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Invited article

# Significance of adverse outcome pathways in biomarker-based environmental risk assessment in aquatic organisms

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ABSTRACT

In environmental risk assessments (ERA), biomarkers have been widely used as an early warning signal of environmental contamination. However, biomarker responses have limitation due to its low relevance to adverse outcomes (*e.g.*, fluctuations in community structure, decreases in population size, and other similar ecobiologically relevant indicators of community structure and function). To mitigate these limitations, the concept of adverse outcome pathways (AOPs) was developed. An AOP is an analytical, sequentially progressive pathway that links a molecular initiating event (MIE) to an adverse outcome. Recently, AOPs have been recognized as a potential informational tool by which the implications of molecular biomarkers in ERA can be better understood. To demonstrate the utility of AOPs in biomarker-based ERA, here we discuss a series of three different biological repercussions caused by exposure to benzo(*a*)pyrene (B*a*P), silver nanoparticles (AgNPs), and selenium (Se). Using mainly aquatic invertebrates and selected vertebrates as model species, we focus on the development of the AOP concept. Aquatic organisms are suitable bioindicator species whose entire lifespans can be observed over a short period; moreover, these species can be studied on the molecular and population levels. Also, interspecific differences between aquatic organisms are important to consider in an AOP framework, since these differences are an integral part of the natural environment. The development of an environmental pollutant-mediated AOP may enable a better understanding of the effects of environmental pollutants in different scenarios in the diverse community of an ecosystem.



## Introduction

The aquatic environment is continuously loaded with diverse xenobiotics such as organic compounds, heavy metals, nanoparticles, and a host of other organic and inorganic chemical pollutants. Aquatic organisms are increasingly being exposed to chemicals released from a wide spectrum of sources during all stages of their life cycles. Moreover, multi-generational effects of chemicals have even been observed (Van der Oost et al., 2003; Ankley et al., 2010; Hutchinson et al., 2013) (Fig. 1). A variety of toxic effect endpoints such as immunotoxicity, neurotoxicity,

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reproductive toxicity, cancer, and death of aquatic wildlife are closely linked with the significant adverse impact of chemicals on the aquatic ecosystem (Adams, 2002). To protect aquatic environmental health and integrity, several countries have enforced specific regulations in the last two decades that restrict the loading of chemicals into the aquatic environment. These regulations have had a significantly positive effect on the level of environmental pollution, especially for aquatic pollutants such as nonylphenol, tributyltin (TBT), and terbutryn (Díez et al., 2002; Quednow and Püttmann, 2009). Pieces of legislation such as the Food Quality Protection Act (FQPA) and the Registration, Evaluation and Authorization of Chemicals (REACH) regulations are also impactful in that they increase awareness of the potential risk of the growing number of chemicals and the need to minimize or control this risk (Ankley et al., 2010; Caldwell et al., 2014).

Environmental risk assessment (ERA) is an important tool for examining the adverse effects of chemicals on various biological responses in target and nontarget species (Van der Oost et al., 2003). In the 20th century, ecological risk assessors have studied the effects of environmental pollutants on the individual, population, community, and ecosystem levels (Choi, 2005; Villeneuve and Garcia-Reyero, 2011; OECD, 2012). For example, the sediment quality assessment triad was conceived as an effect-based approach for ecological/environmental risk assessment. This triad covers sediment chemistry, *in situ* studies (*e.g.*, research on the benthic organism community), and bioassays (toxicity tests) (Chapman, 1986; Chapman, 1996; Chapman and McDonald, 2005). In general, bioassays include direct measurements of adverse outcomes *in vivo* (*e.g.*, mortality and failure to grow or reproduce). However, these kinds of approaches are costly, time-consuming, and unfocused. Moreover, conclusions are often derived from many assumptions and several arbitrary uncertainty factors have been found to influence the outcomes. Additionally, extrapolation from these data is not sufficient to determine interspecific differences or to discriminate controlled tests from uncontrolled real environmental situations (Villeneuve and Garcia-Reyero, 2011). In conventional ERA, it is often insufficient to assess non-lethal effects of low concentrations of pollutants and to detect early biological responses (Van der Oost et al., 2003; Maier et al., 2004; Choi, 2005).

The effects of toxicants begin at the molecular level and then progress to the biochemical, subcellular, cellular, tissue, organ, individual, and population levels (Van der Oost et al., 2003). Thus, a precise understanding of the effects of toxicants on the molecular or biochemical level can provide valuable early warning signals, as opposed to higher level adverse effects that occur later in this chain of progression. Early detection of sublethal effects would be useful to highlight pollution in need of remediation before catastrophic effects occur. Detection of these sublethal events is also useful for monitoring the recovery site after management has been implemented (Van der Oost et al., 2003; Berninger et al., 2014). High-throughput technologies such as transcriptomics, proteomics, and metabolomics have helped us understand the modes of action of many toxins on the individual level (Hook, 2010). However, the biological response observed on the suborganism level does not provide reliable results in the context of environmental risk assessment, since the response on the suborganism level is based on an extensive volume of biological information controlled by physiological compensatory responses and repair pathways (De Kruijf, 1991; Choi, 2005). Thus, these studies have been received with some skepticism, since they do not take the environment into account. Moreover, exposed organisms potentially interact within their own population and with other populations, such as competitors, predators, and prey. Exposed organisms also interact with biotic and abiotic factors of their environment (Kramer et al., 2011). Therefore, it is important to have a linkage framework on the subindividual level by which the response can be connected to potential adverse outcomes (*e.g.*, population, ecological levels). Such a framework highlights the usefulness of biomarkers in mapping the risk of chemical exposure on all the biological levels at which a chemical is likely to act.

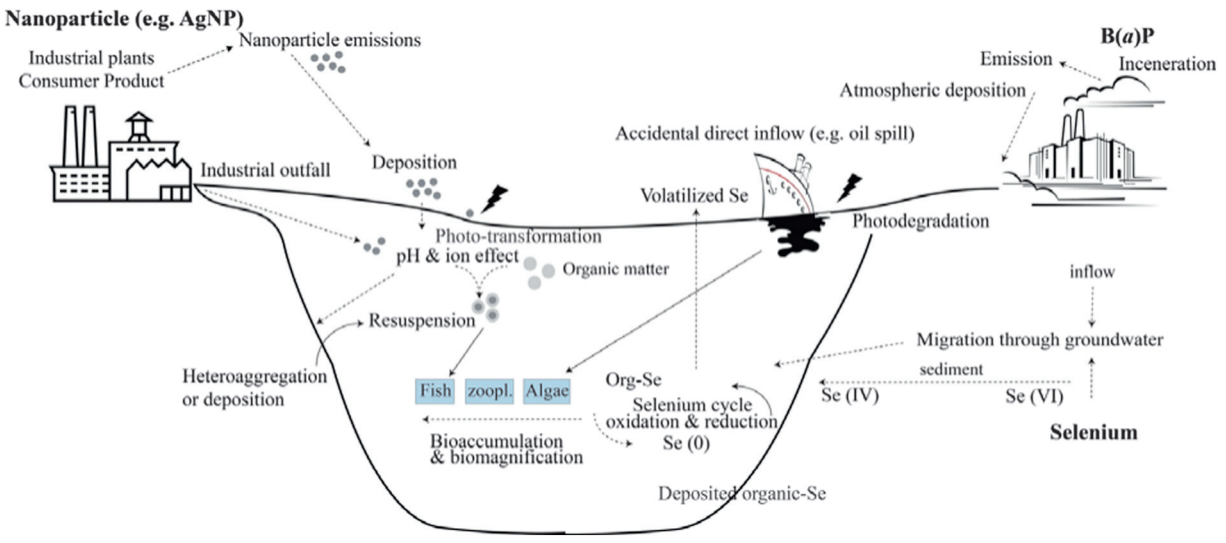


**Fig. 1 – Fates of nanoparticles, B[*a*]P, and Se in the aquatic environment.**

The overall goal of ERA is to protect the status of organisms, populations, communities, and ecological habitats from the adverse effect of environmental contamination. Therefore, to enhance the objectivity of risk assessment, biomarker studies are required to link predictions on the individual level, the population level, and other levels in a conceptual framework (De Kruijf, 1991; Ankley et al., 2010; Kramer et al., 2011). Conventional bioassay approaches for environmental pollutants are costly, time-consuming, and involve extensive animal use. These approaches also yield little information on mechanistic toxicity (Ankley et al., 2010; Kramer et al., 2011; Volz et al., 2011; Hutchinson et al., 2013). Alternative bioassay-based risk assessments, including *in vitro*, *in vivo*, and *in silico* methods, are often used to obtain reliable endpoints. However, these methods also have their weaknesses. For example, their results have been shown to lack relevance to apical endpoints (*e.g.*, adverse outcomes) (National Research Council, 2007; Ankley et al., 2010; Volz et al., 2011; Villeneuve and Garcia-Reyero, 2011; FitzGerald and Wilks, 2014). Therefore, an objective, robust, and accurate framework is needed to accurately predict toxicity outcomes on the various levels of biological organization at which an aquatic pollutant can interact. In this context, adverse outcome pathways (AOPs) have been proposed as a new paradigm to link direct molecular initiating events (MIEs) with adverse outcomes, thus integrating molecular events into the framework of ERA (Ankley et al., 2010; OECD, 2012; Berninger et al., 2014; Vinken et al., 2014). Generally, AOPs comprise modes of action (MOAs) that have been defined as "functional and anatomical changes at the cellular levels that commonly characterize as adverse biological responses" (Borgert et al., 2004; ECETOC, 2007; Ankley et al., 2010). This definition indicates that the existing information is sufficient to predict adverse outcomes to create a framework to improve risk assessment decisions, even when the exact chain of events is unknown (Ankley et al., 2010; Dellarco and Fenner-Crisp, 2012). Thus, the formulation of AOPs that incorporate MIE and MOA concepts is an efficient and targeted approach to maximize the utility of existing knowledge and is an attractive alternative that minimizes the reliance on resource-intensive testing approaches (Ankley et al., 2010; Krewski et al., 2010). These links can provide a greater use of predictive approaches in ERA (Ankley et al., 2010). Therefore, AOPs are a potentially useful tool for predicting adverse outcomes on the individual, population, community, and ecological levels according to biomarker responses, thus yielding a better understanding of the implications of a given molecular event in ERA (Berninger et al., 2014).

In this review, we present case studies of three distinct chemicals: benzo(*a*)pyrene (B*a*P), silver nanoparticles (AgNPs), and selenium. We discuss the biological responses to these chemicals and integrate this information into the AOP framework. This review highlights the utility of AOPs for biomarker-based ERA and examines the use of AOPs in ERA using aquatic organisms.

## 1. Case study 1: benzo(a)pyrene-induced cancer in fish

B*a*P is a polycyclic aromatic hydrocarbon (PAH) and is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC). B*a*P and other PAHs are formed from the incomplete combustion of organic matter and are ubiquitous environmental contaminants (USEPA, 1991). B*a*P binds to the aryl hydrocarbon receptor (AhR) and upregulates the production of cytochrome P450 (CYP1A, B, C) enzymes for its metabolism (Lin et al., 2003b; Boelsterli, 2007; Wang et al., 2010). Microsomal CYP1 converts B*a*P to a carcinogenic intermediate metabolite, B*a*P-r-7,t-8-dihydrodiol-t-9,10-epoxide (±) (BPDE), which can form DNA adducts that cause errors in DNA replication during DNA repair (Boelsterli, 2007; Phillips and Arlt, 2007). Other studies have also shown that B*a*P induces *CYP1A* expression, modulates its enzymatic activity in teleost fish (*Pleuronectes vetelus* and *Fundulus heteroclitus*), and exhibits a positive correlation with tumor formation (Myers et al., 1998; Reichert et al., 1998; Wang et al., 2010; Wills et al., 2010). However, an AOP of B*a*P-induced cancer occurrence has not yet been clearly examined in teleost fish.

Here we propose an AOP for B*a*P exposure that links known molecular alterations to the induction of cancer (Fig. 2). The MIE of B*a*P exposure is the binding of B*a*P to the AhR (Hahn, 2002; Lin et al., 2003b; Wang et al., 2010), leading to AhR activation and *CYP1A* expression in teleost fish (Haasch et al., 1993; Levine and Oris, 1999; Hahn, 2002; Bo et al., 2010; Wang et al., 2010; Lee et al., 2012). In the red sea bream *Pagrus major*, expression of *Pm-CYP1A1* and *Pm-AhR2* is induced simultaneously in a time-dependent and dose-dependent manner (Bo et al., 2010). In a recent study, B*a*P induced both *CYP1A* mRNA and protein expressions in liver and gills of four well known model species (freshwater minnow *Zacco platypus*, zebrafish *Danio rerio*, Japanese medaka *Oryzias latipes*, and common carp *Cyprinus carpio*) (Lee et al., 2015). Moreover, a positive correlation has been observed between CYP1A protein levels and DNA adduct formation in the teleost fish *Z. platypus* upon exposure to B*a*P (Lee et al., 2012). In B*a*P-exposed killifish (*Fundulus grandis* and *F. similus*), a dose-dependent increase in the formation of DNA adducts has been reported, along with significant modulation of *CYP1A* genes (Willett et al., 1995). In feral English sole (*Pleuronectes vetulus*), Reichert et al. (1998) observed a positive correlation between DNA adduct formation and tumor generation. The comparison of B*a*P-induced DNA adduct formation in cancer-prone (brown bullhead *Ameirus nebulosus*) and -resistant fish (channel catfish *Ictalurus punctatus*) suggests that DNA adduct formation is linked with cancer occurrence (Ploch et al., 1998). Moreover, several studies have demonstrated that B*a*P exposure leads to carcinoma and adenoma in fish (Corrales et al., 2014; Lerebours et al., 2014). Wang et al. (2010) showed that B*a*P induces hepatocellular carcinoma, cholangioma, and hepatocellular adenoma in *F. heteroclitus*. Observations of these disorders on the cellular level can be linked to mortality, which is useful for predicting adverse outcomes on the individual level that ultimately affect populations. Thus, B*a*P exposure can cause cancer in fish through AhR-induced *CYP1A* expression and DNA adduct formation, and a series of these adverse outcomes lead to adverse effects on the population level.

B*a*P-induced CYP1A production has also been linked with immunosuppression (Wang et al., 2010). In B*a*P-exposed sea bream, modulation of cortisol levels and antibacterial activity has been associated with the induction of adverse outcomes on the individual organism level (Bo et al., 2012, 2014). B*a*P also

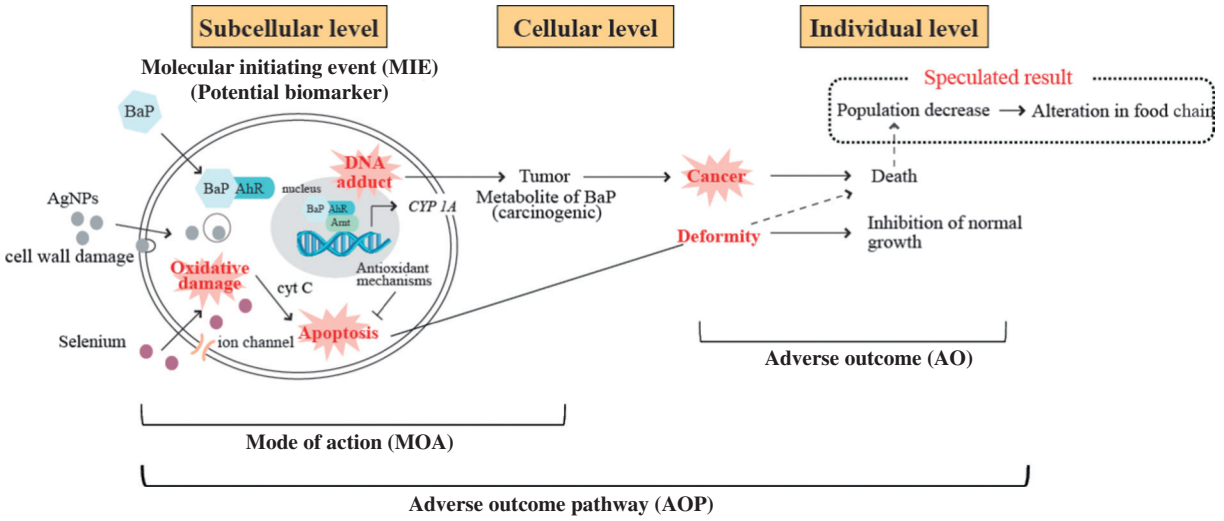


**Fig. 2 – Adverse outcome pathways for silver nanoparticles, B[*a*]P, and Se in various organisms.**

causes AhR2-dependent pericardial edema, yolk sac edema, and the upregulation of CYP1A and other related genes in fish (Cook et al., 2003; Chikae et al., 2004; Ortiz-Delgado and Sarasquete, 2004; Li et al., 2011). Thus, exposure of fish to B*a*P can induce various hypothetical AOPs in the context of cancer induction and the immune response. In initial risk assessments of B*a*P, conventional bioassays have focused on the bioaccumulation of B*a*P and its lethal effects in mammals and aquatic vertebrates (Hofelt et al., 2001; Van der Oost et al., 2003). However, carcinogenesis studies using B*a*P-related biomarkers have revealed a correlation between the mixed function oxidase (MFO) system and metabolites generated by B*a*P metabolism, making a B*a*P-mediated AOP a reliable, fast, and precise approach for assessing risk (Van der Oost et al., 2003).

## 2. Case study 2: silver nanoparticle-induced fish embryo toxicity

Engineered nanoparticles (NPs) have been applied to a wide range of industrial fields according to the physicochemical properties of the different NPs (Kwok et al., 2012; Maurer-Jones et al., 2013). It has been estimated that the production of NPs will exceed half a million tons by 2020 (Robichaud et al., 2009; Stensberg et al., 2011). No data have yet been obtained regarding the consequences of NP disposal, which remains an area of substantial concern. The release of NPs into the aquatic environment is of particular concern due to their potential ecological and environmental health risks (Robichaud et al., 2009; Maurer-Jones et al., 2013). Out of all the NPs in use, silver nanoparticles (AgNPs) are of the greatest concern due to their extensive use for their antimicrobial properties and their release into the aquatic environment, which poses a hazard (Kashiwada et al., 2012; Kwok et al., 2012; Maurer-Jones et al., 2013). In recent study, AgNPs have been shown to be lethal to fish embryos in a concentration-dependent manner and AgNP-treated fish embryos exhibit delayed hatching (Asharani et al., 2008; Bar-Ilan et al., 2009). Also the fate of AgNPs in the aquatic environment demonstrates that ROS (*e.g.*, superoxide and hydroxyl radicals) was generated in response to several light sources such as Xenon or UV lamp (Li et al., 2013).

Here we outline a proposed AOP for AgNPs that focuses on the following endpoints: oxidative stress, apoptosis, and fish embryo malformation. Of them, the MIE of AgNPs exposure is ROS production (Garcia-Reyero et al., 2014). Several *in vitro* assay systems showed the significant ROS induction in response to AgNPs (Arora et al., 2008; Foldjerg et al., 2009; Carlson et al., 2008). In AgNP-exposed zebrafish embryos, the ROS production was increased and followed by delayed hatching, physical deformities, and depressed heart rate (Massarsky et al., 2013). Placing AgNPs into the context of an AOP, exposure to AgNPs has been shown to upregulate the expression of various antioxidant-related genes for up to 28 days in Japanese medaka (*O. latipes*) (Pham et al., 2012). Moreover, polyvinlypyrrolidone-coated-AgNPs (PVP-AgNPs) have been shown to induce oxidative stress in Japanese medaka embryos (Wu and Zhou, 2012). AgNP-exposed zebrafish (*D. rerio*) have been shown to exhibit upregulation of apoptosis-related biomarker genes (*e.g.*, *Bax*, *Noxa*, *p21*) along with increased levels of malondialdehyde and total glutathione, indicating that AgNPs induce oxidative stress and that zebrafish activate an antioxidant defense program in response to AgNPs. AgNP-exposed zebrafish also exhibited upregulation of the expression of a number of apoptosis-related genes, including *p53*, bcl2-associated X protein, phophatidylinositol glycan C, phosphatidylinositol glycan P, and insulin-like growth factor binding-protein 3 (Yeo and Pak, 2008). Embryo deformities have also been reported in AgNP-exposed Japanese medaka, in addition to altered expression patterns of six oxidative stress-related, embryogenesis-related, and morphogenesis-related genes (*ctsL*, *tpm1*, *rbp*, *mt*, *atp2a*, and *hox6b6*) (Kashiwada et al., 2012). Zebrafish also show AgNP-induced phenotypic abnormalities, including spinal deformities, cardiac malformation, yolk sac edema, head edema, and eye malformation (Lee et al., 2007; Asharani et al., 2008). AgNP-exposed zebrafish embryos exhibit morphological malformations and up to 100% mortality when exposed postfertilization (Bar-Ilan et al., 2009), indicating that AgNPs

induce oxidative stress, apoptosis, and embryo malformation. In turn, these effects potentially lead to adverse outcomes such as embryo lethality.

The toxicity of AgNPs varies according to the coating material and silver speciation (Garcia-Reyero et al., 2014). In sea bass and zebrafish, a different AOP for PVP (polyvinylpyrrolidone)-AgNPs has been described in which the PVP-AgNPs antagonize the dopamine receptor (Irons et al., 2013; Leal et al., 2013). Thus, PVP-AgNP-mediated antagonism of the dopamine receptor (the MIE of this AOP) is associated with decreased food intake and locomotion activity in the sea bass *Dicentrarchus labrax* and in zebrafish, consequently leading to death (Irons et al., 2013; Leal et al., 2013). In addition to this AOP scenario, PVP-AgNPs have also been shown to cause disturbances of the egg chorion, developmental malformation, and oxidative stress in Japanese medaka embryos (Wu and Zhou, 2012). Furthermore, chromosomal aberrations were recently reported in AgNP-exposed immortalized medaka cells (Wise et al., 2010). Taken together, these molecular biomarkers in AgNP-exposed fish can help identify different MOAs on the subcellular level and thus lead to different AOPs in ERA.

## 3. Case study 3: Selenium-induced fish embryo toxicity

Trace metals and their various forms have their own specific chemical characteristics, interact with living organisms, and are toxic to different extents (Lin et al., 2003a; Ra et al., 2006). Of them, selenium (Se) recently has emerged as one of the most promising environmental contaminants causing toxicity, although the minimal concentration of these trace elements is required for normal growth and development in organisms as an essential metal (Lemly, 2002). The Se is usually released into the waterways in inorganic forms (selenite or selenate), subsequently absorbed by microbes, and then converted into organic forms such as selenomethionine (SeMet) (Fan et al., 2002; Kupsco and Schlenk, 2014; Thomas and Janz, 2014). The toxicity of Se, the accumulation of Se in the food chain, and the diverse biological effects of Se have been described in vertebrates and in the aquatic ecosystem (Spallholz, 1994; Hamilton, 2004). Both bluegills (*Lepomis macrochirus*) and daphnids (*Daphnia magna*) accumulate significant amounts of Se through their diet (Ingersoll et al., 1990; Cleveland et al., 1993). Specifically, Se concentrations exceeding the essential concentration by 7 to 30-fold can cause embryotoxicity (Lemly, 1997; Kupsco and Schlenk, 2014). Exposure of rat embryos to selenite and selenate has been shown to result in malformation and growth inhibition (Usami et al., 2002). Moreover, exposure of fathead minnow embryos to 16 μg/g Se has been shown to result in reproductive failure (Schultz and Hermanutz, 1990). The ROS induction can be considered as MIE of Se exposure in fish. Rainbow trout (*Oncorhynchus mykiss*) embryos exposed to SeMet have been shown to exhibit increased loads of superoxide radicals (Palace et al., 2004). Specifically, Se and/or converted forms of Se induce oxidative stress and apoptosis (Palace et al., 2004; Selvaraj et al., 2013). Regarding Se-induced apoptosis, Se has been shown to significantly upregulate ROS production and the activity of caspase 3 *in vitro* in the fish cell line PLHC-1 (Selvaraj et al., 2013). In rainbow trout (*O. mykiss*) hepatocytes, selenite elicited an intracellular ROS increase followed by an induction of catalase and superoxide dismutase enzymatic activities and significant increases of caspase 3 and 7 activities as indicators of apoptosis occurrence (Misra and Niyogi, 2009). This finding indicates that Se-induced apoptosis may deleteriously affect embryogenesis, a finding supported by other studies (Nijhawan et al., 2000; Cole and Ross, 2001; Iijima and Yokoyama, 2007). Indeed, Japanese medaka embryos exposed to SeMet have been shown to exhibit significantly increased oxidative stress and impaired embryo hatching (Lavado et al., 2011). Se-exposed zebrafish also exhibit abnormal development and irregular neuron growth accompanied by apoptosis in a dose-dependent and time-dependent manner (Ma et al., 2012). In addition to these findings, selenium toxicity was linked with fish embryo-larval deformities including lordosis, kyphosis, scoliosis, deformities of gills, and mouth as well-documented biomarkers of adverse effects of selenium (Hamilton, 2004). Consistent with these studies, two weeks of exposure to SeMet resulted in reduced growth and increased mortality of juvenile green sturgeons (Riu et al., 2014), indicating that exposure to Se can induce oxidative stress-mediated apoptosis and embryo malformation. Thus, exposure to Se can significantly affect the status of target species in aquatic ecosystems.

## 4. Summary of AOP case studies and future directions

A cumulative body of data suggests that exposure to B*a*P, AgNPs, and Se can lead to adverse outcomes such as cancer, embryo malformation, and death (Fig. 2). Of these AOPs, cancer and embryo malformation can be associated with *CYP1A* expression, DNA adduct formation, and ROS induction in response to B*a*P, AgNP, and Se exposure. Although some biomarkers have been reported to serve as indicators of exposure to and/or effects of these three environmental pollutants (Table 1), few studies have focused on AOPs related to these pollutants. Similarly, diverse species have been used as bioindicators in studies of the ecotoxicity of these chemicals (Table 2). The conceptual evaluation of an AOP in response to environmental chemicals can help to characterize the MOA of the chemicals on the organisms and facilitate the choice of a reliable bioassay using robust biomarkers. In toxicity testing of B*a*P, AgNPs, and Se, the concept of an AOP can help contribute to the development of an "Integrated Approach to Testing and Assessment" and to evaluate an "Integrated Testing Strategy." These approaches and strategies are used to obtain the hazardous endpoint, leading to the refinement, reduction, and/or replacement of conventional *in vivo* testing (OECD, 2012). However, the AOP framework does still have some limitations in terms of environmental monitoring and predictive assessment, since an AOP requires *a priori* assumptions to choose reliable molecular endpoints (*e.g.*, key events, MIEs) and to predict the potential effects of a pollutant in a field study (Berninger et al., 2014). The advantages and drawbacks of AOP *vs.* conventional ERA are highlighted in Tables 3 and 4. To overcome the various limitations of AOPs, the use of "-omics" tools has been suggested to fill this missing link (Berninger et al., 2014; Martinovic-Weigelt et al., 2014). In the past decade, the

application of genomics to toxicology has reaped many rewards, including the successful identification and comparison of the modes of action of various toxic chemicals (Hook, 2010; Biales et al., 2013). For example, microarray analysis of amphipods exposed to copper *via* the food and/or water source demonstrated that different individual adverse outcomes are closely associated with different modes of action of copper (Hook et al., 2014). Similarly, microarray-based transcriptomic analysis of fathead minnows has revealed different types of molecular events in response to pollutant exposure and has helped predict AOs on the population level that can be linked with the initial molecular events (Berninger et al., 2014). In summary, the benefit of omics-based approaches is that they can help with the initial choice of reliable molecular markers that can be anchored to AOs. These approaches are also useful later in the process by helping to fill the gap between molecular markers and individual effects. Omics approaches facilitate the precise definition of MIEs, which serve as key anchors for chemical–biological interactions as the starting point of the AOP. Also, publically available omics-based datasets can also be used to identify MIEs in future risk assessments. Thus, integrating knowledge-based MIEs with omics approaches can be a useful method for finding the initial targeting parameters in mechanistic studies.

## 5. Challenges for establishing AOPs in aquatic invertebrates

The extrapolation of AOPs to various species is closely linked with the increased application of subcellular biomarkers in ERA. For example, B*a*P-induced DNA adduct formation is a highly reliable biomarker in diverse species including humans, rodents, and fish. Also, B*a*P-exposed vertebrates (*e.g.*, rodents, fish, human) exhibit increased *CYP1A1* expression and DNA adduct formation, the latter of which can cause cancer (Huang et al., 1992; Poirier and Beland, 1992; Ross et al., 1995). However, the simultaneous observation of molecular responses and adverse outcomes is technically difficult, since apparent adverse outcomes on the individual level usually appear only after a long period of exposure. One recent study identified several aquatic invertebrates as potential model organisms due to their abundant populations and short life cycles (Dahms et al., 2011). Some invertebrate species that are potentially excellent model organisms in ecotoxicological risk assessment include (but are not limited to): water flea (*D. magna*), monogonont rotifer (*Brachionus koreanus*), and intertidal copepod (*Tigriopus japonicus*) (Choi, 2005; Raisuddin et al., 2007; Colbourne et al., 2011; Dahms et al., 2011; Kim et al., 2011; Rhee et al., 2011, 2013b). The extensive genomics information available for these species may facilitate the simultaneous understanding of MOA and AO analyses on both the population and molecular levels in response to diverse classes of environmental pollutants. Furthermore, extensive genomic DNA and RNA-seq databases give these species an advantage over other aquatic invertebrate species with less complete genomics databases (Raisuddin et al., 2007; Lee et al., 2010; Colbourne et al., 2011; Dahms et al., 2011; Lee et al., 2011; Hwang et al., 2013a,b; De Coninck et al., 2014). Thus, these aquatic invertebrates are suitable species for the development of AOPs. For example,

**Table 1 – Identified biomarkers for benzo(a)pyrene, silver nanoparticles, and selenium exposure and their end points in different species.**

| Environmental pollutant | Type of biomarker (exposure/effect) | Biomarker level (molecular/cellular/tissue) | Biomarker/end point | Predicted pathway | Reference |
|---|---|---|---|---|---|
| A. Benzo(a)pyrene | Exposure | Molecular | Aryl hydrocarbon receptor expression | CYP1A expression | Bo et al. (2010) |
| | | Molecular | Antimicrobial peptide hepcidin (PM-hepc) expression | Immune-associated | Bo et al. (2012) |
| | | Molecular/subcellular | Cortisol level and DNA integrity, CYP4501A | Immune-associated | Bo et al. (2014) |
| | Exposure | Molecular | CYP1A expression | DNA adduct formation | Lee et al. (2012); Willett et al. (1995) |
| | Effect | Molecular/subcellular | DNA adduct formation | Tumor generation | Wang et al. (2010); Reichert et al. (1998) |
| | Effect | Tissue | Tumor generation | Cancer | Wang et al. (2010) |
| B. Silver nanoparticles | Exposure | Molecular | ROS generation | Oxidative stress | Li et al. (2013) |
| | Exposure | Molecular | Antioxidant expression | Oxidative stress | Pham et al. (2012); Wu and Zhou (2012), |
| | | Molecular | Chromosomal aberrations and aneuploidy | Cytotoxicity | Wise et al. (2010) |
| | Effect | Cellular | Apoptosis occurrence | Embryo malformation | Yeo and Pak (2008), |
| | Effect | Individual | Embryo malformation | Mortality | Lee et al. (2007); Asharani et al. (2008) |
| | Effect | Individual | Mortality | Population decrease | Bar-Ilan et al. (2009) |
| C. Selenium | Exposure | Molecular | Superoxide radical increase | Oxidative stress | Palace et al. (2004) |
| | Effect | Cellular | Oxidative stress | Oxidative stress | Lavado et al. (2011) |
| | Effect | Individual | Embryo malformation | Mortality | Ma et al. (2012) |
| | Effect | Individual | Mortality | Population decrease | Riu et al. (2014) |

**Table 2 – Bioindicator species used in the biomonitoring of benzo(*a*)pyrene, silver nanoparticles, and selenium.**

| Environmental pollutant | Ecosystem (marine/freshwater/estuaries) | Bioindicator species | Place in food chain | End point | Reference |
|---|---|---|---|---|---|
| A. Benzo(*a*)pyrene | Marine | *Brachionus koreanus* | 1st consumer | CYP family expression | Kim et al. (2013) |
| | Freshwater | *Zacco platypus* | 2nd consumer | CYP1A expression, DNA adduct formation | Lee et al. (2012) |
| | Marine | *Oryzias melastigma* | 2nd consumer | CYP family expression | Kim et al. (2014) |
| | Freshwater | *Oryzias latipes* | 2nd consumer | Gonadosomatic index | Chikae et al. (2004) |
| | Marine | *Pagrus major* | 2nd consumer | CYP1A expression | Bo et al. (2014) |
| | Estuaries/freshwater | *Fundulus grandis* | 2nd consumer | CYP1A expression, DNA adduct formation | Willet et al. (1995) |
| | Freshwater | *Fundulus similus* | 2nd consumer | DNA adduct formation | Willet et al. (1995) |
| | Estuaries | *Pleuronectes vetulus* | 2nd consumer | Neoplasm | Reichert et al. (1998) |
| | Estuaries | *Fundulus. Heteroclitus* | 2nd consumer | Carcinoma, adenoma | Wang et al. (2010) |
| | Freshwater | *Ameriurus nebulosus* | 2nd consumer | DNA adduct, EROD | Ploch et al. (1998) |
| | Freshwater | *Ictalurus punctatus* | 3rd consumer | DNA adduct, EROD | |
| | Freshwater/marine | *Oncorhyncus mykiss* | 3rd consumer | CYP1A expression | Levine and Oris (1999) |
| B. Silver nanoparticles | Freshwater | *Oryzias latipes* | 2nd consumer | Antioxidant expression, embryogenesis and morphogenesis-related gene expression | Pham et al. (2012); Wu and Zhou (2012); Kashiwada et al. (2012) |
| | | | | Chromosomal aberrations and aneuploidy | Wise et al. (2010) |
| | Freshwater | *Danio rerio* | 2nd consumer | Antioxidant expression, apoptosis gene expression, morphological malformations, mortality | Yeo and Pak (2008); Lee et al. (2007); Asharani et al. (2008); Bar-Ilan et al., 2009; Choi et al. (2010) |
| C. Selenium | Freshwater | *Daphnia magna* | 1st consumer | Se accumulation | Ingersoll et al. (1990) |
| | Freshwater | *Lepomis macrochirus* | 2nd consumer | Se accumulation | Cleveland et al. (1993) |
| | Freshwater | *Danio rerio* | 2nd consumer | Cortisol, glycogen, abnormal development, growth, apoptosis | Thomas and Janz, (2014) Ma et al., 2012 |
| | Estuaries | *Acipensor medirostres A. transmontanus* | 2nd consumer | Growth inhibition, mortality | Riu et al., 2014 |
| | Freshwater/marine | *Oncorhynchus mykiss* | 3rd consumer | Superoxide radical expression | Palace et al., 2004 |

**Table 3 – Comparison of conventional ERA vs AOP with respect to their applications in environmental monitoring.**

| Sl. | Parameter | ERA | AOP |
|---|---|---|---|
| 1. | Precision of analysis | Moderate | Moderate |
| 2. | Accuracy of prediction | Moderate | High |
| 3. | Time involved in analysis | Long | Short |
| 4. | Cost factor | High | Low |
| 5. | Human resources involved | Many | Few |
| 6. | Prediction value | Low | High |
| 7. | Ecosystem level involved (individual/population/community) | Individual | Individual to population |
| 8. | Chances of harmonization | High | High |
| 9. | Regulatory acceptability | Accepted with uncertainty factors | Yet to be explored |

the rotifer *B. koreanus* is an ideal species for the development of an AOP, since these organisms are widely distributed along marine and estuarine coastal lines. These rotifers also possess other desirable characteristics such as an abundant population, ease of cultivation, small size, and short generation cycle (Yoshinaga et al., 2003; Dahms et al., 2011; Kim et al., 2013) (Fig. 3). Rotifers play a critical role in the aquatic food web; thus, risk assessment using an AOP framework can be used to systematically interpret community-wide effects in response to chemical exposure. Genomic DNA and RNA-seq databases of rotifers provide a wealth of information from which diverse MIEs in response to environmental pollutant exposure can be obtained (Lee et al., 2011; Kim et al., 2013; Rhee et al., 2013b). Moreover, the complete *CYP* gene sequences are known for rotifers. Thus, this information could be applied to metabolomics-based studies of risk assessment for waterborne xenobiotics, such as B$\alpha$P and other hazardous and persistent aquatic pollutants (Kim et al., 2013). Cumulatively, these data indicate that rotifers have great potential as bioindicator species to study xenobiotic metabolism and the effects of xenobiotics on detoxification and oxidative stress (Rhee et al., 2011, 2013b; Yang et al., 2013a, 2013b, 2013c) in AOP development. The potential of rotifers as an indicator species was also demonstrated in various studies in which they were exposed to gamma-radiation, pharmaceuticals, metal, and endocrine disruptors (Rhee et al., 2013b; Han et al., 2014). Thus, rotifers may help the development of an AOP that links the molecular response to effects on the individual and population levels in ERA.

However, due to the absence and/or low affinity of the AhR in several aquatic invertebrates, only limited predictions can be made regarding the toxicity of B*a*P using a vertebrate AOP with Phase I reactions (Livingstone, 1998; Hahn, 2002; Rewitz et al., 2006). Although weak EROD activity and CYP1A-immunoreactivity have been observed in invertebrates (Livingstone, 1998; Peters et al., 1998; Chaty et al., 2004; Rewitz et al., 2006; David et al., 2012), the AhR ligand is unable to signal *via* TCDD or $\beta$NF in a number of aquatic invertebrates (*e.g.*, molluscs and *Caenorhabditis elegans*) (Hahn, 2002). The different profiles of EROD activity imply that the presence of different *CYP* isoforms generates diverse PAH metabolic processes in rotifers, copepods, fish, and the crustacean *Aristeus antennatus* (Koenig et al., 2012; Kim et al., 2013; Rhee et al., 2013a; Han et al., 2014). Another relevant issue is interspecific differences of nuclear receptors. For instance, the estrogen receptor (ER) binds to estrogen and plays a critical role in vertebrate reproduction, but several invertebrates (*e.g.*, mollusks, cephalochordates) have been reported to be insensitive to estrogen. Invertebrate endocrine function is also affected by endocrine-disrupting pollutants (Janer and Porte, 2007; Oehlmann et al., 2007; Keay and Thornton, 2009), indicating that estrogenic effects can be caused by non-ER-mediated pathways in several invertebrates. Thus, applications of established AOPs to some species without considering species diversity could lead to false predictions of adverse effects and thus incorrect selection of reliable bioindicators, biomarkers, and bioassays in ERA. Therefore, extensive studies are required to fully understand toxicity mechanisms across species to obtain the best AOPs, particularly in aquatic invertebrates.

## 6. Conclusions

This review discussed the effects of exposing aquatic organisms to B*a*P, AgNPs, and Se in the context of AOPs with the goal of better understanding the implication of biomarker responses in ERA. AOPs have great promise as a useful tool for predicting adverse outcomes (*e.g.*, cancer, embryo malformation, death) when accompanied by a complete analysis of the molecular event. Also, AOPs have the potential to help characterize and classify chemical MOAs and to choose suitable

**Table 4 – Comparison of conventional ERA vs alternative AOP-based ERA with respect to various parameters of environmental analysis.**

| Conventional ERA | Alternative AOP-based ERA |
|---|---|
| High relevance to adverse outcomes, but costly and time-consuming (apical individual end point)<br>Assumption and uncertainty factors | Enhances the utility of biomarkers (short term test) as molecular initiating events (MIEs) that connect molecular to individual level adverse outcomes<br>Increases the predictive value of biomarker assays<br>Allows the categorization of chemicals based on toxicological mechanisms<br>Facilitates the development of qualitative and quantitative predictive models based on structure-activity relationships |

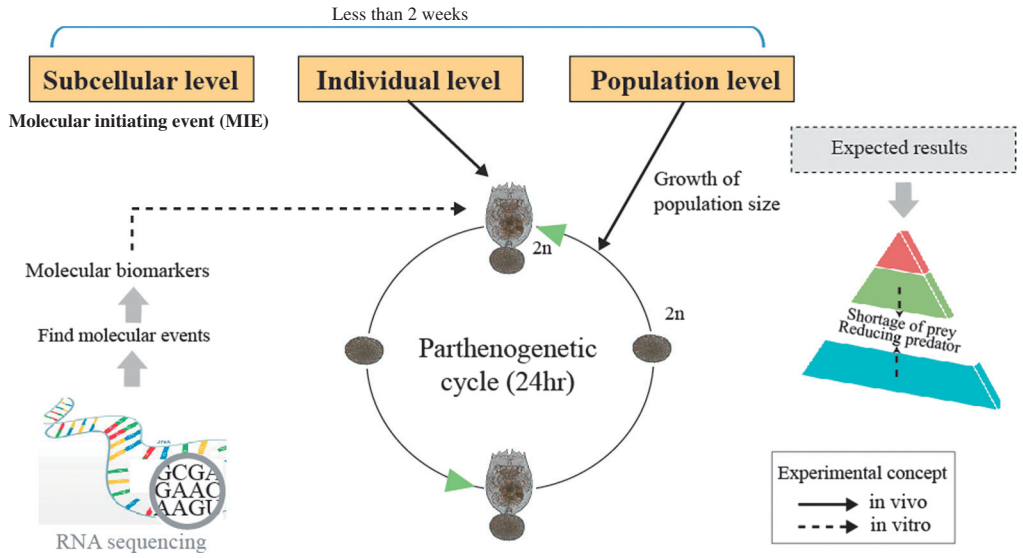


**Fig. 3 – Advantages of rotifers as a model species for developing an AOP concept-based study.**

bioassays for emerging chemicals. Thus, AOPs can help link chemicals with their effects on the molecular, cellular, or higher levels in ERA. However, interspecific differences regarding chemical MOAs are a formidable challenge in the development of AOPs, since different species can vary widely in their susceptibilities to chemicals. However, since a wealth of toxicity data has been generated regarding specific biochemical, molecular, and physiological endpoints, it is an opportune moment to develop the concept of AOPs in the context of ERA. Specifically, AOPs can be applied to biomarkers as early warning signals. In particular, the enhanced utility of MIEs in a conceptual AOP approach can connect adverse outcomes from the molecular to the individual level. In addition, the categorization of chemicals based on MOA, MIE, and AOP facilitates the development of qualitative and quantitative predictive toxicity models. Successful implementation of AOPs will require a concerted effort throughout the scientific community.

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