemical structures, so a receptor-based lock-and-key mechanism is unlikely. The lipid portion of the neuronal membrane has been proposed as a target site for narcosis (Fig. 2a), with simple partitioning of narcotic chemicals causing membranes to swell, increasing lipid membrane fluidity, reducing the transition temperature between phases, and affecting ion permeability [8]. Ferguson [13] proposed a thermodynamic relationship for narcosis, in which chemical activity is tied to an equilibrium between the media and the organism, and the toxic action is caused by the physical presence of the chemical at the target site. The narcotic effect is observed when the target site is swollen beyond some critical volume.

Although other mechanistic details of the narcosis AOP are not resolved (Fig. 2a), the importance of hydrophobic interactions within membranes provides the foundation for risk assessment approaches focused on a chemical property—the octanol/water partition coefficient (K_{OW})—that forms the basis of models for the partitioning of chemical from water into the membrane lipids of a receptor organism. The use of K_{OW} has resulted in very reliable quantitative structure–activity relationship (QSAR) models for estimating acute toxicity of narcotic chemicals [14,15]. However, key to the development of these robust QSARs was the grouping of chemicals causing baseline toxicity apart from other pathways. To this end, methods were developed to sort chemicals so that narcosis chemicals could be effectively distinguished from chemicals that operated via other AOPs [16–18]. The narcosis QSAR is the basis of the nonspecific toxicity models included in the EpiSuite software used by the U.S. Environmental Protection Agency (U.S. EPA) for actions under the Toxic Substances Control Act [19] (http://epa.gov/oppt/newchems/tools/21ecosar.htm). As part of an ongoing analysis to assess the validity of models for use under REACH, the EpiSuite narcosis QSAR developed using fathead minnow data has been shown to perform well in estimating toxicity for other fish species [20]. Accordingly, the EpiSuite narcosis model has been included in the Organisation for Economic Co-operation and Development (OECD) Application Toolbox [18] to address the REACH legislation. The model has also been used in risk assessment screening exercises, in which large lists of chemicals, such as the Canadian Domestic Substances List [21], are prioritized and ranked based on potential hazard, in part to help in identifying substances that exhibit more toxic than baseline toxicity.

To date, most applications of models based on the narcosis AOP relate toxicity to chemical concentrations in the water, but this baseline toxicity is better explained by a common chemical activity independent (at least to useful approximation) of the chemical identity [10]. The understanding of baseline toxicity serves as a useful reference point for identifying

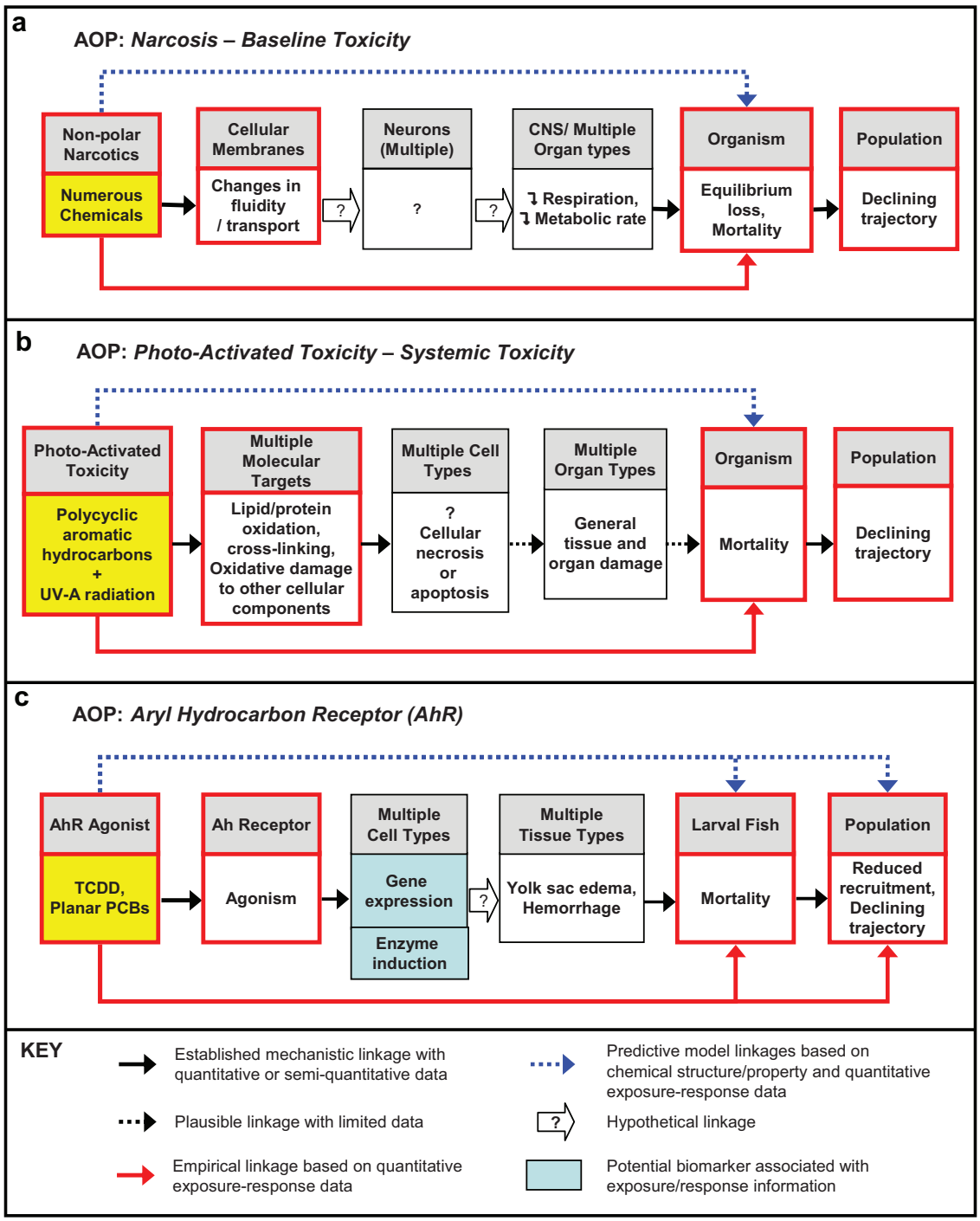

Fig. 2. Representations of three adverse outcome pathways (AOPs) in which chemical structures/properties triggering molecular initiating events are well established, but specific linkages to all steps leading to an adverse outcome are not fully defined. Depicted are AOPs for narcosis or baseline toxicity (**a**), photoactivated toxicity (**b**), and activation of the arylhydrocarbon receptor (AhR; **c**) resulting in fish early life-stage mortality. Compounds listed in the yellow boxes are examples of chemicals that can elicit toxicity via a particular pathway. Further technical detail concerning the pathways is found in the accompanying text. CNS = central nervous system; UV = ultraviolet; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

specific toxicity, that is, AOPs initiated by biological–chemical interactions more specific than hydrophobicity alone [10]. This aspect of the AOP also supports the use of additive joint toxicity models, which provides a foundation for predicting toxicity from critical body burdens of narcotic chemicals [22,23] and for proposed water and sediment quality criteria for both single compounds and mixtures of narcosis chemicals [24,25].

AOP case example 2: Photoactivated toxicity

In the 1980s, studies demonstrated heightened lethality of polycyclic aromatic hydrocarbons (PAHs) when fish or daphnids were simultaneously exposed to sunlight or, more specifically, radiation at ultraviolet (UV)-A wavelengths [26,27]. These initial findings spurred considerable follow-up research to understand the photoactivated toxicity (PAT) of PAHs and

other chemicals [28,29]. This work showed that UV radiation enhances the toxicity of a variety of PAHs to aquatic species, sometimes causing lethality at water concentrations an order of magnitude or more below those that are toxic in the absence of UV radiation.

Photoactivated toxicity occurs when light of specific wavelengths is absorbed by a compound with an appropriate molecular structure such that electrons are elevated to higher energy orbitals. When these electrons return to lower energy orbitals, this energy can be transferred to other molecules [30]. Photoactivated toxicity occurs primarily when sufficient energy is transferred to molecular oxygen, forming reactive singlet oxygen, a powerful oxidizing agent that can damage a wide variety of biological molecules. This oxidative damage to cellular components is the initiating event for this AOP (Fig. 2b). This damage is nonspecific in that it will affect a variety of tissues, although tissues (and organisms) will be differentially affected as a result of differences in contaminant distribution, UV radiation penetration, and the ability of an organism to cope with reactive oxygen. In the PAT AOP, sufficient molecular damage leads to cell death, organ failure, organism mortality, and population effects (Fig. 2b).

This understanding of the AOP provides a foundation for formulating dosimetry relationships for PAT. For a given chemical and organism, the rate of accumulation of oxidative damage is proportional to the product of chemical concentration in the organism and the intensity of UV radiation at the appropriate wavelengths, resulting in reciprocal relationships between chemical concentration and radiation intensity as related to time to death [31,32]. Absolute sensitivity to PAT varies among organisms but can be accounted for empirically by measuring the sensitivity of an organism under a given set of conditions, then using modeled relationships to make predictions for other conditions. Because the initiating event for the PAT AOP is the production of reactive oxygen, the effects from mixtures of photoactive compounds should be additive, an expectation that has been confirmed experimentally for mixtures of PAHs [33]. Also, QSAR models to predict the relative PAT potential of different PAHs have been developed, based on the relationship of electron orbital energies of a chemical to the potential for formation of reactive oxygen [29,34,35].

Practical applications for the PAT AOP already exist in site-specific risk assessment, such as the widespread need to evaluate legacy PAH contamination of aquatic sediments [28]. In these applications, knowledge of the AOP is combined with PAH and UV exposure data to make relative risk predictions. In addition, the potential for PAT could be included in chemical screening programs, for example, in Europe, where QSAR-predicted PAT potential based on calculated physicochemical characteristics of test chemicals would contribute to meeting the goals of the REACH legislation.

AOP case example 3: Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a gene transcription factor that binds 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related planar aromatic chemicals. Activation of the AhR induces expression of xenobiotic-metabolizing enzymes and can lead to toxicity, including reproductive dysfunction, cancer, immunosuppression, and early-life-stage mortality [36,37]. A preponderance of both indirect and direct evidence demonstrates that the molecular initiating event for this AOP is binding to the AhR, although the events leading to toxicity have not been fully characterized. Direct evidence that the toxic effects of TCDD are initiated through the AhR has been demonstrated by studies with AhR knockout mice that are resistant to TCDD-induced toxicity [38] and by demonstration of reduced and delayed TCDD toxicity in zebrafish embryos injected with morpholino oligonucleotides targeted to suppress translation of AhR protein [39]. Biomarker responses (such as induction of certain enzymes), although not causal to producing toxicity, nonetheless can help identify chemicals operating via the AhR AOP. Activation of the xenobiotic-metabolizing enzymes is considered an adaptive response distinct from the cellular and physiological events that lead to toxicity, which are uncertain [40]. Knowledge of the nature of this AOP has contributed in a number of ways to the risk assessment process.

Chemicals structurally related to TCDD that bind the AhR almost always occur as complex mixtures in the environment; therefore, to assess the risk posed by exposure to mixtures of these chemicals, their combined toxicity must be determined. Because binding to AhR and the consequent gene expression are a common step in the AOP for all chemicals, mixture toxicity should be concentration additive. This provides the basis for the toxicity equivalence approach used for assessing the ecological risk of these chemicals relative to early-life-stage mortality in fish (Fig. 2c). To achieve this, potencies of individual chemicals in the mixture are characterized relative to that of TCDD, the most potent AhR activator known, to derive the TCDD toxicity equivalence concentration (TEC) contributed by each chemical [41]. The sum of the TECs for all chemicals in the mixture gives a total TEC used to predict the toxicity of the mixture. The utility of this approach for fish early-life-stage mortality has been confirmed in a retrospective analysis of the lake trout population in Lake Ontario (USA, Canada) throughout the last century [42] and has been shown to predict accurately the toxicity of a mixture of polychlorinated-biphenyls, -dibenzofurans, and -dibenzo-*p*-dioxins reflective of that found in salmonids from Lake Michigan [43].

In addition to providing a theoretical basis for assessing mixture toxicity, an understanding of the AhR AOP helps in addressing another challenge to ecological risk assessment, interspecies extrapolation of chemical effects. Differences in species sensitivity to TCDD-like chemicals may be attributed in large part to the specific characteristics of their AhR and its capacity to bind these chemicals [44]. Vertebrates, in general, are sensitive to the effects of these chemicals, whereas invertebrates tend to be insensitive, consistent with the fact that vertebrates possess an AhR that binds TCDD-like chemicals, whereas invertebrate AhR homologs do not. Frogs may be less sensitive than other vertebrates because of the relatively low affinity of their AhR for TCDD-like chemicals [45]. Within vertebrate classes, large sensitivity differences could be related to AhR structure [46]; for example, the difference in sensitivity for early-life-stage mortality caused by TCDD is nearly 40-fold between lake trout and zebrafish [47], and sensitivity between the domestic chicken and other avian species to TCDD can vary by an order of magnitude or more [46]. This understanding of the central role of AhR binding in the AOP potentially allows interspecies extrapolations to be based on short-term in vivo biomarker responses, in vitro assays, or structure–activity

relationships, reducing the need for long-term, whole-animal testing.

AOP case example 4: Activation of the estrogen receptor

The AOP for estrogen receptor (ER) agonists is anchored by the molecular initiating event of binding of an estrogen-mimicking chemical to the ER and altered reproduction (embryo production) at the whole-organism level, with consequent effects on populations (Fig. 3). Several other, more specific biological responses or biomarkers associated with ER activation have been identified at intermediate levels of biological organization (Fig. 3), including changes in expression of estrogen-responsive genes, alterations in plasma sex steroid and vitellogenin (VTG; egg yolk protein) concentrations, gonad abnormalities (such as intersex), and changes in secondary sex characteristics and reproductive behavior [48]. However, the mechanistic linkages among these specific responses and production of fertile eggs are not completely understood (Fig. 3). For example, although production of VTG is a specific and sensitive response to ER activation, the relationship of VTG induction (at least in male fish) to reproductive success is obscure [49], although correlations with reduced testicular size have been noted in developing fish [50].

Chemicals that bind to and activate the vertebrate ER have been postulated to cause a wide range of effects in fish and wildlife [51]. In vitro and in vivo studies have shown that many different environmental contaminants can activate the ER [52,53]. One of the best-characterized examples of endocrine disruption in the environment involves feminization of male fish (e.g., induction of VTG, production of intersex gonads, sex reversal) by ER agonists present in discharges from municipal wastewater treatment plants (WWTP) [51]. Estrogenic chemicals in WWTP discharges include contaminants such as octylphenol, nonylphenol, and their ethoxylates and carboxylates, but of most concern are potent natural and synthetic steroids such as 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) [51]. As a consequence, substantial amounts of toxicity data have been generated for aquatic species, especially fish, exposed to

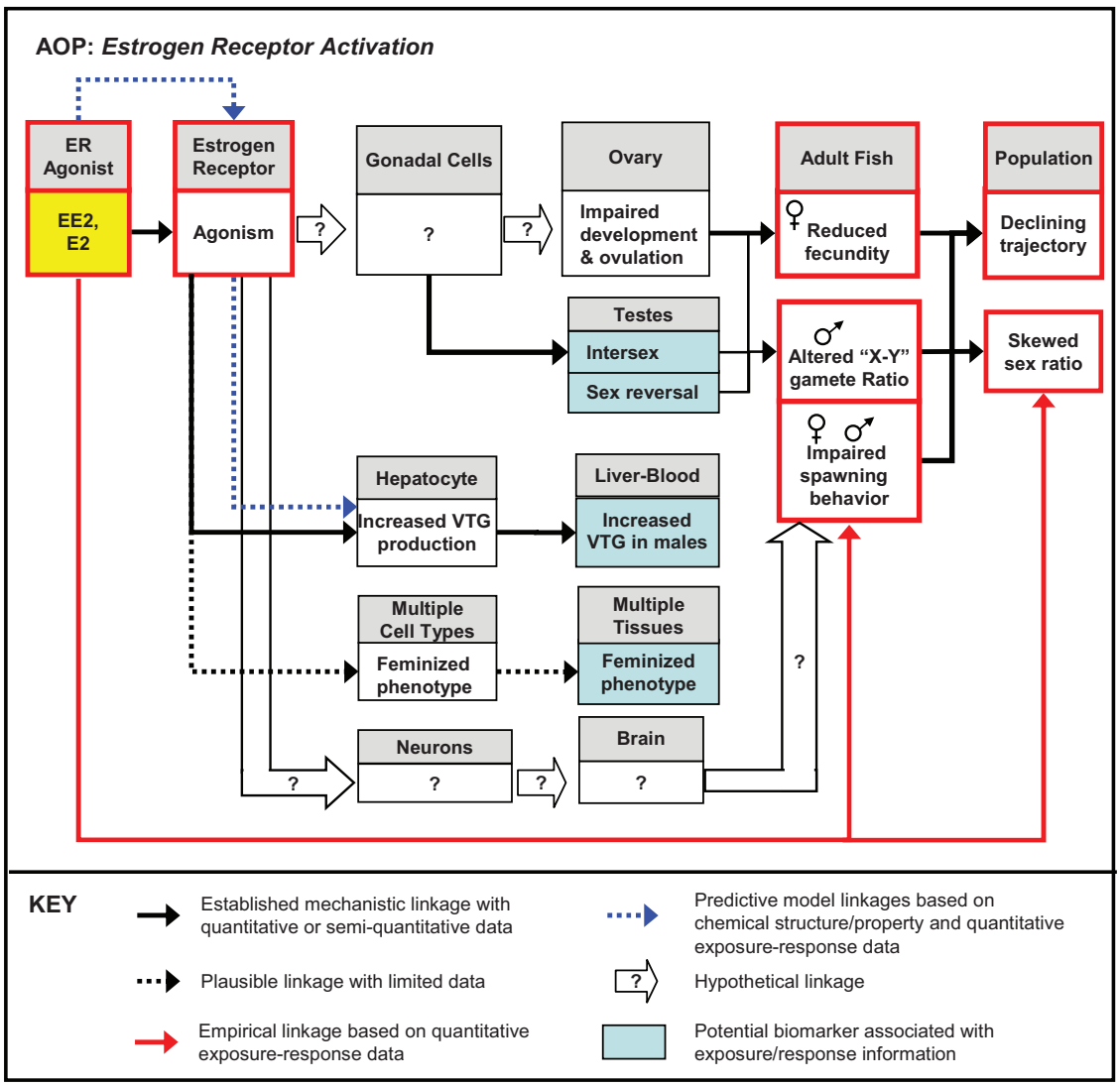

Fig. 3. Representation of an adverse outcome pathway (AOP) for estrogen receptor (ER) activation in fish. Although not all mechanistic linkages between the molecular initiating event (ER activation) and an adverse outcome (reduced reproductive success) are fully understood, several potential biomarkers are depicted that could be used to indicate exposure to a chemical operating via this pathway, such as 17α-ethinylestradiol (EE2) or 17β-estradiol (E2). Further detail concerning the pathway can be found in the text.

EE2 [48,54]. In life-cycle studies with a variety of fish species in the laboratory, EE2 consistently causes adverse effects on production of fertile eggs at low (ng/L) concentrations in the water [48,54]. Consistent with this, Kidd et al. [55] found that EE2 concentrations in the range of approximately 2 to 8 ng/L (over three years) resulted in collapse of a native fathead minnow population in a whole-lake dosing study.

Although not all responses to ER agonists can be causally related to one another in a linear manner, what we know about this AOP can nonetheless guide regulatory decision-making and testing. In vitro assays with fish tissues (ER binding, VTG mRNA transcription) have been developed and used in the context of an AOP framework [56] for systematic testing of chemicals within large inventories [57]. Data collected in such a manner and correlated with specific chemical structural features and physical–chemical properties are being used to develop a QSAR-based expert system to prioritize chemicals and to select those most likely to initiate the AOP for priority testing [4]. These tools directly support programs such as REACH in Europe and the Endocrine Disruptor Screening Program (EDSP) mandated by the FQPA and SDWA in the United States.

Knowledge that ER binding is the key initiating event for impaired reproduction and reproductive development also can help to guide test selection and design in regulatory efforts. An example of this is recent U.S. EPA recommendations regarding developing aquatic life criteria for EE2 [48]. Because the initiating event for AOPs involving the ER is binding to the receptor, knowledge of which taxa actually possess the ER allows risk assessors to focus testing on those with the ER and forego the need for in-depth testing of species without the receptor. Given that most (if not all) invertebrate organisms do not have a functional ER analogous to that of vertebrates, it may not be necessary to evaluate this large group of organisms when deriving water-quality criteria for estrogenic chemicals [48]. Knowledge of the AOP can also help in guiding test design. For example, although fish are quite sensitive to estrogens during portions of their life cycle, traditional fish early-life-stage tests (commonly used as surrogates for chronic tests in criteria derivation) would not provide data suitable for assessing the ecological risks of EDCs, because the test design does not include endpoints indicative of endocrine function [48].

AOP case example 5: Impaired vitellogenesis

In contrast to the previous AOP examples, this set of three interrelated AOPs—all causing impairment of vitellogenesis—has relatively well-defined biological responses linking molecular initiating events to an adverse outcome, reproductive dysfunction in fish (Fig. 4). Vitellogenin is synthesized in the liver and transported through the blood for uptake into developing oocytes [58]. Hepatic production of VTG is regulated by E2-mediated activation of the ER. Inhibition of E2–ER binding by estrogen antagonists results in reduced circulating VTG in fish [59]. The enzyme aromatase catalyzes the synthesis of E2 from the androgen precursor testosterone, so that chemicals inhibiting aromatase activity reduce circulating E2 concentrations in females, with concomitant decreases in plasma VTG concentrations [60]. Additionally, strong androgen receptor (AR) agonists (e.g., 17β-trenbolone) inhibit VTG production in female fish [61]. However, in this case, the chemical does not appear directly to affect E2 synthesis or binding to the ER; rather, the androgen agonist seems to trigger a negative feedback response resulting in reduced testosterone synthesis, which in turn limits the synthesis of E2. These three different molecular initiating events all lead to reductions in circulating VTG concentrations and reduced deposition of VTG to oocytes [60]. Because VTG is critical for oocyte development [58], reductions in VTG availability can be translated into reductions in fecundity [62]. In addition, it is possible to use the relationship between reduced VTG and fecundity to model population-level impacts for fish exposed to chemicals that affect vitellogenesis [62]. Overall, this chain of events, spanning multiple levels of biological organization, depicts a plausible linkage between several discrete molecular initiating events through a common node (VTG production) and an outcome (fecundity) relevant to population-level risk assessments (Fig. 4).

Definition of these AOPs provides a basis for the development of focused in vitro assays or QSAR models specific to ER antagonism, aromatase inhibition, and AR agonism to assist in the hazard evaluation of large chemical inventories to support efforts such as the REACH program. Various in vitro methods and computational models for detecting ER and AR agonists and antagonists or inhibition of steroidogenic enzymes already exist, and these AOPs inform how data from these types of tools can be translated to the tangible biological outcomes needed for regulatory applications.

Knowledge of these three related AOPs already has affected the development and validation of test methods for the EDSP in the United States and similar efforts elsewhere. Testing programs throughout the world typically include assays with fish to detect and assess the biological consequences of EDCs [63]. Initially these tests emphasized only the physiologically abnormal process of VTG induction in juvenile or male animals as a sensitive, relatively specific biomarker of chemicals with estrogenic activity (see previous AOP). However, as information has emerged concerning the mechanistic basis for, and biological consequences of, inhibition of vitellogenesis in female fish, VTG status in females has become a routine biomarker measurement in EDC screening assays with a variety of species [63].

The AOPs depicted in Figure 4 also can lend insights into the use of biomarkers in retrospective assessments. For example, monitoring programs in Canada have focused on the impacts of complex pulp and paper mill effluents on aquatic communities, including fish. A frequently observed biomarker response is decreased plasma sex steroids in fish (for review see Hewitt et al. [64]), which, based on these AOPs, would be predicted to decrease VTG and, subsequently, egg production and quality. Consistent with this prediction, McMaster et al. [65] found that female fish (white suckers) exposed to mill effluent exhibited reduced plasma steroid concentrations and had smaller eggs than females from a reference site.

IMPLMENTING AND ENHANCING THE AOP FRAMEWORK

Use of AOPs in risk assessment

The examples given above illustrate the general logic and the practical aspects of AOPs and highlight how this conceptual construct helps to establish the relevance of molecular initiating events and intermediate endpoints to outcomes used in ecological risk assessment, outcomes that typically concern apical

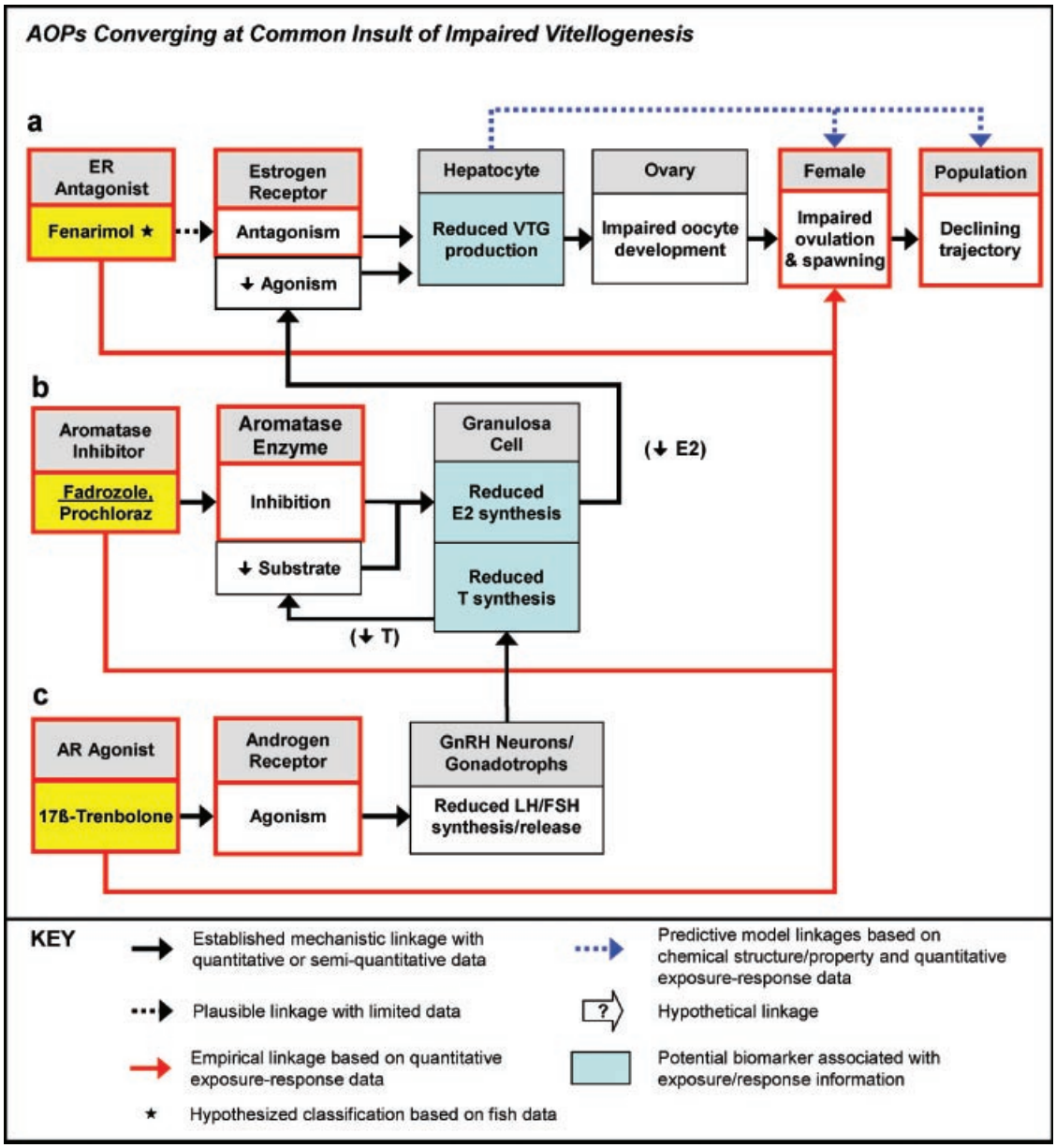

Fig. 4. Representation of three adverse outcome pathways (AOPs) that share reduced vitellogenin (VTG) production in fish as a common factor resulting in adverse impacts on egg production. Production of VTG can be inhibited by antagonism of the estrogen receptor (ER; **a**), direct inhibition of 17β-estradiol (E2) synthesis (**b**), or indirect reduction of E2 production by feedback inhibition of steroidogenesis (**c**). Compounds listed in the yellow boxes are examples of chemicals that could elicit toxicity via a particular pathway. Further technical detail concerning the pathways can be found in the text. T = testosterone; AR = androgen receptor; GnRH = gonadotrophin receptor hormone; LH = lutenizing hormone; FSH = follicle-stimulating hormone.

endpoints related to fitness. As noted above, an AOP would be a key part of the conceptual model for a risk assessment, providing the framework by which ecotoxicological information at various levels of biological organization is applied and integrated to assess outcomes more effectively.

The utility of information other than apical responses is readily apparent in AOPs that comprise a mechanistic chain of events leading from an initiating event to an adverse outcome. The case of inhibition of VTG synthesis (example 5) illustrates this point clearly; several pathways converge to reduce circulating VTG, a necessary component for viable oocyte production. Other AOPs may be sparsely populated in terms of mechanistic information that link an initiating event to an adverse outcome but can still be very useful to risk assessors because of the strength of the correlation or weight of evidence connecting the two. This is true in the case of the narcosis pathway (example 1), in which mechanistic detail is unnecessary to support the use of chemical K_{OW} in risk assessment decisions. In other instances, AOPs may include endpoints whose mechanistic significance is uncertain but that are nonetheless informative as specific markers of pathway activation or perturbation. An excellent example is the induction of VTG production in male fish by ER agonists (example 4) [4]. Although there is no known role for VTG in male reproduction, there is strong evidence that VTG induction in males is a sensitive and specific indicator of activation of the ER signaling

pathway, a pathway whose perturbation has been associated with reproductive impairment of fish. The AOP for activation of the ER pathways shows why this seemingly tangential marker can have relevance to risk assessments.

As AOPs become populated with greater mechanistic detail, it may be possible to discover endpoints that can be related to an adverse effect and that have diagnostic value for identifying the responsible initiating event. The example 5 AOPs illustrate how VTG reduction in female fish collected from the field can be interpreted in the context of potential impacts on reproductive capacity as well as how this information can be used to identify chemicals responsible for this effect. As such, the AOP framework provides a means to organize information from different levels of biological organization to help scientists make more effective use of the diversity of data types that may be available to support a risk assessment.

In the future, it will be possible to use a well-defined set of AOPs to ask informed questions about chemicals for which little is known. Molecular and biochemical endpoints usually do not, in and of themselves, provide sufficient information for a quantitative risk assessment for any one chemical. With this type of information, however, it may be possible to place a chemical within an established AOP and allow some level of evaluation with minimal data. If necessary, it would then be possible to perform hypothesis-driven testing along the AOP to evaluate the chemical's similarity to compounds that are better studied in terms of adverse outcomes. For example, extensive knowledge of how a potent ER agonist such as EE2 behaves (example 4) informs how one would approach evaluation of other potential estrogenic compounds in a focused, cost-effective manner [4,48,56].

The AOP framework also illustrates how effects caused by mixtures of chemicals that act via the same molecular initiating event (example 3) or affect pathways that converge at common intermediate steps (example 5) can be aggregated for risk characterization. In addition, the AOP framework can be used to address the issue of interspecies extrapolation by helping to identify points of convergence or divergence within pathways and the extent to which pathways are conserved across taxa of interest. For example, phylogenetic lack of operative AhRs and ERs in invertebrates renders these phyla relatively insensitive to agonists for those receptors (examples 3 and 4). Similarly, the AOP is a powerful concept for extrapolation among species in which conserved mechanisms are operative, but issues of relative sensitivity have to be addressed. Indeed, AOP development, in conjunction with knowledge concerning the significance of an AOP relative to an organism's life history, provides the basis for improving our understanding of the phylogenetic differences in species sensitivity.

Some of the case examples (examples 1, 2, and 4) illustrate how the AOP framework supports the use of QSARs for chemical extrapolation, by linking a compound's ability to interact with specific molecular targets to defined adverse effects. Finally, the AOP framework supports the use of biomarkers in risk assessment by putting these endpoints in the context of a mechanism-based pathway that, depending on exposure, could lead to ecologically meaningful outcomes. This is important in several regards, including understanding how genomic data can be used to develop biomarkers useful for prospective and retrospective risk assessments.

Identifying new AOPs

A relevant question concerns how specific AOPs should be identified and assembled. The AOPs described herein were developed from a largely retrospective analysis of data, informed by specific risk assessment problems. Although independently developed, each of these examples went through phases of descriptive or phenomenological science, followed by a certain amount of reductionism to elucidate mechanisms or develop relevant correlations, which culminated in the development of synthetic generalizations or models. This process is not new to toxicology. What is arguably different today, however, is that recent advances in the biological sciences have provided a means by which AOPs can be developed more efficiently than in the past. In particular, the emergence of genomic technologies, combined with advances in biological systems modeling, creates the opportunity to hypothesize, a priori, AOPs for toxicological processes of interest. This prospective use of the AOP concept may, in turn, shape the science used to support future development and use of AOPs.

The problem-formulation phase of ecological risk assessments is critical to defining AOPs. That is, for the approach to be useful, AOPs must present a clear articulation of pathway information regarding a specific risk issue. In addition, AOPs must be anchored to endpoints that are relevant to a specific risk issue. For ecological receptors, these endpoints are generally factors affecting fitness, such as fertility, development, growth, survival, and reproduction. Each of these general components of fitness can be further refined as more detailed, specific endpoints, depending on the nature of the AOP. Among the case examples, the narcosis and PAT AOPs are focused primarily on acute lethality, the steroid hormone AOPs are focused on regulatory pathways associated with reproduction, and the AhR AOP is focused on development. All of these endpoints are of relevance to ecological risk assessment and ensure that the respective AOPs address endpoints of ecotoxicological concern.

Finally, as additional AOPs are developed, it is critical that we continue to evaluate the gold standard used for characterization of adverse effects: apical responses from whole-animal testing. In this context, it is important to recognize that AOPs may extend to effects at the population level and that in this instance the whole organism is a single reference point in the overall pathway [66,67]. Results from standardized whole organism testing have provided most of the data used for population-level risk assessments, but the results from these tests are not always adequate to estimate changes in demographic parameters used in population assessments. If currently accepted test designs are not compatible with linkages in either direction (organ or population), then those protocols should be revisited in order to maximize their utility [68].

Integrating exposure

The AOP concept directly addresses questions regarding whether and how a particular initiating event can cause an adverse outcome. Adverse outcome pathways do not, however, address the question of what dose of chemical will cause sufficient perturbation to drive the pathway to the adverse outcome. Common expressions of dose include the total applied concentration (e.g., chemical concentration in the exposure

water) or administered dose (e.g., that injected or consumed within a defined period of time). In either case, it may be difficult to translate these dose metrics into an estimate of the chemical concentration time course at the site of toxic action because of issues related to uptake, tissue distribution, biotransformation, and elimination [69]. Without this knowledge of internal dose, it becomes difficult to compare results from different studies or to interpret apparent differences in species sensitivity [70]. In an analogous manner, in vitro studies are often performed with little knowledge of dose beyond the amount of material introduced into the system. Chemical binding to components of the system may substantially reduce the amount of compound available to cause observed effects [71], complicating comparisons among studies and making it difficult to extrapolate test results to the intact animal. Additional uncertainties arise in relating laboratory-derived toxicity test results to the environmental systems that we aim to protect because of issues such as bioavailability, animal life history, and the fluctuating nature of many exposures.

Although the AOP concept does not directly address the issue of chemical dosimetry, it provides a rational basis for conducting the science needed to address this question. In short, full implementation of the AOP concept requires methods and modeling approaches to relate seamlessly chemical dose at one level of biological organization, or within one experimental system, to other levels of biological organization and within other experimental systems relevant to a chemical's mechanism of action and expressed effects. Additional methods and models are then needed to place these exposures within an environmental context. The AOP concept also may require consideration of which dose metric provides the most meaningful basis for interpreting observed effects. For an AOP that involves long-term accumulation of tissue damage, the area under the plasma concentration–time curve may provide an informative dose metric. In other cases, the maximal achieved concentration in plasma or the target tissue (if known) may provide a useful dose metric. Finally, it is important to characterize both environmental and internal exposures in terms of free (unbound) chemical, because this is the chemical form that is most available to be taken up by the organism and to interact with specific cellular targets [72].

Considered in isolation, an AOP also does not provide guidance on the issue of relative potency. Many compounds are likely to act via different AOPs and cause different adverse effects depending on dose. Because, in general, risk assessors have to know the lowest concentration of a chemical that causes adverse effects, it may be necessary to characterize the dose–response relationship for several AOPs as a means of focusing attention on the pathways that are most relevant for a particular compound. Alternatively, it may be important to evaluate the relative potency of several compounds acting via the same AOP.

Role of systems biology

In and of themselves, AOPs cannot take into consideration all of the potential biological interactions that may determine whether an initiating event will drive the system, uninterrupted, to the adverse outcome. To the extent possible, therefore, AOPs should be considered in the context of broader systems biology. Here we refer to systems biology as a field of study concerned with understanding and modeling the integrated, interacting networks of genes, proteins, biochemical reactions, or higher order biological units that give rise to function and support life (http://www.systembiology.org/Intro to ISB and Systems Biology/Systems Biology the 21st Century Science). In the sense that it considers how molecular interactions can be translated into adverse outcomes that threaten individual or population survival, the AOP concept draws upon a systems biology perspective. Adverse outcome pathways can be viewed as cutting across, intersecting or interacting with, or wholly contained within various biological systems, depending on how they are defined. Regardless of whether they are contained within or simply intersect with surrounding systems, AOPs are distinct in that they are relatively sequential depictions focused on portraying existing knowledge concerning the connectivity between a particular initiating event and a defined adverse outcome. However, consideration of the more focused AOPs within the context of the surrounding systems provides a foundation for considering higher order questions such as those related to adaptive or compensatory responses and cross-talk among various pathways and multiple AOPs. Thus, the continued characterization of both AOPs and broader systems biology has an important role to play in the ultimate success of predictive toxicology.

CONCLUSIONS

In the present paper, AOPs are described as an organizing framework to help facilitate ERAs (and the research supporting these assessments) for toxic chemicals. Consideration of the linkage of adverse outcomes to molecular initiating events and intermediate endpoints allows a diversity of ecotoxicological information from different levels of biological organization to be more effectively applied to risk assessment goals. As illustrated in the case examples, considering assessment problems in terms of AOPs can help the development of methods for extrapolating effects among species and chemicals and for relating organism-level endpoints to biomarkers, cellular assays, and other suborganismal information. Although these examples derive from work not explicitly using the AOP terminology and framework, they demonstrate the value of recognizing such mechanistic linkages and the need to better formalize and pursue their consideration in risk assessments.

As also illustrated in some of the case examples, the utility of the AOP perspective does not require complete elucidation of all the steps in the AOP or all possibilities for different AOPs. In fact, an AOP perspective can help in identifying what information would have utility for assessing the adverse outcomes relevant to risk assessors and thus help efficient assignment of limited resources. For example, an attempt to elucidate all mechanisms without concern for their relevance to specific outcomes will result in an inefficient use of resources. By using the AOP approach, it is possible to identify endpoints of regulatory concern and to ask which toxicity mechanisms are most likely to lead to these outcomes. By combining this approach with an understanding of chemical exposure and potency, it will be possible to focus research efforts on the questions of greatest relevance to a specific assessment problem.

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