Pure Appl. Chem., Vol. 75, Nos. 11–12, pp. 2435–2444, 2003.
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Workshop 3.1

Need for establishing integrated programs to monitor endocrine active compounds*

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Abstract: Environmental monitoring programs on endocrine active compounds (EACs) have been used to document the level of exposure and to assess the possible association to the occurrence of developmental and reproductive disorders in wildlife. The establishment of causal links between exposure and effect data, however, was found to be difficult due to, for example, the presence of confounding factors or limited understanding of EAC mechanisms and interactions, but also because of conceptual and methodological limitations of current monitoring strategies. In order to provide plausibility of an EAC etiology for a developmental or reproductive alteration in a wildlife population, integrated monitoring programs are needed that will use a combination of complementary approaches: methods for a targeted search for suspected EACs in an environmental mixture, analysis of internal EAC doses instead of external EAC concentrations, utilization of mechanism-based endpoints in bioanalytical and effect monitoring, investigation of the basic biology and physiology of wildlife sentinel species, laboratory replication of field effects, as well as consideration of epidemiological and weight-of-evidence criteria in the design and data evaluation of monitoring programs.

INTRODUCTION

A number of synthetic as well as natural chemicals are capable of interfering with the endocrine system of wildlife, including fish, amphibians, reptiles, birds, and mammals. These so-called endocrine active compounds (EACs) may lead to activational or organizational alterations of endocrine homeostasis, development, and reproduction in exposed organisms. The consequences of EAC exposure have attracted much attention due to the potential impact on survival and sustainability of wildlife populations. In fact, monitoring studies on a range of wildlife species have shown the vulnerability of developmental and reproductive processes, and exposure to chemicals has been suspected to be the causative factor in many studies [e.g., 1–3]. However, to date a chemical etiology could be unequivocally demonstrated only in a few cases. In a review of field monitoring studies, Monosson [4] identified eight cases where scientists felt confident on a chemical etiology of the observed developmental and reproductive disturbances, while in the majority of the studies there was only some suspicion that contaminants are causative for the adverse effects. Thus, the proof of causative links between environmental chemical exposure and adverse biological alterations in endocrine-related parameters of wildlife continues to be a subject of debate.

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

source: https://doi.org/10.7892/boris.115312 | downloaded: 26.4.2024

EXAMPLES OF MONITORING STUDIES ON DEVELOPMENTAL AND REPRODUCTIVE DISTURBANCES IN WILDLIFE WITH SUSPECTED CHEMICAL ETIOLOGY

In most of the field research, wildlife populations dependent upon aquatic food webs such as fish and fish-eating birds have been in the focus. The aquatic environment is a sink for many chemicals that can be bioaccumulated from water or sediment or can be bioconcentrated along the food webs. The release of EACs into the aquatic environment occurs, e.g., via sewage treatment plants (STPs), and the observation that STP effluents possess endocrine activity [5], was one of the hallmarks that led to public concern on EACs.

One of the best studied examples of endocrine disruption in aquatic organisms is the phenomenon of imposex in prosobranch mollusks, particularly neogastropods. The term imposex has been coined to describe the superimposition of male characteristics such as penis and vas deferens onto female mollusks. Available evidence shows that worldwide more than 100 species of prosobranchs are affected by imposex [6]. Abundant field data indicate a correlation between this irreversible sexual abnormality and environmental tributyltin (TBT) contamination [7–9], pointing to TBT as the causative agent of imposex. Further support for a TBT etiology comes from the observation that with declining environmental concentrations of TBT, subsequent to TBT bans in many countries, a recovery of affected field populations of mollusks did occur [9]. Finally, laboratory experiments demonstrated that TBT is able to induce imposex in mollusks at environmentally realistic concentrations and in a concentration-dependent way [10,11]. The TBT example satisfies most of the criteria of Gray et al. [12], for a cause–effect relationship between an EAC and observed effects in wildlife, in particular correlation between effect and exposure, presence of the same effect in related species, induction of a specific pathognomic effect, disappearance of the effect with chemical cleanup, and ability to replicate the effect in the laboratory using relevant concentrations of the suspected EAC. One criterion that is not fulfilled in the TBT example is the identification of the mechanism of action. To date, the precise mechanism by which TBT leads to imposex formation has not been proven unequivocally.

The importance of understanding the mode of action in the diagnostic assessments of EAC-suspected field cases may be illustrated by the studies on the impact of pulp- and paper-mill effluents on fish populations. A number of studies have shown that pulp- and paper-mill effluents can induce alterations in endocrine homeostasis and reproductive fitness in exposed feral fish [13]. Perhaps the most extensive of these studies deal with white sucker (*Catostomus commersoni*) exposed to bleached kraft pulp-mill effluent (BKME) in Jackfish Bay, Lake Superior, USA. These fish displayed an array of altered physiological and reproductive parameters, including cytochrome P4501A induction, elevated apoptotic DNA fragmentation in ovarian follicular cells, alterations of circulating sex steroid levels, reduced gonad size, reduced fecundity, and delayed sexual maturity [13–15]. Examination of white sucker collected during their spawning migration demonstrated that a number of functions within the pituitary-gonad axis were affected by exposure to BKME [16]. Laboratory studies with fathead minnows confirmed it is the effluent exposure that induces the reproductive changes [17]. However, identification of the chemicals responsible for the BKME effects has been not achieved yet. Plant-derived compounds such as β-sitosterol have been discussed as causative agents for some of the effects associated with BKME exposure. Treatment of fish with β-sitosterol showed that this compound binds to the estrogen receptor and has estrogenic properties, however, it appears that the β-sitosterol effects are mediated not only through the estrogen-receptor pathway but involves other mechanisms as well [18,19]. Further, these studies indicated that it is unlikely that β-sitosterol is solely responsible for the endocrine and reproductive changes. However, the full identification of the responsible chemicals requires that the mechanisms underlying the BKME-induced responses are known, so that a directed search for those chemicals in the BKME which interfere with the relevant molecular targets and processes in exposed organisms will be possible.

The double-crested cormorants (*Phalacrocorax auritus*) at the U.S. Great Lakes may illustrate the difficulty in assessing the role of EACs relative to other factors in determining recruitment and dynam-

ics in widlife populations. Great Lake populations of this fish-eating bird fell dramatically throughout the 1950s and 1960s, and by 1970, the double-crested cormorant was nearly extirpated from the Great Lakes [20]. DDT-induced eggshell thinning is believed to have been the primary cause of this cormorant decline in the Great Lakes. With the banning of DDT in 1972, cormorant populations in the Great Lakes began to recover and increased from approximately 100 breeding pairs in 1973 to more than 10 000 breeding pairs in the 1990s [20]. However, in the post-DDT era, cormorant populations continued to suffer from embryo mortalities and birth defects [21,22]. In contrast to the DDT-induced syndrome, which is characterized by normal embryonic development with mortality induced by eggshell breakage, the post-DDT syndrome is characterized by in ovo mortality, edema, and deformities (GLEMEDS: Great Lakes embryo mortality, edema, and deformities syndrome; [23]). Epidemiological investigations, findings from laboratory studies with other avian species, as well as analytical studies on the correlation between embryo deformities and their contaminant burdens indicated that GLEMEDS is caused by the accumulation of dioxin-like compounds, i.e., compounds that act through the arylhydrocarbon-receptor pathway [21,22,24,25]. Possibly, the GLEMEDS effects have been present already during the DDT era, but have been masked by the eggshell thinning. The case of GLEMEDS in double-crested cormorants provides an example of a field study where the mechanism of action, the causative chemicals, and the adverse effects at the level of the individual are known. However, the ecological relevance of the dioxin-induced adverse effects is questionable since cormorant populations strongly increased during the 1990s, despite the continued contamination with dioxin-like chemicals. It appears that chemical contamination is just one of the cumulative set of factors that contributed to the previous population decline of cormorants; other factors involved could be changes in human persecution of cormorants, alterations in the abundance of the main forage fish of cormorants, the alewife (*Alosa pseudoharengus*), or altered interspecific competition, for instance, because of different sensitivities of Larids and cormorants to developmental toxicants [22].

CONFOUNDING FACTORS TO THE ESTABLISHMENT OF CAUSE–EFFECT RELATIONSHIPS BETWEEN EAC EXPOSURE AND RESPONSES IN WILDLIFE

Monitoring provides an approach to assess the association between the scale and magnitude of environmental EAC contamination, and the biological responses in wildlife organisms and populations. The principal questions that have to be answered by impact monitoring are whether an impact does occur, by which factor, and how "large" the impact is [26]. As exemplified by the case studies above, the most compelling challenge hereby is perhaps not finding out what is wrong, but in finding out what is causing the effects. The difficulty in linking cause and effect at either the population or individual level in monitoring programs on endocrine disruption may stem from a number of factors, some of them indicated in the following:

- Exposure assessment of wildlife to EACs is a critical component in evaluating cause–effect relationships. While some compounds that interfere with the endocrine system, e.g., PCBs, are persistent and bioaccumulative, other EACs such as alkylphenols or estrogens are biodegradable and/or metabolizable. Very little is known about the fate of natural and synthetic EACs in the environment [27]. For the natural estrogen, 17β-estradiol (E2), it has been shown that the steroid was degraded by river microorganisms with half-lives between 0.2 to 9 days at 20 °C, while the synthetic estrogen, ethynylestradiol (EE2), was found to be more recalcitrant to catabolism [28]. Larsson et al. [29], observed in a study on a Swedish sewage treatment work, that both natural estrogens and EE2 were present in unconjugated form although humans primarily excrete the conjugates. This suggests that deconjugation occurred within the sewage system. The ratio between EE2 and natural estrogens was higher than the theoretical value based on human secretion ratios of natural and synthetic estrogens, indicating a faster degradation of the natural estrogens. The findings from chemical analytical investigations on environmental concentrations of EE2 are

variable, with some studies reporting no detectable concentrations of EE2 in the environment, while other studies found EE2 levels in the low ng/l-range [27,29,30]. These concentrations are sufficient to induce biological effects [31,32], but are close to the current limits of chemical analytical detection. Considering the lipophilicity of EE2, bioaccumulation is to be expected, however, to date few studies have addressed this question. Larsson et al. [29] found that bile of fish caged downstream to the sewage plant contained estrogenic substances at concentrations 10^4–10^6 times higher than water levels, indicating a strong bioaccumulation of these substances. Lai et al. [33] calculated EE2 bioaccumulation factors for a range of aquatic organisms. The calculations predicted that bioaccumulation would occur in all organisms, with the bioaccumulation factors ranging from 1.8 to 332. EE2 was predicted to exhibit a higher bioaccumulation than the natural estrogens. Compared to actually measured data as provided in the literature, the predicted values for fish were approximately 1000 times less than the values observed in laboratory tests, while for invertebrates, the modeled values were less than two orders of magnitude below laboratory results.

- The time- and concentration-dependency of EAC-induced biological responses may differ from that of other hazardous substances. There are discussions ongoing whether EACs evoke unusual concentration–response relationships such as U-shaped and inverted U-shaped curves [34]. Further, sensitivity of organisms to EACs can vary for specific periods during their life cycle ("sensitive window" concept). Oviparous species possess complex life cycles that contain transitional periods in which new functions emerge. The transitional life stages are often the critical windows for exogenous perturbation. In mammals, with an early determination of sexual differentiation, the embryonic and fetal period may be the critical period for EAC action, while this may be different in many teleost species with a high degree of plasticity in phenotypic sex throughout their life span [35–37].
- Species differences of endocrine physiology and reproductive strategies are of particular importance for the assessment of EAC effects, since they may lead to different responses under identical exposure situations [27,36]. For instance, sexual differentiation in mammals is primarily controlled by androgens, in reptiles by androgens and estrogens, and by estrogens in birds; hence, it is expected that androgen analogs would be more likely to have an effect on the sexual differentiation of mammal, rather than avian, species [36,37]. The specific reproductive strategy of oviparous species offers several targets for EAC action that do not exist in mammals, including external fertilization, eggshell production, egg yolk production/utilization, hatching, and metamorphosis [3].
- Natural fluctuations of developmental and reproductive rates in wildlife populations can be high, which makes the detection of an—additional—impact of EACs on population dynamics difficult. Further complications arise from possible compensatory responses to EAC impact, for instance, an EAC-induced increase of embryo mortalities may be compensated by reduced mortalities at later stages of development. Not only population-level fluctuations, but also changes at the organism level may be difficult to judge with respect to the role of EACs. For instance, for roach, (*Rutilus rutilus*), it has been shown that intersex, i.e., the simultaneous occurrence of both male and female gametes in the gonad, is inducible by exposure to environmental estrogens [38,39]. This gonadal condition appears to occur also spontaneously, but it is not known at what frequency. Jobling et al. [38] reported that in roach from a laboratory control, 4 % of the fish examined showed intersex condition, and at field control sites, incidence of intersexuality ranged from 4 to 18 %. According to the authors, a "low level of intersexuality can be considered as natural", however, it can be difficult to exactly define what a "low level" is.
- Developmental and reproductive alterations at the organism level as well as alterations of population dynamics in wildlife can be induced by a number of factors other than EACs. In the environment, wildlife is not only exposed to EACs, but to other stressors such as overfishing of populations, habitat loss or change, predation, food availability, water temperature, etc. The

combination of multiple stressors and inherent attributes of wildlife biology makes it difficult to tease out the relative contribution of exposure to EACs in endocrine-disruptive effects. Frequently, locations showing elevated levels of EACs may be also contaminated by other anthropogenic compounds. Further, environmental factors other than chemical contamination, such as pathogens or thermal stress can impair reproduction, growth, and development. A well-known example from fish is the influence of temperature on sexual differentiation, also parasite infection has been shown to modify sexuality in fish [40]. For birds, the Seychelles warbler provides an interesting example as this species can manipulate the sex ratio of their offspring such that a breeding pair produces predominantly males or females, depending on the availability of food [41]. These few examples may illustrate the environmental plasticity of endocrine-regulated processes in many vertebrate species. Therefore, disturbances in endocrine parameters of wildlife species, e.g., skewed sex ratios, are not necessarily an indication of endocrine disruption.

- A similar problem as the multiple stressor issue discussed above is the fact that under real world conditions, organisms typically are not exposed to single but to mixtures of EACs. These can include mixtures of chemicals with identical action, e.g., estrogenic activity, but also mixtures of compounds with differing activity. It has been suggested that the possible adverse effects of estrogenic EACs may be neutralized by the presence of antiestrogenic compounds, but conclusive data are missing. Currently, we have no thorough understanding of the behavior of EACs in mixtures in order to be able to explain and understand endocrine-disrupting effects that may arise from exposure to EAC mixtures.

INTEGRATED CHEMICAL–BIOLOGICAL MONITORING

From the previous sections, the problems of monitoring studies in linking observed biological responses to exposure to suspected EACs are apparent. These difficulties come in part from the lack of current understanding of many important interactions, but they are also due to methodological and conceptual limitations in monitoring programs. Clearly, monitoring has to be more than a "just measuring" activity. Effective monitoring requires understanding of the nature, as well as temporal and spatial scales of both, the disturbance and the responses. Monitoring programs that are limited in their methodological or conceptual approach will be not sufficient to provide comprehensive answers. Also, the purely correlative use of chemical and biological monitoring measures is restricted in its ability to establish causative links. What we actually need are monitoring approaches that can identify the relevant chemicals in a targeted search (i.e., to identify those chemicals having an endocrine potency among the many chemicals present in an environmental sample), and, at the same time, the monitoring approach has to be able to sort out the relevant factors among the many confounding factors which confuse the cause–effect relationship. This asks for the use of modern methodologies of exposure and effects assessment, for instance, combined chemical–bioanalytical techniques or the use of a broad array of biological endpoints. In addition, however, a weight-of-evidence approach will have to be used to reduce the level of uncertainty. Several investigators have proposed criteria which can help to make a case for causal inference and to evaluate whether wildlife populations have been affected by EACs [4,12,57,58]. An example of such criteria is provided by the work of Ankley and Giesy [60], who suggested the following criteria for establishing cause–effect relationships: (1) documentation of effects in individuals, (2) documentation of adverse effects in populations, (3) coherence between effects observed in populations vs. those in individuals, (4) identification of a plausible mechanism of action consistent with effects in individuals (possibly through laboratory studies—see above), (5) positive identification of specific contaminants operating through this mode of action (possibly by combined bioanalytics/chemical analytics—see above), (6) reasonableness of dose–response relationships, (7) evidence of the recovery of populations or individuals upon removal of the stressor. Hill [60], suggested nine epidemiological criteria to be used when a correlation is observed between an exposure and an effect, to help decide whether there is a case for inferring that exposure causes the effect: strength of association, consistency of association, speci-

ficity of association, time order, biological gradient/dose–response relation, biological plausibility, coherence, experimental evidence, and analogy. The usefulness and applicability of the weight-of-evidence approach and epidemiological criteria in an ecological context is demonstrated in the review of Rollands [61], upon the effects of pollution on reproduction and survival of early life stages in teleosts.

An important step for the success of a monitoring study is the definition of which questions the study is actually supposed to answer and in what detail. The precise formulation of the monitoring objective has important implications for the selection of the monitoring design and of the variables and measurement parameters to be monitored. For instance, if the monitoring study is intended as an initial screen to identify potentially impacted sites, the approach has to be different than when the aim is to identify the EACs at an impacted site. This may be exemplified by comparing the approach taken by the European project COMPREHEND, which aimed to examine the distribution of endocrine active STP effluents across European countries [42], or the approach taken by Routledge [43], who aimed to identify the compounds responsible for the estrogenic activity of STP effluents in the United Kingdom. Another question to be considered in the design of a monitoring program is whether we deal with point sources or non-point sources. Critical parameters for the success of a monitoring study are the temporal and spatial scale, i.e., the recognition of cause–effect linkages by comparison the "before-and-after-impact" situation, or by comparing a control and an impact site [26]. To find suitable control sites, i.e., locations that are as similar as possible in all respects to the impact location, except for the presence of the putative impact (EAC contamination), can be difficult. In densely populated areas such as Europe, a location not impacted by anthropogenic activity hardly exists. In riverine systems, often upstream locations are taken as controls, since they are usually less impacted, at least they may have not yet received STP effluents and may be not surrounded by urbanized or agricultural areas. However, upstream sites are often different to downstream site in many aspects other than the chemical impact, for instance, flow and temperature regimes. Therefore, downstream/upstream differences may be due to background spatial changes that are unrelated to EAC impact. In addition, since both the control and the impact site are located in the same stream, it cannot be excluded that the dynamics of a variable at one point influence the dynamics of this variable at the other site, i.e., strictly speaking, the two locations are statistically not independent [26]. Alternatively, no control sites may be used, but the monitoring study may focus on the comparison of different sites with differing levels of EAC impact.

A methodological aspect critical for the success of EAC monitoring is exposure assessment. Usually, this is done by the analytical determination of preselected compounds. However, this approach may easily miss relevant chemicals, which makes the establishment of cause–effect relationships problematic, if not impossible. Integrated monitoring programs should take advantage of recently developed advanced chemical analytical as well as bioanalytical technologies, which can be valuable complements to the classical analytical approach. Mechanistic understanding of the modes of EAC action has allowed the construction of rapid, cost-effective bioassays, e.g., assays such as the yeast estrogen-receptor assay which detects substances acting through estrogen-receptor binding [44], or the enzyme-linked receptor assay [45]. These assays enable to screen large numbers of samples for endocrine potencies that are mediated through the estrogen receptor. The more laborious and costly chemical analysis can then be restricted to samples that were positively identified in the screening bioassay.

Bioanalytical methods can only indicate that an endocrine activity is present in a complex environmental sample or in a tissue extract, however, they cannot identify the individual compounds being responsible for the biological activity. This requires the combination of the bioanalytical method with chemical analytical techniques. The targeted search for EACs exerting a specific mode of action, for instance, binding to the estrogen-receptor, among the many chemicals present in an environmental sample is possible, for instance, by the bioresponse-linked instrumental analysis [45]. Biomolecular components—in this example, estrogen receptors—are used to selectively extract and subsequently analyze estrogen receptor-binding substances from a complex sample. A different approach is the bioassay-directed fractionation [43,46]. In this method, the environmental sample is subjected to chemical fractionation, for instance, according to lipophilicity, and the resulting fractions are assessed for endocrine

activity by means of a specific bioassay. Positive fractions are further separated until a fraction is left which contains one or only few substances that can then be determined by means of chemical analytics. An important part of bioassay-directed fractionation is to confirm that the identified substance, at the concentration present in the environmental sample, is able to induce the observed biological response at a magnitude comparable to that of the sample (confirmation step). An example of the application of bioassay-directed fractionation to identify EACs in environmental samples is the British study on estrogenic STP effluents. Caging experiments with trout had shown that a number of STP effluents were estrogen-active [5]. As causative agents, alkylphenol derivatives were suspected, however, since effluents contain highly complex mixtures of substances, it was difficult to prove this hypothesis. Desbrow et al. [47], therefore applied the bioassay-directed fractionation to search for the estrogenic compounds in the STP effluents. Despite the complexity of the composition of the effluents, only a small number of estrogenic compounds were identified using this approach; namely, the synthetic estrogen EE2, the natural estrogens 17β-estradiol and estrone, and alkylphenolic compounds.

When addressing EAC exposure in monitoring studies, to date the focus has been mainly on the assessment of external exposure, however, more attention should be given to internal exposure and organ dosimetry, i.e., accumulation and disposition of EACs in target tissues. With respect to oviparous vertebrates, xenobiotic residues in endocrine organs, gametes and early life stages may be particularly critical [48]. While there is a good database available concerning the bioaccumulation of highly lipophilic compounds such as PCBs in reproductive organs and gametes, much less is known about the accumulation of more polar EACs. A well-known fact is that maternal steroids and thyroids are deposited in the eggs of oviparous vertebrates, however, the possible additional deposition of hormonally active exogenous substances and the short- and long-term biological consequences of this additional burden have hardly been investigated [49].

In addition to improved exposure assessment, improved effects assessment is essential for future EAC monitoring. The selection of appropriate effect measures depends on the purpose of the monitoring study and can be decisive for its success. One of the more frequently used effect measures is VTG induction in male fish [5,50,51]. This parameter offers a number of advantages, as it is based on a known mechanism, specific for estrogens, sensitive, and it shows a concentration-dependent graded response. The relevance of vitellogenin induction with respect to adverse alterations at the organism or population level, however, remains questionable. What would be important to include, in addition to the molecular response parameter, vitellogenin, are other more individual- or population-related endpoints in order to provide evidence that induction of vitellogenin correlates to alterations at the individual or population level. The concomitant observation of, e.g., reproductive dysfunction and induction of vitellogenin would give a hint to a possible causative role of environmental estrogens in the observed reproductive disturbance.

In the face of multiple exposures, and in the face of multiple modes of endocrine disruption, effects monitoring has to go beyond the measurement of single parameters such as vitellogenin, but should apply multiple response measures. For this purpose, candidate measures can be selected according to their specificity, mechanistic basis, threshold values, effect sizes, and effect quantification. The advantage of using a broader set of mechanistically based effect measures is the capability to differentiate between the impact of EACs and other toxicants, since EACs are often found at complexly contaminated sites with many chemicals showing different modes of action being present [52,53]. Multiple response measurements may be facilitated by recently developed techniques such as gene arrays. Initial attempts to utilize such techniques for EAC assessment have been made in laboratory studies [54,55], however, their potential for assessing EAC impact in field studies still needs to be demonstrated.

Laboratory studies can be an essential component of monitoring programs. Through laboratory studies, it can be confirmed whether biological responses observed in the field are in fact caused by water-borne factors, i.e., when controlled laboratory exposure to environmental matrices is able to induce the biological response [39]. Laboratory studies provide plausibility on observed associations of

EAC exposure and effect in that they reveal underlying mechanisms of action. These experiments establish effect threshold values, and they determine the rate of increase of the response in relation to EAC concentration. Further, laboratory studies are important to establish basic biological traits and response patterns to EAC exposure of the monitored species, e.g., knowledge of developmental patterns of endocrine systems, or recognition of sensitive periods during development [12]. Finally, laboratory studies are essential in studying the mixture issue, i.e., the combined effect of various substances with identical or different modes of endocrine action. Since it will be not feasible to test all possible combinations of EACs as they may occur in the environment, the function of laboratory studies on EAC mixtures is to develop a rational basis to understand EAC interactions in environmental matrices [56].

Monitoring the association between EAC exposure and biological effects will always include some degree of reasonable speculation [62]. It is clear that there will be not the one and only monitoring strategy for all problems, but for each individual case or purpose, different pieces of the puzzle will have to be assembled and utilized to provide plausibility for an EAC-related etiology. Depending on the specific aims of the study, integrated monitoring will have to include detailed demographic information where adverse effects are occurring in wildlife individuals or populations, improved assessment of extent and pattern of EAC exposure and accumulation, consideration of epidemiological criteria, realistic replication of environmental exposure in the laboratory, knowledge upon mechanisms of action, or evaluation of the role of confounders. As such, integrated monitoring is not a new instrument, but the consequent and, importantly, the purpose-driven combination of existing and newly emerging techniques and concepts.

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