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Special Section

In Situ–Based Effects Measures: Considerations for Improving Methods and Approaches

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ABSTRACT

In situ-based effects measures have gained increased acceptance as a means to improve the link between cause and effect in aquatic ecotoxicological studies. These approaches have primarily been employed where more conventional laboratory tests with field collected samples and routine in-field community surveys have failed to provide reasonable answers with respect to causes of toxicity, primary routes of contaminant exposure, and what constitutes ecotoxicologically relevant contaminant levels, at least at a site-specific level. One of the main advantages provided by in situ tests compared to more conventional field-based monitoring approaches is that they provide better control over stressor exposure to a defined population of test animals under natural or near-natural field conditions. In situ techniques can also be used to avoid artifacts related to sampling, transport and storage of contaminated water and sediment intended for laboratory-based toxicity assessment. In short, they can reduce the need for laboratory to field extrapolation and, when conducted properly, in situ tests can provide improved diagnostic ability and high ecological relevance. This paper provides suggestions and considerations for designing in situ studies, choosing test species and test endpoints, avoiding or minimizing test artifacts, best addressing some of the limitations of in situ test techniques, and generally improving the overall quality of the in situ approach chosen.

Keywords: In situ toxicity testing Cause–effect relationships Test design considerations Technique-related artifacts

INTRODUCTION

Defining in situ–based effects measures

In situ–based effects measures, over the past decade or so, have received increased attention and acceptance as ways to improve the ability to link cause and effect in aquatic ecotoxicological field studies. These approaches primarily have been employed where more conventional laboratory tests with field-collected samples and routine in-field community surveys have failed to provide reasonable answers on causes of toxicity, primary routes of contaminant exposure, and what constitutes ecotoxicologically relevant contaminant levels, at least at a site-specific or condition-specific level. In essence, in situ–based approaches provide a valuable link between and complement to laboratory experiments and field surveys.

As implied by the words in situ, these are in place experiments conducted in the field. They do not include experiments conducted with field-collected samples brought back to the laboratory. Some degree of experimental manipulation also is implied, generally related to controlling or confining contaminant exposure to a defined population or community of organisms. Approaches include such methods as caged organisms (Chappie and Burton 2000; Burton et al. 2005), colonization substrates (Liber, Call, et al. 1996; Courtney and Clements 2002), in situ enclosures (Solomon et al. 1990; Liber 1994; Liber et al. 1998; Kline and Stekoll 2001; Trannum et al. 2004), shore-based microcosms or mesocosms (Culp et al. 2003; Pettigrove and Hoffmann 2005), and online automated biotests (Gerhardt 1999). Whole-lake contaminant dosing, such as that performed at the Experimental Lakes Area in northwestern Ontario, Canada (e.g., Palace et al. 2002), also can be considered an in situ testing approach, but because such studies generally have little or no experimental manipulation of the organisms that will be exposed to contaminant stress, they will not be discussed further here.

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Advantages of in situ experimental techniques

One of the main advantages provided by in situ tests compared to more conventional field-based monitoring approaches is that they provide better control over stressor exposure to a defined population of test animals under natural or near-natural field conditions. By partly controlling what environmental compartment(s) a known or standardized number of test animals is in contact with, the investigator can gain improved ability to describe and link cause and effect in the field. In situ techniques also can be used to avoid artifacts related to sampling, transport, and storage of contaminated water and sediment intended for laboratory-based toxicity assessment. In short, they can reduce the need for laboratory-to-field extrapolation and, when conducted properly, in situ tests can provide improved diagnostic ability and high ecological relevance.

In situ approaches allow for some level of control and replication, or at a minimum psuedoreplication, within natural systems. For example, spiked sediment–filled colonization trays have been used successfully to gain better insight on factors influencing metal bioavailability to benthic organisms (Liber, Call, et al. 1996; Trannum et al. 2004; Pettigrove and Hoffman 2005; Piola and Johnston 2006), and macroinvertebrate colonization baskets have aided in evaluating the relative influence of water and substrate quality on benthic macroinvertebrate communities in a metal-polluted mountain stream (Courtney and Clements 2002). Furthermore, in situ tests with caged organisms can serve an important function in the problem formulation phase of larger ecological risk assessments where there is a need to better identify which environmental variables or exposure pathways require further assessment or consideration.

In situ enclosures also have been used to help define environmentally relevant toxicity thresholds and population/community recovery times for pesticides (e.g., Liber, Schmude, et al. 1996), and shore-based stream mesocosms have been used effectively to evaluate confounding factors and estimate acceptable effluent discharge levels (e.g., Culp et al. 2000). However, due to the extensive, existing literature on mesocosms, including large in situ enclosures (e.g., Graney et al. 1994; Hill et al. 1994), these test systems are not discussed specifically in any detail here. Caged organisms, such as bivalves, have been used widely as biomonitors of highly bioaccumulative compounds (Salazar and Salazar 2005). Caged organisms also can be used to evaluate the influence of unpredictable storm run-off events that create pulse contaminant exposure durations of unknown length and severity (Schulz 2005). Because most of these in situ approaches have not been standardized formally (with the exception of a caged bivalve test [ASTM 2003a]), there is a need to identify and summarize various design and test considerations that can have profound influences on test outcomes. However, this lack of standardization does give investigators a certain degree of creative flexibility in designing test systems and approaches to address site-specific issues and potentially minimize the influence of test artifacts, uncertainties, and confounding factors. Nevertheless, eventual development of a generic protocol within which site-specific needs can be incorporated would be advantageous.

Limitations of in situ experimental techniques

Although in situ techniques offer numerous exciting opportunities, they do have limitations and weaknesses that should be recognized. Logistical issues related to acclimation, transport, and transplantation of test animals can be substantial. Furthermore, multiple field trips or extended stays at field locations are often necessary, possibly leading to greater costs and levels of effort than for more conventional field surveys and laboratory studies. Choosing an appropriate control or reference site also can be difficult, especially in urban, coastal, and estuarine environments (although not necessarily any more so than for conventional field surveys), and fully understanding the food and habitat requirements for native, caged species can be challenging. Unpredictable environmental events, such as droughts, floods, climatic extremes, and episodic changes in basic water quality conditions, such as dissolved oxygen concentration, also can impact negatively on the success of a study. Maybe most important, technique- and exposure-related artifacts (e.g., effects of cages on animal behavior and contaminant exposure) can invalidate test results if important methodological and biological aspects are not addressed properly and/or accounted for. Finally, most current in situ techniques are nonconventional in their design and use, and therefore have seen only limited use in regulatory and other decision-making processes. However, this is likely to change. Stream mesocosms, for example, now are considered an acceptable alternative assessment method within the Canadian Environmental Effects Monitoring program (e.g., Dubé et al. 2002) in situations where appropriate reference sites are not available.

The primary objectives of the remainder of this paper are to provide the reader with suggestions and considerations for how to avoid or at least minimize test artifacts, best address some of the limitations of in situ test techniques, and generally improve the overall quality of the experimental approach chosen and the utility of the results generated. The paper is part of a series of 5 papers that collectively aim to evaluate the use of in situ–based effects measures in aquatic ecosystems and offer suggestions and considerations for how to improve the accuracy and relevance of these approaches for research and environmental decision-making processes.

IMPORTANT ISSUES, CHALLENGES AND CONSIDERATIONS FOR CONDUCTING IN SITU STUDIES

General study design considerations

Most of the basic principles for designing a biological test also apply to in situ tests. For example, the study should be designed to evaluate cause-and-effect relationships and the test should be executed for a sufficient period of time to provide a meaningful exposure duration. Seasonal variability in both contaminant exposure and biological population parameters should be investigated, or at least considered, in the study design. For all test organisms, tolerance windows will apply with regard to water quality variables, such as pH, alkalinity, hardness, temperature, dissolved oxygen saturation, salinity, ammonia, etc. These variables may influence the physiological status of test animals and thus test endpoints and should be taken into account and monitored during a test whenever possible. The optimal population density of organisms during testing may vary greatly depending on the species used. Therefore, a density should be chosen that does not stress test animals or create unnatural competition for space and resources. Appropriate controls, replication (or at least pseudoreplication where true replication is not possible), and reference samples should be included in the test protocol.

Traditional biological/toxicological endpoints, including survival, individual and population growth, reproduction, maturation, colonization, contaminant tissue concentrations, and physiological and biochemical alterations also are applicable to in situ assessments. Though more difficult to use in regulatory decision making, the use of sublethal indicators of organism exposure or stress (e.g., subcellular biomarkers) may be used as tools to monitor organism condition (well being, stress) and inform data interpretation (Galloway and Handy 2003; Galloway et al. 2004).

Biological, ecological, and behavioral considerations

Choice of test species—The choice of test species is central to the success of in situ studies and there are both advantages and disadvantages of using either indigenous or surrogate test species, depending on the nature of the study site and the objectives of the investigation. It could be argued that, for most habitats and situations, the availability of a suitable surrogate species for which sufficient biological information is available outweighs the identification of an appropriate indigenous species for which less may be known of its resilience to handling, acceptance to caging, potential and speed of acclimation, ability to recover from stress, propensity to bioaccumulate contaminants, and range of measurable toxicological responses. It is possible to culture many standard test species including fishes, mollusks, chironomids, amphipods, oligochaetes, and daphnids in the laboratory to provide a constant supply of test organisms of defined age, size, and condition. However, long-term mass culturing may lead to reduced genetic variability and either increased or decreased sensitivity to natural environmental fluctuations, handling, and toxicant stress, possibly resulting in either an overestimation or underestimation of toxicity (Gerhardt et al. 2004). An additional problem is that some groups, such as meiobenthic invertebrates, are not easy to culture, handle, or identify. For meiobenthic organisms, a general lack of understanding of their biological and ecological requirements has limited their use in in situ experiments. Regardless, when surrogate species are used, effort should be made to ensure that they are representative of the system studied. Where native, field-collected organisms are to be used, enough should be known about the biology, physiology, behavior, and dietary requirements of the species to appropriately execute the test and interpret the results. The use of species with overlapping polyvoltine life cycles allows for availability of organisms of all size classes in different seasons.

Another consideration is that resident species may have pre-exposure histories (i.e., they are accustomed or acclimated to a certain level of background contamination from the study site) that could influence their response (i.e., lower toxicity) relative to naïve (e.g., true reference site or laboratory-cultured) organisms. This may or may not be a problem depending on the objectives of the study (e.g., such organisms would represent current, site-specific conditions). However, individuals of the same species from clean reference sites in the same catchment, or at least from the same type of ecosystem within the same ecoregion, generally are preferred because they should not show habituation and adaptation effects. In short, careful consideration should be given to both the choice and source of test organisms.

The choice of test species also will depend on the selection of test endpoints. For example, for many smaller species, survival and growth may be most appropriate, whereas for larger organisms that can be more amenable to handling and have longer generation times, a greater range of endpoints may be incorporated. Furthermore, some endpoints might be more difficult to assess in small species, such as bioaccumulation (i.e., getting sufficient tissue quantities for analysis) and reproduction (i.e., finding small offspring in an in situ chamber). The choice of test endpoints is discussed further in the section *Choice of test endpoints and test duration*.

Handling and density of test organisms—General handling, transfer, and transport of test organisms and the number of test organisms per cage or experimental unit (density) are important considerations (Chappie and Burton 1997, 2000). Clearly, the density of test organisms within a chamber should not be so great that it affects the endpoints measured (Ringwood and Keppler 2002; den Besten et al. 2003; Bervoets et al. 2004). Food and habitat (space) limitation, as well as intraspecies predation (cannibalism), can create further problems. Intuitively, organisms should be handled as little as possible, and care should be taken during introduction to test chambers (Sibley et al. 1999; Bervoets et al. 2004). For example, a common method for introducing amphipods and chironomids to test containers is via careful syringe injection though container ports (Kater et al. 2001; Castro et al. 2003). Acclimation to key site-specific water quality conditions (e.g., pH, hardness) also should be considered. Food quantity and quality further may affect test endpoints, especially when food needs to be added to exposure chambers (Chappie and Burton 1997; Pereira et al. 2000; Ringwood and Keppler 2002; Castro et al. 2003; Robertson and Liber, in press). Predation and/or competition from indigenous species are other important considerations (Sibley et al. 1999; Crane et al. 2000; Castro et al. 2003; Bervoets et al. 2004). Multispecies exposures may be useful for assessing indirect effects of contaminants that could occur due to the presence of other organisms (Ciarelli et al. 1999; Greenberg et al. 2002; Ciutat and Boudou 2003; Goodfellow 2005).

In bioassays using benthic organisms such as chironomids, sediments may have to be sieved to remove indigenous species, however, this also likely will alter contaminant concentrations and bioavailability (Crane et al. 2000; Simpson and Batley 2003; Bervoets et al. 2004). Freezing has been used successfully as an alternative means to remove (kill) indigenous macroinvertebrates in sediment prior to use of that sediment in colonization trays (Kline and Stekoll 2001; Trannum et al. 2004; Liber et al. 2005; Pettigrove and Hoffman 2005). It should be recognized, however, that freezing likely will change the physical structure of sediment and thus possibly influence the response of subsequently introduced test organisms if the sediment is to be used in sediment toxicity studies. In such situations, it would be advisable to investigate how changes in sediment chemistry might affect test outcomes (Beiras et al. 1998; Geffard et al. 2004; Jung and Batley 2004).

Choice of test endpoints and test duration—The choice of test endpoints and test duration to some extent will depend on the choice (including size and longevity) of test species (see discussion above). However, the choice of endpoints largely should be determined by the nature and scope of the study, and the length of time planned for deployment (i.e., the test duration should be adequate to address the study objectives and for expression of the endpoints measured). Rapid cellular changes, inflammatory responses, induction of stress proteins, alterations in enzyme activities, and changes in behavior all can occur quickly, and offer early indications of exposure or

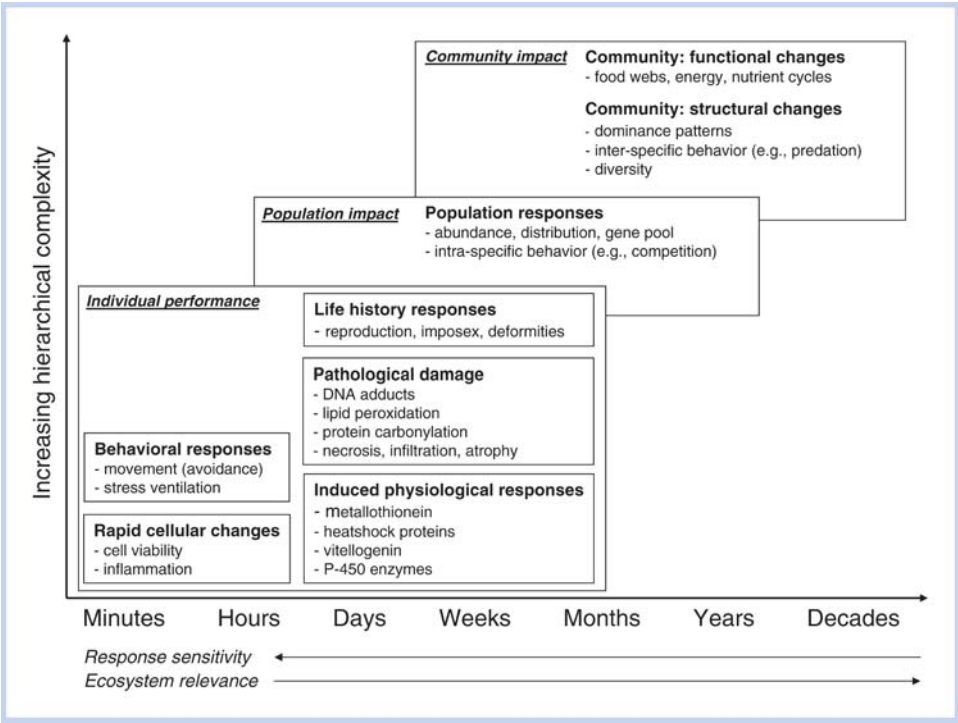

Figure 1. Relationship between level of biological organization (hierarchical complexity) and response time for selected assessment endpoints (biochemical, cellular, whole-organism, population, and community responses) to contaminant stress.

sublethal effects, but they may not necessarily lead to pathological change, reduced fitness, or population-level effects (Galloway and Depledge 2001). Therefore, these endpoints often are less useful in regulatory situations. Most of these endpoints are also more difficult to use with small organisms (e.g., many freshwater invertebrates). Further complicating the issue is that many stress responses will decline with time (i.e., adaptation), even when the level of stress remains constant (Wu et al. 2005). Cellular and/or histological changes (e.g., protein carbonyl adducts, lipid peroxication, DNA adducts, infiltration, atrophy, necrosis) typically take longer to develop, but provide a definitive indication of pathology and organ or tissue damage (Handy et al. 2003).

The time of year during which a study is conducted also could influence test results through differences in site conditions and contaminant exposure, and seasonal differences in physiological status of test organisms. For example, tests conducted at colder temperatures often require a longer test duration. Furthermore, some physiological responses (e.g., changes in respiration and heart rate) have been shown to relate to artifactual changes associated with handling, test chamber deployment, and general stress on the test animals. Silting of cages may reduce oxygen availability and hence cause a compensatory tachycardia in caged bivalves.

Behavioral endpoints recently have received increased attention due to their rapid response times, which are optimal for diagnostic site screening; their apparent sensitivity to many types of pollutants, i.e., ideal early warning parameters for pollution pulse studies and in situ on-line biomonitoring systems; and their nondestructive nature, which allows for repeated measures, longer-term toxicity testing, and on-line biomonitoring. The choice of behavioral endpoints depends on the purpose and design of the study and the choice of test species, and includes avoidance for single-species tests (recorded as increased locomotion in cage experiments or as downstream drift) and inter- or intraspecific interactions such as predation, competition, and cannibalism for multispecies tests (Gerhardt et al. 1994; Dell'omo 2002; Sprugeon et al. 2005).

Overall, a more complete understanding of results from in situ studies where organisms may be exposed to varying levels of pollution, either continuously or periodically, can, where feasible, best be achieved by taking an integrated approach that considers molecular, cellular, physiological, behavioral, histological, whole organism (e.g., survival, growth, reproduction), and population endpoints, and the timeframe over which they occur (Galloway et al. 2002, 2004; Figure 1). That said, further research is required to determine whether the cause–effect linkages commonly observed in laboratory studies also are observed in in situ studies, which sublethal and subcellular responses are best evaluated using in situ methods, and whether relationships can be established between biochemical endpoints (biomarker responses) and population-level effects using in situ techniques.

Habitat considerations for tests with benthic species—Most benthic species require the presence of both water and sediment habitats, either simultaneously or subsequently, for normal behavior during different phases of their life cycle. Therefore, testing ideally should offer a design with both compartments present (Chappie and Burton 2000; Gerhardt and Schmidt 2002; Robertson and Liber, in press). Reasons for the importance of sediment for benthic species in both lentic and lotic habitats include provision of refuge and shelter against flow, predators, etc.; suitable habitat for burrowing, tube building, and other essential behavior, and for early life stage development; a feeding environment and source of food; and a location for egg deposition. In addition, contaminant exposure is more realistic if both sediment and water is present, because bioavailability of contaminants and water

Table 1. Potential technique-related artifacts that can be associated with different in situ test systems and thus require careful consideration during the design and application of experiments. NS = not significant; X = minor concern; XX = moderate concern; XXX = major concern

Artifact[a]	Caged organisms: water column	Caged organisms: sediment	Land-based systems[b]	Open water enclosures	Colonization substrates
Reduced water flow and exchange	XX	XXX	X	XX	X
Fouling	XX	XXX	XX	XX	X
Contaminant adsorption	X	XX	XX	XX	X
Accumulation of sediment and waste materials	XX	XX	XX	X	X
Emigration and immigration	X	XX	XX	X	XX
Altered light regime	XX	XX	XX	X	NS
Increased temperature	X	X	XX[c]	XX	NS
Reduced dissolved oxygen	XX	XXX	X	X	X
Species interactions	X	XX	XX	X	XX
Altered behavior	XX	X	XX	X	X
Food quality and quantity	XX	X	XX	X	NS

[a] Artifact and severity can be due to improper test system or experimental design. The severity of most artifacts can be reduced with careful design and deployment of test units to suit the specific ecosystem, compartment, contaminant(s), or species investigated.
[b] For example, shore-based artificial stream mesocosms.
[c] Including greater temperature fluctuation.

flow are influenced by sediment (Burton 1991; Greenberg et al. 2002; Luoma and Rainbow 2005).

Disturbance to sediments (e.g., sieving) prior to transfer into test containers may significantly alter the concentrations (e.g., for volatile organics) and speciation (e.g., for metals) of contaminants (Pereira et al. 2000; Anderson et al. 2001; Bull and Williams 2002; Simpson and Batley 2003). If significantly disturbed, adequate time should be allowed for re-equilibration of the sediment (i.e., to at least partly restore chemical conditions, although the physical structure remains altered) before test organisms are added to the test containers (Kater et al. 2001; Lee et al. 2004; Simpson et al. 2004; Burton et al. 2006). Alternatively, the use of intact sediment cores within test chambers may be a useful approach for minimizing disturbance to the sediment profile and physicochemical conditions, thus maintaining more realistic sediment contaminant exposure conditions (Jung et al. 2003; Robertson and Liber, in press). Regardless of the approach chosen, it is important to ensure compatability between sediment particle size and the chosen test species (some species have specific requirements), to create an environment within the test chamber that adequately resembles the habitat conditions of the original sediment, and to appreciate the pros and cons of each sediment sampling approach, especially with respect to competition/predation by native species and alteration in contaminant exposure.

Design considerations for optimizing in situ exposure systems

One of the primary justifications for conducting in situ–based experiments is to improve realism of contaminant exposure within the test system, while maintaining control over exposure duration and the number and type of organisms exposed under natural environmental/climatic conditions. Thus, in situ test systems should be designed to ensure that organisms experience environmentally realistic conditions and contaminant exposure, and to minimize technique-related artifacts that can occur when confining organisms to chambers, cages, or shore-based mesocosms. General considerations include the importance of incorporating all appropriate environmental compartments (e.g., sediment and overlying water for benthic organisms) and conducting experiments in microhabitats where the organisms naturally would occur. A critical aspect in the design of in situ tests therefore is to identify those variables that are most likely to influence the outcome of a test, either by altering contaminant exposure or by modifying organism responses and to consider these influences in the test design and data interpretation. The significance of these artifacts will vary among test systems, test duration, and test species, but are generally a more serious issue in experiments involving caged organisms or communities (Table 1). The following section (*Technique-related artifacts*) focuses largely on design considerations for aqueous exposure chambers. Additional design considerations for sediment exposure chambers and colonization studies are presented in the sections *Design considerations for sediment exposure chambers* and *Design considerations for sediment colonization experiments*, respectively.

Technique-related artifacts

Scale—The limited spatial and temporal scales of many in situ experiments are considered among the most serious limitations of this approach. Although the question of

appropriate scale is central to the debate over the usefulness of in situ techniques (because of the uncertainty associated with extrapolation across spatial and temporal scales), surprisingly few studies have tested the hypothesis that experiments conducted at 1 scale can be used to predict responses at a different (usually larger) scale. The small spatial scale of most in situ chambers greatly restricts the numbers and types of organisms that can be included. If larger or longer-lived organisms are an essential component of the natural system (e.g., in systems regulated by top down predators), or have a disproportionate influence on its structure (e.g., a keystone species), results of in situ experiments may not be fully relevant to the natural world. However, because top predators or keystone species control relatively few natural, aquatic communities, our inability to include large, highly mobile species in in situ test systems may rarely be a critical issue. The possible importance of seasonality on the expression of in situ toxicity also should be considered in the study design, especially when using short-term tests for assessing long-term/chronic field contamination.

Water exchange—Probably the most serious artifact associated with in situ experiments involving caged organisms and some land-based microcosms and mesocosms usually is reduced water flow and associated limited water exchange with the natural environment (Chappie and Burton 2000). In lotic ecosystems, the delivery of food, nutrients, dissolved oxygen, and other materials is essential for organism growth and survival. Flowing water also removes and prevents the accumulation of waste products such as ammonia. The mesh surrounding organisms in cages can significantly reduce water flow and dissolved oxygen renewal rates, and therefore indirectly cause stress (Chappie and Burton 2000). Depending on the specific container design, reduced flow and water exchange also may be an issue during substrate colonization studies (i.e., reduced colonization through drift). Although water movement may be less critical in colonization studies in lentic and many marine ecosystems, adequate exchange between organisms and the natural environment may be impeded when organisms are placed in cages or chambers. Consequently, effort should be made to use the maximum screen size that will safely confine the test organisms to the test vessel, and maximize the vessel surface area covered by screens.

Fouling—Fouling of test chambers by sediment, biofilm, or other organisms can be a significant artifact for many in situ experiments, particularly those involving caged organisms. Fouling of the fine mesh used in many in situ experiments with small invertebrates can significantly decrease water flow and exchange, contributing to accumulation of waste materials and potentially to anoxic conditions. Occasional cleaning of the outside surface of the mesh, if possible, therefore may be necessary to maintain good water exchange, light transparency, and flux of solutes and fine particles (Pereira et al. 2000). Accumulation of fine particles that are deposited in test chambers also may smother organisms or alter behavior, contaminant bioavailability, and contaminant exposure routes. In other test systems, such as shore-based mesocosm systems, the accumulation of biotic and abiotic materials on container walls may greatly complicate assessments of exposure, especially if contaminants are removed either by bioaccumulation or adsorption in relatively static water column exposures. Regardless of the test vessel design used, water quality variables such as pH, temperature, dissolved oxygen, salinity, turbidity, and light transmission should be monitored regularly, both inside and outside of test vessels, if at all possible (Pereira et al. 2000; Ringwood and Keppler 2002).

Immigration, emigration, and predation—The degree of immigration of exotic organisms into test chambers, or emigration of test organisms out of test chambers, is a concern in cage, recolonization, and many land-based systems, and is dictated by organism size relative to mesh size (Pettigrove and Hoffmann 2005). The loss of organisms by emigration and predation, which are often difficult if not impossible to quantify, complicates the ability to correctly estimate mortality. The addition of organisms by immigration may confound mortality estimates, especially for in situ experiments using indigenous species (e.g., recent immigration by individuals of the same species would off-set estimates of mortality). In an effort to distinguish test animals from indigenous organisms of similar species, Crane et al. (2000) successfully marked *Chironomus riparius* with small paint spots before their use in an in situ experiment. Documentation of the number and type of nontest species removed from test chambers at test termination always should be made.

Light and temperature—Reduced light can be a significant artifact in many in situ chamber studies and some land-based mesocosm experiments, especially if chambers do not allow adequate light penetration. Changes in light regime will affect primary producers (phytoplankton, periphyton) and may alter behavior of light sensitive and phototrophic organisms. Because the toxicity of photoreactive contaminants, such as some polycyclic aromatic hydrocarbons (PAHs), increases under ultraviolet light exposure, reduced light levels may also significantly affect test results (Ireland et al. 1996; Boese et al. 2000). Photoinduced toxicity of PAHs has been observed during in situ studies and confirmed in a parallel laboratory study (Ireland et al. 1996). Thus, in some situations, in situ assessments should consider whether shaded versus unshaded treatments might be needed to fully characterize in situ exposure and effects. Because light influences primary producers, animal behavior, and toxicity of some chemicals, in situ chambers should, as much as possible, be constructed of materials that allow penetration of natural wavelengths of light. The choice of construction materials (e.g., plastic or glass) also should be influenced by the nature of the primary contaminants of concern at the test sites. Depending on the amount of water exchange with the natural system, temperature in land-based test systems (e.g., stream mesocosms built on shore next to a river) may increase relative to the natural habitat, or display greater diurnal fluctuations. This can be a significant artifact for these systems because temperature can influence both persistence and bioavailability of toxicants, and behavior and susceptibility of organisms.

Confinement—Restricting organisms to small in situ chambers both may alter their behavior and affect contaminant exposure. For example, confining organisms to sediment or the near-sediment habitat when they normally make vertical migrations into the water column can artificially alter exposure to contaminated sediments. Alterations in food availability and feeding behavior resulting from artificial conditions in test chambers also may modify dietary exposure to contaminants. Because of organism confinement, species interactions such as competition and predation can be important issues in enclosures or other systems that contain natural assemblages of organisms. Thus, the choice of in situ test vessel design

should be strongly influenced by the choice of test species and the contaminant exposure routes being evaluated.

Chamber size—Few studies have examined the relationship between chamber size and shape and the responses of organisms to contaminants. Because of surface area–to–volume relationships, some container artifacts are diminished with increased size of the in situ chamber. Perez et al. (1991) reported that fate and effects of kepone on phytoplankton communities varied with chamber size and that results of experiments conducted in small microcosms would underestimate effects. Solomon et al. (1989) showed that the aqueous dissipation of methoxychlor was faster in small (20 m^3) enclosures than in large (1,000 m^3) enclosures and suggested that the higher dissipation rate may have been related to processes dependent on the periphyton community associated with the enclosure walls. The influence of chamber size on the toxicological response of test organisms when exposure is unaltered does not appear to have been investigated. Additional experiments are necessary to document the relationship between chamber size, organism response, and the significance of artifacts, especially for caged organisms.

Physicochemical conditions—When conducting in situ experiments, it often is assumed, and always desired, that the physicochemical conditions within the chambers are similar to those of the surrounding environment. Because such conditions can greatly influence contaminant bioavailability, organism behavior, and organism performance, and because they are likely to change at least somewhat during in situ experiments, it is essential that physicochemical conditions be measured throughout an experiment, both inside and outside of test vessels. Chambers therefore should be designed and deployed in such a way as to allow for periodic recording of physicochemical conditions and measurement of contaminant concentrations inside the test vessel. Alternatively, additional chemistry chambers (also containing test organisms) could be installed in parallel with the primary test chambers. These chambers could be used to record variables such as dissolved oxygen, temperature, ammonia, salinity, and contaminant concentrations without worrying about the influence of inserting probes into or collecting samples from chambers on the health and confinement of test organisms. Measurement and reporting of important water quality variables such as temperature, salinity, pH, dissolved oxygen, and ammonia, are needed for a complete understanding of cause-and-effect relationships. Decisions concerning how much variation from natural external conditions can be tolerated should be made a priori, if possible. Such decisions will require a good understanding of the life history characteristics and the tolerance test species have for container-related artifacts, information that may or may not be readily available.

Design considerations for sediment exposure chambers

Design considerations for in situ sediment exposure chambers need to consider the dynamics of the environment at the test site, the natural behavior of the test organisms, and the sensitivity of test species to naturally occurring stressors (Chappie and Burton 1997; Baudo et al. 1999; Ciarelli et al. 1999; Sibley et al. 1999; Chappie and Burton 2000; Kater et al. 2001; Greenberg et al. 2002; Castro et al. 2003; Ciutat and Boudou 2003). Exposure chamber design generally will be specific for the chosen test and site conditions (e.g., flow, suspended solids, sedimentation, food availability, predation; Pereira et al. 1999; Chappie and Burton 2000; Crane et al. 2000; Meregalli et al. 2000; dos Santos et al. 2002). Typically, exposure chambers should provide the organisms with access to the sediment and allow the overlying water conditions and sediment fluxes to be controlled by the site conditions. A common design involves the insertion into the sediment of a tube that is transparent to light and has mesh walls above the sediment surface, to maintain bidirectional flux of solutes and fine particles while preventing organism escape (Chappie and Burton 1997; Baudo et al. 1999; Kater et al. 2001; Greenberg et al. 2002; Castro et al. 2003; Moreira et al. 2006). Below the sediment surface, the tube should extend to a sufficient depth to allow organisms to burrow and ideally contain mesh-covered walls or openings to allow exchange of interstitial waters. The tube may or may not be closed at the bottom to prevent organism escape or immigration of predatory organisms. Advective water flows (e.g., currents, groundwater upwelling) inside the test chamber, when possible, should be similar to flows in the surrounding environment because these flows greatly will affect dissolved oxygen, planktonic food, suspended solid concentrations, and the flux of contaminants from the sediment (Ziebis et al. 1996; Ciarelli et al. 1999; Greenberg et al. 2002; Ciutut and Boudou 2003). Transparent plastics (e.g., polycarbonate) and glass are considered suitable for in situ chamber construction, but the choice of materials should consider the type of site-specific contamination (e.g., organic contaminants adsorb more strongly to plastic and metals more readily to glass; Ireland et al. 1996; Sibley et al. 1999; Chappie and Burton 2000; Greenberg et al. 2002).

Design considerations for sediment colonization experiments

Design considerations for conducting recolonization (sediment transplant) experiments should carefully consider the system being monitored (e.g., type, source, concentrations and dispersal of contaminants, site hydrodynamics, geomorphology) and include a basic understanding of the ecology of the system investigated. Recolonization of sediment by benthic organisms may occur through settling of planktonic organisms from the water column, or through migration of species above or below the sediment–water interface (Lange-zaal et al. 2003; Lu and Wu 2003; Schratzberger et al. 2003; Trannum et al. 2004; Pettigrove and Hoffman 2005). The design and position of test containers (above, at, or below the sediment–water interface) will greatly influence which species can migrate most easily into test containers. Many benthic organisms spawn and exist as plankton during early life stages and will colonize sediments directly from the water column. Recolonization experiments may target specifically these species by placing containers above the sediment surface. Container walls in or above the sediment will inhibit the migration of many benthic and epi-benthic life stages (Mistri et al. 2001). Containers with mesh-covered openings below the sediment surface will allow for the colonization of some benthic species, however, the mesh size chosen will determine which species or life stages can enter (Liber, Call, et al. 1996). Container size and surface area also can influence which species recolonize the sediments.

Another consideration is that test containers can become buried, or adjacent sediment eroded, by a variety of hydrodynamic forces within the water body. In addition, in marine systems in particular, protection of containers from predation by nontarget organisms (e.g., fish, crabs, octopus) may be necessary and can be achieved using strong, large-mesh cages

surrounding the colonization containers (Schratzberger et al. 2003; Pettigrove and Hoffmann 2005). Temporal and spatial variation in water column conditions (e.g., dissolved oxygen, high turbidity, strong currents) will favor different species and may result in mass migration from containers, or potential mortality. Seasonal variability in reproduction will influence population abundance (Morrisey et al. 2003; Wu et al. 2005). Because organisms readily can migrate out of or into colonization substrates, relatively sedentary species generally make for better assessment organisms (i.e., care should be taken when interpreting the presence/absence of highly mobile species from test substrates). The length of time that colonization substrates are placed in situ also will influence the abundance and diversity of species colonizing those substrates. Timing and method of substrate retrieval are also important considerations.

Collection of resident test organisms for in situ experiments

Both active and passive approaches have been employed to collect benthic organisms for in situ tests. Active methods involve the use of quantitative sampling devices such as Surber samplers or Hess samplers. Passive methods generally involve colonization of substrates (e.g., trays or baskets containing stones or gravel) placed for a predetermined length of time at a field reference site prior to transfer to a test site (Clements 2004). Although there is generally less handling stress associated with passive methods, this approach offers different challenges. Passive methods require separate trips to the field for deployment and subsequent collection. In addition, loss of colonization substrates from vandalism, spate, or other events during colonization can be a concern. A key limitation of both passive and active approaches for establishing in situ–colonized test communities is that densities at the start of the experiment are unknown and only can be estimated. Consequently, variability among replicates at the start of the experiment may complicate the ability to accurately measure subtle responses to contaminants. Initial community composition can be compared to controls at the end of the experiment, but data cannot be expressed using conventional toxicological endpoints (e.g., percent mortality).

Logistical considerations and test site understanding

Before initiating an in situ test, consideration must be given as to whether or not the physicochemical conditions (e.g., salinity, pH, hardness, alkalinity, temperature, oxygen saturation, conductivity, ammonia) and physical factors (e.g., suspended solids load, current velocity) are suitable for the test organisms utilized. However, field sites to a large extent are chosen for reasons other than the applicability of the test. Therefore, it is crucial to understand the dynamics of the test sites and design the experimental approach accordingly. Examples of site-specific conditions that may occur (and change over time) include

- Unexpected changes in hydrodynamics (e.g., currents just above the sediment);
- Tidal flows;
- Groundwater/surface water interactions (e.g., upwelling, downwelling);
- Shifting sediment conditions;
- Heterogeneity of the sediment, substrate, or water composition;
- Diural variation in dissolved oxygen saturation, temperature, water level, pH, etc.;
- Temporal changes in light conditions; and
- Temporal changes in effluent release patterns and volumes.

These factors can influence test results and lead to artifacts if not accounted for and/or interpreted correctly. Most of these factors can be eliminated, or their influence reduced, from the effect measurement by carefully choosing the exact location of test sites and by choosing reference sites that resemble test sites as much as possible. In addition, the location of test chambers relative to effluent plumes and mixing zones in rivers and estuaries, and the depth of test chamber location in lakes in relation to limnological characteristics such as currents and thermoclines, also should be given careful consideration.

With regard to logistics, a critical element is the handling of test organisms. When test organisms need to be transported to a test site, acclimation and suitable transportation can be crucial. It is strongly encouraged to demonstrate that these steps have no influence on the endpoints measured (e.g., through the use of transportation controls for evaluation of biomarker responses). For example, the more the endpoint is related to sublethal effects, the more critical handling of the test organisms will be to success of the test. Factors that need to be considered are

- Transport of aquatic organisms should not lead to excessive stress and physical damage;
- Water temperature and basic water quality should be maintained at the acclimation or cultured level (and similar to the field site);
- Food may be required during long transport periods, but many fish as well as other species do not travel well when fed (Bardach et al. 1972);
- Some organisms (e.g., bivalves) may be transported out of water, but this may make them highly vulnerable to temperature changes, etc.;
- Handling of organisms for reference sites should be identical to that for test sites in all respects including transport time, temperature changes, etc.; and
- Methods used for deployment should result in minimal stress to test organisms.

Statistical considerations

Considerable discussion has occurred in the literature concerning the relative merits of various statistical approaches for analyzing results of microcosm and mesocosm experiments (e.g., Liber et al. 1992). Many of these same arguments apply to the design of in situ experiments. Most studies employing in situ approaches simply compare responses at multiple reference and contaminated sites and, in these cases, analysis of variance designs are appropriate. Assemblage-level experiments can be analyzed using a variety of multivariate approaches (e.g., multivariate analysis of variance) that characterize differences among locations based on the presence and abundance of numerous species. In some situations, in situ chambers can be deployed along a gradient of expected contamination. In these instances, it may be appropriate to use regression analyses to establish concentration–response relationships. Regardless of the statistical design, estimates of sampling variability ideally should be obtained before in situ chambers are deployed in the field,

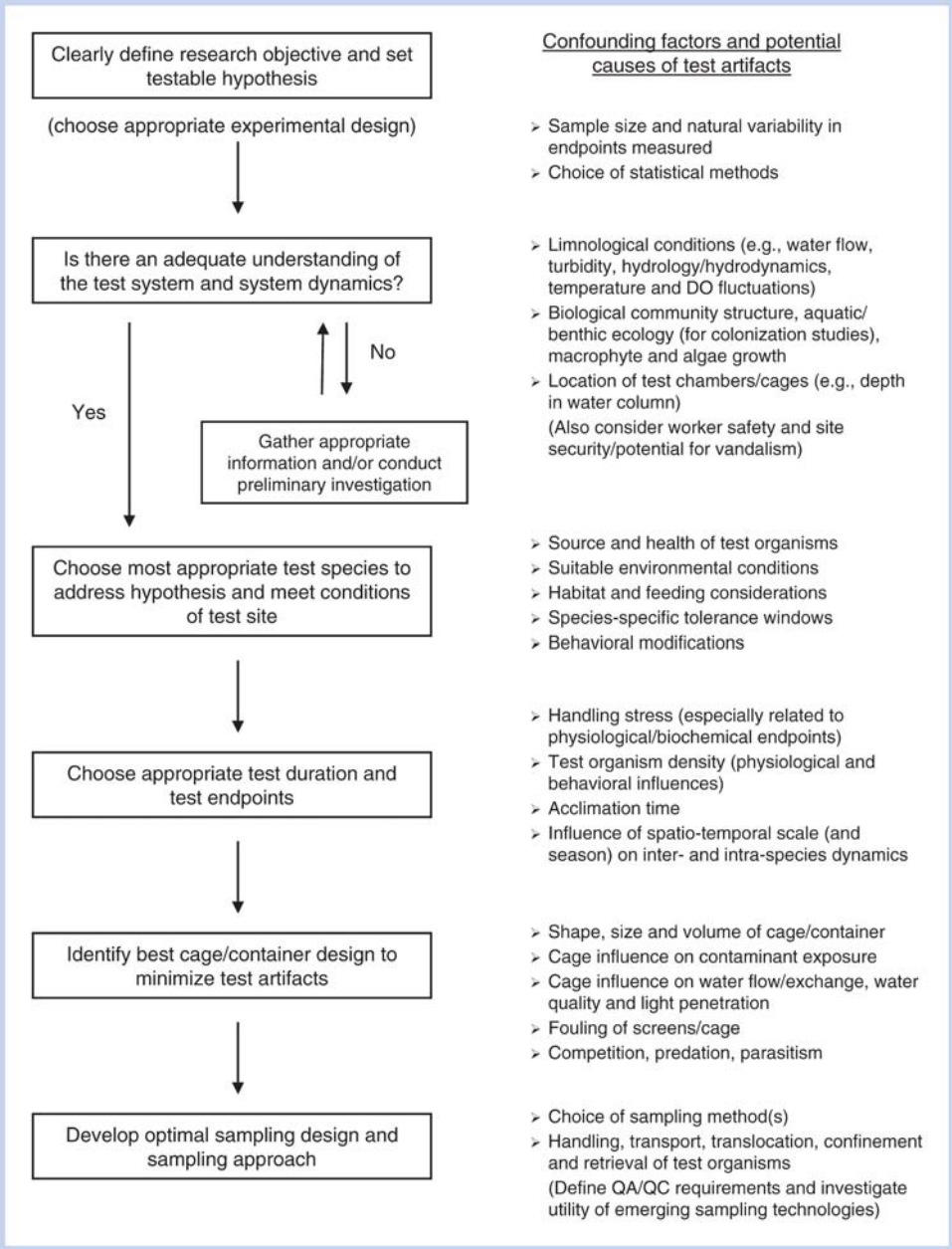

Figure 2. Considerations for the design and execution of an aquatic in situ effects study using caged organisms.

or at least based on existing data from similar experiments. Standard power analyses are recommended to determine the number of replicates necessary to detect specified differences among treatments. At a minimum, the statistical power of the performed test should be reported.

Another statistical consideration in the design of in situ studies relates to the appropriate terminology used to define experimental units. Although all in situ studies should deploy multiple test chambers at reference and contaminated field sites, it is important to recognize that these chambers generally do not represent true replicates. Because treatments (e.g., contaminated sites or reference sites) generally are not replicated, multiple chambers within a single location represent subsamples or pseudoreplicates (Hurlbert 1984). The major limitation of such pseudoreplicated designs is the inability to extrapolate findings to other systems. However, because most in situ studies are designed to assess conditions at specific sites, this may not be a serious concern (Stewart-Oaten et al. 1986).

OTHER CONSIDERATIONS

Emerging technologies

Many emerging scientific technologies are becoming used in in situ testing, but extensive coverage of such technologies was not the objective of this review. Thus, only a brief discussion of selected technologies is provided.

On-line biosensors, bioprobes, and biomonitors can be operated on a real-time basis either directly in the ecosystem, or in a bypass-system where water is pumped through a biomonitor placed streamside. On-line biomonitors, also called continuous (dynamic) biotests, biotest automates, alert systems, or biological early warning systems (BEWS) are composed of a test organism placed in a cage or flow-through

test chamber; an automated detection system recording a sensitive response (e.g., behavioral, physiological) of the test organism; and an alarm system consisting of software for data evaluation, decision making, and data transfer (Gerhardt 1999). Biomonitor systems have been developed for bacteria, algae, mussels, fish, cladocerans, and other species, including the multispecies freshwater biomonitor (a detailed review is presented by Gerhardt 1999). The mosselmonitor and the multispecies freshwater biomonitor have been applied directly in aquatic ecosystems (Gerhardt et al. 2004), whereas most other instruments still have to be used in a streamside housing with electricity supply. Further development is needed towards simplification and hardiness of the complex instruments (e.g., waterproof, robust, easy to use) and provision of self-supporting power supply systems (e.g., solar or wind energy based) before long-term on-line biomonitoring at remote field sites can be accomplished. Chemical sensors (e.g., gel probes, voltametric electrodes) recently have been developed and allow for improved understanding of the biogeochemistry of a site (Viollier et al. 2003; van Leeuwen et al. 2005). These tools can be integrated with in situ toxicity tests, thus providing a weight-of-evidence approach for use in decision-making frameworks (Gerhardt et al. 2004).

Vandalism and safety

When conducting in situ tests, one needs to consider and plan for potential vandalism of the test site and/or apparatus. The possibility of this occurring generally can be estimated based on the location of a site and the frequency of people being in the area. Typical strategies to help address this issue include camouflage to prevent the discovery of the experiments, fencing off the test area, placing notification/warning signs to keep people away, and site monitoring (e.g., electronic or human).

One also needs to consider safety issues when conducting in situ tests. Potential issues to consider and plan for include physical hazards associated with the site (e.g., high or fast-flowing water, difficult access), inclement weather, animals (e.g., mammals, reptiles, parasites, microbes), hunting, and site access issues such as private property, urban areas associated with high crime levels, and access to emergency medical care in remote areas. Careful consideration of possible site-specific hazards should be taken into account and preventive measures implemented along with having appropriate safety equipment (e.g., first-aid kit) and communication devices on hand.

QUALITY ASSURANCE/QUALITY CONTROL CONSIDERATIONS

Quality assurance/quality control (QA/QC) is an important aspect of a successful in situ study. In this brief discussion, the focus is on QA/QC issues that are unique or particularly important to in situ experiments. The researcher also should consult QA/QC recommendations for conventional aquatic toxicity testing and other biological testing programs, including the importance of adequate replication and the use of duplicate samples (e.g., OECD 1993; APHA, AWWA, WEF 1998; USEPA 2002; ASTM 2003a, 2003b, 2003c). In addition, it is recommend that a study plan be developed that defines the study objectives, experimental approach, assessment and measurement endpoints, and test methods to be used, and establishes performance criteria goals for the study prior to the initiation of the in situ study.

Organism-specific considerations are among the most important aspects dictating the success of in situ tests. If cultured organisms are selected, organism health should be validated by the use of reference toxicant testing. Additionally, the organisms should be from known lots of similar age and size to facilitate interpretation of test results. If field-collected organisms are used, investigators should pay attention to the selection of collection gear to ensure that only healthy and uninjured organisms are used. It is also recommended that a voucher collection be maintained to document the species and/or community of organisms used as part of the in situ study. Regardless of the source of test organisms, it is important that animals are acclimated to the test conditions and test chambers, when appropriate.

Because in situ testing typically is nonstandardized, it may be of assistance to use standardized procedures for other aspects of the in situ experiment when possible. Furthermore, in the absence of standardized protocols, it is important to use broadly accepted procedures whenever possible to gain greater approval of the procedures used. For example, water quality monitoring could follow standardized protocols (i.e., APHA, AWWA, WEF 1998). This gives the evaluator of the program the sense that care and attention to overall QA/QC was performed when specific guidelines were available. It is also important that the investigator understands the variables that the study is attempting to control and how the results are going to be analyzed (e.g., statistical analysis). Observation and maintenance of test chambers and equipment, cleaning of screens, and removal of excess food and dead organism (if possible) are also important aspects of successful in situ tests that are not always considered during the planning phase of a program, but when not performed can result in an unsuccessful test.

CONCLUSION

Recommendations

In situ–based experiments for effects assessment can take many forms, but the overriding reason for conducting most in situ tests is to improve the connection between stressor exposure and biological effects under actual field conditions. Most in situ approaches are not standardized and therefore offer a substantial degree of flexibility for the investigator to custom-tailor the test design to site-specific or objective-specific needs. In situ tests do have potential problems and are susceptible to experimental artifacts, so care must be taken to address or at least recognize as many of these possible issues in the general test design and the physical design of the experimental units (Figure 2). Because all possible artifacts and confounding effects likely can not be eliminated, emphasis must be directed towards those that could have the greatest negative impact on the overall test results. To aid in that decision-making process, and to help ensure that the study design is optimized and that the in situ test is a success, the following general recommendations are made:

- Develop a solid a priori understanding of each test site and the limnological conditions at those sites (e.g., flow dynamics and hydrological parameters, temporal and seasonal variations in general water quality variables).
- Be aware of possible test artifacts related to choice of technique and test system design, and design the experimental test units (e.g., cages, containers) and the

test/deployment protocol to minimize the influence of such artifacts.
- As much as possible, ensure that the conditions inside the test units are similar to those outside the test units that the study is designed to address (i.e., ensure that contaminant/stressor exposure and habitat conditions inside and outside of the test vessels are similar). Measure exposure conditions in all relevant matrices both inside and outside of test units when feasible.
- Ensure that test organisms are healthy and that their potential test response is due to the stressors being investigated and not due to confounding effects (e.g., handling, transportation, transplantation, confinement, modified habitat, suboptimal environmental conditions).
- Design and implement a proper QA/QC protocol to address, at a minimum, general water and habitat quality, contaminant/stressor exposure, and animal health and response.

Research needs

Most types of in situ test systems still are used only by a relatively small number of researchers and their design and use are not standardized. As a result, specific research needs into in situ chamber designs and optimization of experimental techniques are great and largely beyond the scope of this paper. However, the following general areas and issues do require further research before in situ toxicity and colonization tests will gain broader use and acceptance in risk assessment and regulatory decision making:

- Develop a broader understanding of the biological and habitat requirements for many of the test species utilized in in situ tests. This is particularly an issue when nontraditional species (e.g., native, indigenous) are utilized.
- Investigate and describe how various possible artifacts can influence the outcome of in situ tests. Of specific interest are test chamber size and design; food availability and food quality; contaminant exposure; behavioral modifications, including avoidance; and handling, transport, and transplantation stress.
- Continue to improve on test chamber designs to optimize conditions for commonly used test species, minimize exposure and habitat differences between the inside and outside of test chambers, and limit general test artifacts.
- Explore how emerging techniques such as electronic sensors, probes, data recording devices, and automated on-line biomonitors/biosensors can aid or improve current in situ test methods.
- Advance the development of defensible QA/QC protocols for field-based research utilizing various types of in situ test systems. This should include an emphasis on documentation and assurance of animal health and response, and representative contaminant exposure.

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