

Review

Higher-tier laboratory methods for assessing the aquatic toxicity of pesticides

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Abstract: Registration schemes for plant-protection products require applicants to assess the potential ecological risk of their products using a tiered approach. Standard aquatic ecotoxicity tests are used at lower tiers and clearly defined methodologies are available for assessing the potential environmental risks. Safety factors are incorporated into the assessment process to account for the uncertainties associated with the use of lower-tier single-species ecotoxicity studies. If lower-tier assessments indicate that a substance may pose a risk to the environment, impacts can be assessed using more environmentally realistic conditions through the use of either pond mesocosms, artificial streams or field monitoring studies. Whilst these approaches provide more realistic assessments, the results are difficult to interpret and extrapolation to other systems is problematic. Recently it has been recognised that laboratory approaches that are intermediate between standard aquatic toxicity tests and field/mesocosm studies may provide useful data and help reduce the uncertainties associated with standard single-species tests. However, limited guidance is available on what tests are available and how they can be incorporated into the risk-assessment process. This paper reviews a number of these higher-tier laboratory techniques, including modified exposure studies, species sensitivity studies, population studies and tests with sensitive life stages. Recommendations are provided on how the approaches can be incorporated into the risk-assessment process.

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1 INTRODUCTION

Registration schemes for plant-protection products require applicants to assess the potential ecological risk of their products using a tiered approach. Standard tests are used at the lower tiers and clearly defined methodologies are available for assessing the potential environmental risks (eg toxicity exposure ratios or risk quotients). For a lower-tier single-species study to be representative of the wider environment the following criteria would need to be fulfilled:¹ the response of selected organisms in single-species laboratory tests should correspond to those of a larger array of organisms in natural systems and the chosen endpoints in single-species tests should be more sensitive than any other at any level of organisation. As standard test organisms do not necessarily meet these criteria, uncertainty factors are incorporated into the risk-assessment process. In order to refine the assessments, it will be appropriate to evaluate critically the base data set. However, if these assessments indicate that a compound is likely to pose a risk to the environment, impacts can be determined using more environmentally realistic conditions.

A number of approaches have been used to address concerns arising from the preliminary assessments, including the use of pond mesocosms,² artificial streams,^{3,4} field monitoring studies³ and experimental ditches.⁵ These approaches have been applied to pesticides and surfactants, and have a number of advantages over single-species investigations, including the ability to assess: (1) endpoints at higher levels of biological organisation, (2) species interactions and (3) indirect effects. The approaches also allow the assessment of population and community recovery.

There are, however, a number of limitations associated with model ecosystem approaches, most notably that the results are difficult to interpret and extrapolate to other situations. Reasons for this include the fact that no-effect concentrations are dependent on the choice of test concentrations; studies are performed at different times using different starting conditions; studies vary in duration; sensitivities may vary across experimental systems; and measured endpoints are not always consistent or comparable. The high background variation associated with the studies means that the discriminatory

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power of the tests can be low. Moreover, in order to simulate natural conditions and to ensure the maintenance of basic ecological characteristics and functions (ie different trophic levels, possibilities for organisms to find new habitats, energy input and nutrient cycles), the test systems need to be of a sufficient size and complexity.⁶ This means that the studies can be costly.

Approaches that are intermediate between standard aquatic toxicity tests and field and microcosm studies can provide valuable data for use in the risk assessment of plant-protection products. The objectives of these laboratory-based higher-tier studies include one or more of the following: to account for the effects of non-continuous exposure; to improve the assessment of species sensitivities to pesticides; to assess the effects of contaminants on sensitive life stages and populations; to determine the potential for organisms, populations and systems to recover after exposure to a pesticide; and to determine indirect effects of compounds on biological communities. There have been significant developments in the design and application of higher-tier laboratory toxicity tests, primarily over the last 5–10 years. It is thus timely to review the current position and opportunities for future development.

2 REALISTIC EXPOSURE SCENARIOS

Preliminary acute-risk characterisation methods typically use the initial predicted environmental concentration (PEC) along with the results from standard ecotoxicity studies. However, the method of entry and rate at which a compound dissipates in the environment may have a significant impact on the relevance of the standard ecotoxicity endpoints used. A number of approaches have been proposed that account for differences in exposure scenarios, dissipation and bioavailability between natural systems and standard toxicity studies.

2.1 Time-to-event analyses

Both exposure concentration and duration determine the effect of a toxicant. However, the main approach to quantifying the effects of a toxicant currently focuses on the exposure concentration (eg determination of LC₅₀). As the tests are performed over a set time period, they can neglect the effect of exposure duration. Time-to-event (TTE) approaches characterise the toxicity of a compound by considering both the intensity and duration of exposure.⁷ TTE analyses have been used since the earliest days of toxicity testing. The approaches draw upon experimental designs where groups of exposed individuals are monitored through time and the intervals to some event, usually death, are recorded for each individual. The resulting data can then be analysed using a range of approaches, including: Kaplan–Meier methods, life table methods, the semi-parametric Cox proportional model and fully parametric models.⁸ Each of the approaches can be used to predict time-to-event and to

test the significance of a co-variate (eg exposure concentration) on a time-to-event.

The use of TTE methods explicitly includes exposure duration in the assessment, but still allows conventional endpoints (eg LC₅₀) to be estimated. In addition, TTE information enhances the power of statistical tests as more data are extracted per test treatment, the effects of covariates can be measured and included in predictive models, and results can be incorporated directly into ecological, epidemiological and toxicological models. Whilst summary data from standard toxicity tests are usually reported and used in the risk-assessment process, the use of standard methods (eg those recommended by OECD) means that additional data will have been recorded throughout the duration of the test. It may often be possible to use this supplementary data for TTE assessments without the need for further experimental study.⁹

2.2 Variable and pulsed-exposure studies

Pesticide contamination of surface waters typically occurs in pulses as a result of spray drift, overland flow and drainage inputs,¹⁰ and exposure of aquatic organisms may be short for compounds that dissipate rapidly. Continuous exposure, as used in standard tests, may therefore not provide a true estimate of the ecotoxicity of compounds and could result in both over- and under-estimations of ecotoxicity.^{11,12} Possible reasons for this include: (1) organisms are able to detoxify or depurate any accumulated test compound during the exposure interval;^{13,14} (2) induced individual tolerance—the first pulse may strengthen survivors through acclimation or induction of detoxification enzymes;^{15,16} (3) individual selection—weaker individuals may be removed by the first pulse, resulting in selection of more robust individuals and an apparent reduction in responses to future exposure; and (4) ecological recovery—after exposure, an impacted population may or may not recover, depending on the characteristics of the species that are affected.

For compounds that dissipate rapidly, the duration of exposure to a pesticide may be very short. Studies can be performed with varying exposure intervals to determine the impact, if any, of such short exposures. For example, *Gammarus pulex* (L) were exposed to lambda-cyhalothrin for either 1, 3, 6, 12 or 96 h.¹⁷ After exposure, organisms were transferred to clean water for 96 h and after this time the effects were observed. There was a highly significant relationship between effects and duration of exposure. The numerical value of the effect concentration after exposure for 1 h was 18 times larger (ie less toxic) than that for 96 h.¹⁷ Similar results have been obtained for *Hyalella azteca* using the pyrethroid cypermethrin.¹⁷

Whilst data from standard toxicity studies can be used to provide an initial assessment of the effects of intermittent exposure to a contaminant,¹⁸ the approaches used assume that the toxicity of an inter-

mittent event is the same as that of a continuous exposure test of equivalent dose. To overcome this problem, the effects of intermittent exposure can also be assessed using 'custom-designed' tests. A number of studies have investigated the effects of pulsed exposure on a range of organisms (including daphnids, amphibian tadpoles, caddis flies, chironomids and fishes) for a range of pesticides (including fenoxycarb, fenitrothion, tebufenozide, chlorpyrifos and fenvalerate).^{11,19,20}

Two approaches have generally been used, namely static exposure studies and flow-through studies. Static renewal studies are particularly useful for generating 'square pulses' (ie constant exposure for a set duration). This situation is most relevant when real-world exposure is expected to be dominated by water flow—for example, where a pulse of chemical travels quickly down a small stream. The use of flow-through exposure systems allows pulsed exposure to be more realistically simulated with gradual changes in concentration. This approach has been used to simulate the effects of degradation of a herbicide on *Selenastrum capricornutum*.²¹ Three compounds were used in the simulation, namely the parent compound and two major metabolites, and the pulses of each mimicked the fate of the compound in the natural environment. Pulsed-exposure experiments can provide information on the potential for organisms to recover, the development of resistance to pesticide exposure and any latent effects.

As a wide range of possibilities exists for the intensity and duration of exposure to pulsed release of contaminants, no testing regime can cover all pulse discharge scenarios. Mechanistic models have therefore been proposed and tested for predicting the toxicity of time-varying exposures (Table 1). Investigations into the effects of monochloroamine on rain-

bow trout (*Oncorhynchus mykiss* Walbaum) and common shiners (*Notropis cornutus* Mitch) demonstrated that concentration \times time and Mancini-type models can predict an LC₅₀ to within $\pm 50\%$.²² The Mancini model performed better for longer-term pulse exposures.

2.3 Inclusion of dissipation processes

Once a compound has entered the aquatic environment, its distribution, and hence potential to cause adverse effects, will depend on a number of factors, including the potential for abiotic (eg hydrolysis, oxidation, photolysis) and biotic (aerobic and anaerobic) degradation and the partitioning behaviour of the compound to sediment and air. A number of studies have investigated the incorporation of one or more of these processes into laboratory ecotoxicity studies. The processes simulated have included spray drift, effects of suspended sediment, effects of pesticide entering the environment in run-off and the effects of bed sediment (Table 2).^{17,24}

By adding sediment to the standard test system, the degradation and adsorption processes occurring in the environment can be simulated.²⁷ Studies using the hydrophobic synthetic pyrethroids, lambda-cyhalothrin (log K_{ow} 6.8), esfenvalerate (log K_{ow} 6.22) and fenvalerate (log K_{ow} 5.0) have demonstrated that the presence of soil or sediment in the test system results in a significant reduction in the observed effect of a pesticide (Table 2). This observation can be explained by the fact that very little pesticide (ie $<1\%$ for lambda-cyhalothrin) is available in the water column due to partitioning of the compound from the aqueous phase to sediment.²⁸ The data in Table 2 indicate that the addition of sediment had less effect on the hydrophilic compounds isoproturon (log K_{ow} 2.5) and pirimicarb (log K_{ow} 1.7).

Table 1. Mechanistic models for assessing the effects of intermittent exposure to contaminants

Model	Generalized model	Inputs
Concentration \times time ²²	$A_m = C_w^y \cdot t_d$	A_m = a species specific constant for $m\%$ mortality ($\mu\text{g litre}^{-1}\text{h}^{-1}$) C_w = toxicant concentration in exposure water ($\mu\text{g litre}^{-1}$) t_d = time to death (exposure time required for $m\%$ mortality; h) y = a power term
Mancini uptake-depuration model ²³	$\frac{dC_{soa}}{dt} = K_u C_w^y - K_{dep} \cdot C_{soa}$	C_w = toxicant concentration in exposure water ($\mu\text{g litre}^{-1}$) C_{soa} = concentration of toxicant at site of action ($\mu\text{g g}^{-1}$) K_u = uptake rate constant (h^{-1}) K_{dep} = depuration rate constant (h^{-1}) t = exposure time (h) y = a power term
Breck damage-repair model ¹⁴	$\ln\left(\frac{M}{1-M}\right) = d\left[y \cdot \ln(C_w) + \ln\left(\frac{1 - e^{-k_{rep}t}}{k_{rep}}\right) + \ln\left(\frac{K_{dam}}{D_{L,50}}\right)\right]$	M = the observed mortality proportion D = slope of logit response as a function of $\ln(C_w)$ C_w = toxicant concentration in exposure water ($\mu\text{g litre}^{-1}$) K_{dam} = damage rate constant (h^{-1}) K_{rep} = repair rate constant (h^{-1}) $D_{L,50}$ = lethal damage at 50% mortality t = exposure time (h) y = a power term

Table 2. Changes in observed toxicity caused by a range of modifications to water-only exposure studies^{17,23,25,26}

Compound	Test species	Reduction factor in toxicity or BCF relative to water-only study					
		Simulation of spray drift	Simulation of drift and suspended sediment	Application to soil prior to addition of water	Simulation of suspended sediment	Simulation of bed sediment (not stirred)	Simulation of sediment (stirred)
lambda-Cyhalothrin	<i>Daphnia magna</i>	3	40	175	—	120–140	81–280
Glyphosate	<i>Daphnia magna</i>	—	—	—	3.3	—	—
Esfenvalerate	<i>Daphnia magna</i>	—	—	—	—	3.0	—
	<i>Lepomis macrochirus</i>	—	—	—	—	5.0	—
Fenvalerate	<i>Limephilus lunatus</i>	—	—	—	10–100	—	—
	<i>Palaemonetes pugio</i>	—	—	—	—	—	9700
Pirimicarb	<i>Daphnia magna</i> (BCF)	—	—	—	—	0.7	—
Isoproturon	<i>Scenedesmus subspicatus</i>	—	—	—	—	—	2
Herbicide 1	<i>Selenastrum capricornutum</i>	—	—	—	—	>17	—
		—	—	—	—	>900	—
Herbicide 2	<i>Navicula pelliculosa</i>	—	—	—	—	>17	—
		—	—	—	—	>900	—

In the natural environment, water contains dissolved or colloidal organic matter (DOM). These substances have been shown to interact with organic compounds, resulting in effects on bioavailability because only freely dissolved compounds are generally assumed to be accumulated by organisms. Previous studies using pesticides, surfactants and polycyclic aromatic hydrocarbons have demonstrated that the presence of DOM reduces accumulation and toxicity.^{24,29–31} However, at low concentrations of DOM (ie less than 10 mg litre⁻¹) bioaccumulation may be enhanced.³²

In order to simulate the effects of natural concentrations of DOM on ecotoxicity, studies have also been performed using water samples collected in the field.³³ Studies on a range of compounds have demonstrated that the apparent toxicity of chemicals is only slightly reduced in tests using natural waters (Table 3).³³ This suggests that the use of laboratory water does not necessarily introduce a significant source of uncertainty.

Standard ecotoxicity studies usually require that the test concentration is maintained at >80% of the starting concentration throughout the test. For compounds that are volatile or degraded abiotically (eg via hydrolysis) this can mean that the test system is covered, that a flow-through test system is required or that the test solution is replaced regularly. Thus the impact of abiotic degradation processes on toxicity

could readily be assessed using a static exposure system with no replacement of test solution.

The results of modified exposure studies can be used in conjunction with toxico-kinetic models to assess toxicant effects under non-steady state conditions. Compartment-based models such as PULSETOX describe the movement of toxicants between compartments;^{34,35} physiologically based codes describe accumulation, elimination and distribution, and bioenergetically based models such as FGETS and DEBtox simulate accumulation and loss in terms of the organism's energy requirements.^{36,37} To apply the models, information is required on body residue levels and toxic effects. This is a neglected area in ecotoxicology, and further work is required before it can be incorporated into the risk-assessment process.

3 STUDIES USING ADDITIONAL SPECIES

There is no species that is 'most sensitive' to all classes of contaminant.^{38–40} Species can vary in their sensitivity to chemicals by orders of magnitude.⁴¹ Preliminary assessments of pesticide risk to the aquatic environment therefore incorporate factors of either 10 or 100, in part to cover uncertainties regarding the relative sensitivities of the standard test organisms compared with the range of species that are likely to be present in the field. One approach to reducing these uncertainties is to test additional organisms in order to

Table 3. Mean ratios of EC₅₀ values obtained using tests on 15 natural waters to EC₅₀ values obtained using ISO standard media³³

	Log K _{ow}	<i>Raphidocelis subcapitata</i> microplates	<i>Raphidocelis subcapitata</i> erlens	<i>Brachionus calyciflorus</i>	<i>Daphnia magna</i>
Zinc sulphate	na	5.7	3.0	2.1	1.2
4-Nonylphenol	5.92	0.6	0.7	1.2	0.9
Phosalone	4.30	0.7	1.2	1.1	1.7
Pentachlorophenol	5.12	2.7	2.1	1.2	3.1
2,4,5-Trichloroaniline	3.45	1.9	2.2	2.1	1.0
Mean		2.3	1.8	1.5	1.6

gain a more precise estimate of the most sensitive endpoint and/or to derive a distribution of sensitivity.⁹ The testing of additional organisms also generates more ecological information for use in site-specific analyses.

3.1 Selection of additional test species

A number of approaches have been proposed for selecting additional test species. The preferred approach will depend on a number of factors, including the nature of the compound being tested and the availability of data for compounds with a similar mode of action. For compounds that do not have a mode of action that is specific to a particular taxon, the Aquatic Dialogue Group⁴² proposed that the test species should include at least two species of fish, one invertebrate and one aquatic plant (macrophyte or algae) plus four other species. The other four test species should be selected from the group(s) that is/are shown to be the most sensitive after the initial studies have been completed.⁴² If chronic studies are to be performed, it is recommended that one fish, one invertebrate and one plant be used. A further organism should be selected from the most sensitive group identified in acute tests.⁴² Opportunities for extrapolation should not be overlooked for compounds with a strong similarity to an existing substance. For example, the number of additional organisms required to show the range of species sensitivity might justifiably be reduced on the basis of data for a similar compound.

The USEPA⁴³ have recommended that additional data should include invertebrate acute and chronic tests, sediment toxicity tests, rooted plant testing and amphibian testing. The increase in the number and type of invertebrate toxicity tests reflects an attempt to provide some consideration of the range of potential responses. In assessing impact on aquatic invertebrates, risk assessments generally focus on *Daphnia magna* Straus, a parthenogenic, short-lived crustacean. The following should be considered when selecting additional species: (1) different and often variable life spans; (2) unpredictable lengths of life stages and different metamorphic stages; (3) indeterminate growth in some species; and (4) significant differences in adipose stores.⁴³ The USEPA suggest that additional species for invertebrate acute toxicity tests could include stoneflies and amphipods, whereas chronic tests should address effects on sexually reproducing invertebrate species including copepods and chironomids. The addition of an acute amphibian test reflects the need to consider effects on amphibian species.

The sensitivity of individual species is primarily dependent on the compound's mode of action, and species from the same taxonomic group (either phylum or class) generally respond to contaminants in a similar manner.⁴⁴ For chemicals with a non-polar mode of action, variation in species sensitivity is small. However, for compounds that are either reactive or have a specific mode of action, the variation can be as

large as 10^5 – 10^6 . Based on an understanding of the mode of toxic action of a compound, it may be possible to identify and group sensitive and less sensitive organisms. This allows the testing strategy to be focused on groups at high risk.⁸ For example, Cuppen *et al.*⁴⁵ recommended the use of non-arthropod macroinvertebrate species at an advanced stage of the risk assessment of fungicides. This is based on the assumption that the spectrum of effects of fungicides in aquatic systems is similar to results obtained for carbendazim and pentachlorophenol. Similarly, tests on insects would be recommended for substances with an insecticidal activity. It should be noted that mode of toxic action is often not well understood, particularly for new compounds. Whilst the mode of action of a pesticide is an important criterion for grouping of organisms, habitat, reproductive strategy and life cycle may also affect the sensitivity of species.

Recent studies^{46,47} have assessed the suitability of a battery of ecotoxicity tests that evaluate the sub-lethal and lethal effects of chemicals on algae, protozoans, rotifers, crustaceans and dipterans. The aim of the studies was to identify tests that could be adopted in the risk-assessment process. Comparison of the results from the tests with those from stream and pond mesocosm studies for lindane, 3,4-dichloroaniline, atrazine and copper compounds demonstrated that lowest observed effect concentrations obtained in the laboratory were generally similar to results obtained using pond mesocosms and artificial streams (ie within a factor of 6). The exception to this was 3,4-dichloroaniline, for which the pond mesocosm was 200 times more sensitive. Similar studies on isoproturon⁴⁸ demonstrated that single-species tests using a range of organisms resulted in a more conservative assessment than a microcosm study.

Campbell *et al.*⁹ suggest that, for compounds that do not have a mode of action specific to a particular taxa, eight species should be tested as a minimum to describe the distribution of sensitivities of aquatic organisms. In cases where it is known that a specific group of organisms is particularly sensitive, the additional test species should be selected from the most sensitive group. However, if fish are the most sensitive group, fewer test species are required and five species are probably sufficient. This is for animal welfare reasons and because distributions of sensitivity are usually narrow for fish. By using at least eight additional test species (five for fish), there is a high probability that a good estimate (ie within an order of magnitude) of the most sensitive endpoint will be obtained.⁹ The use of eight additional species also allows a probabilistic approach to be used for effects characterisation.⁴²

3.2 Analysis of data

One of the objectives of laboratory ecotoxicity testing is to assess the potential for impact of a chemical on natural ecosystems. Rather than using uncertainty factors, the data from single-species studies can be

extrapolated to field conditions using either regression techniques or distribution models.⁴⁹ A wide range of approaches are available that are based on the same general principle (ie they assume that experimental ecotoxicity data fit a given distribution), but differ in the statistical distributions that they use.^{10,37,39,50–52}

Outputs from the methods include: (1) hazardous concentrations for a specified percentage of species (HC_x where x is the specified percentage);^{51,53} (2) concern levels—levels that can be expected to cause an environmental effect at least 95% of the time⁵⁴ and (3) final chronic values—a threshold concentration for unacceptable effects that is calculated from the lowest of three chronic values (the final chronic value, the final plant value and the final residue value). The final chronic value is calculated from the no observed effect concentrations (NOECs) for at least eight families.⁵⁵ There may be problems with availability of internationally accepted methods for this number of chronic tests (eg for invertebrates).

The results of single-species distribution methods have been compared with those from multi-species studies (Table 4).^{56,57} In most cases, the extrapolation procedures lead to lower ‘safe’ values than NOECs from multi-species studies and demonstrate that field effects can be predicted if uncertainties related to mode of action (eg species groups tested and exposure regimes) are accounted for. The studies have demonstrated that the larger the number of species, the smaller the difference between the NOECs derived using multi-species studies and the ‘safe’ values derived using statistical techniques.

There is debate over which value from a range of species sensitivities is most appropriate to protect the aquatic environment. Van den Brink *et al*⁵⁷ suggest that the 5th percentile value that is based on acute

$L(E)C_{50}$ data is a generically applicable criterion that is protective for exposed communities (the higher the number of species tested, the lower the difference between the NOEC for an ecosystem and the 5th percentile value). Whilst the 5th percentile is therefore the accepted norm, previous studies with pyrethroids⁶⁰ and atrazine¹⁰ have proposed that the 10th percentile effect concentration is adequate.

More recently, the use of bootstrapping to derive hazardous concentrations has been investigated.⁶¹ The approach involves random sampling (with replacement) of an ecotoxicity data set for a compound to create a resampling data set (eg of 100 observations). The resulting data set is then ranked and the 5th percentile value is selected as the HC_5 . The resampling is performed a number of times (eg 10 000) to produce a number of HC_5 estimates. These are then ranked, and the value corresponding to 50% is taken as the best estimate of HC_5 . Estimates at 2.5% and 95% are used as the 95% bootstrap confidence limits. Large data sets are required to derive reliable HC_5 estimates because knowledge of the mode of action of the substance and most sensitive species is not accounted for.

3.3 Current limitations to the approach

Whilst the use of additional test species provides useful data, there are a number of limitations to the approaches compared to standard ecotoxicity studies. These include: large datasets are required to ensure that the resulting analyses are as representative as possible; there is disagreement about the number and taxonomic distribution of species to be tested; test results may not be directly comparable; test guidelines are only available for a few test species; organisms may not be from the same sensitivity distribution and there

Table 4. Comparison of multi-species NOEC values with results obtained using extrapolation techniques on single-species toxicity data.^{46,56,57} All values are $\mu\text{g litre}^{-1}$

Compound	MS NOEC ⁵⁸	Aldenberg and Slob ⁵⁹ 50%	Aldenberg and Slob ⁵⁹ 95%	Wagner and Lokke ⁵³ 50%	Wagner and Lokke ⁵³ 95%	SSD (method not reported) ^a 95%	USEPA ⁵⁴
Lindane	0.22	0.12		0.13			
Dichloroaniline	1.0–12	120		120			0.067
Atrazine	<3.0–5	0.82–2.2	0.12	0.87–2.1	0.17		0.15
Copper compounds	1.1	0.28		0.30			
Isoproturon	2					0.63	
Atrazine	20					13	
Diflubenzuron	0.3					0.18	
Chlorpyrifos	0.1					0.044	
Diflubenzuron	0.1	—	—	—	—		0.001
Parathion	0.1	0.013	0.00013	0.011	0.00023		0.002
Azinphos-methyl	0.20–0.25	0.15	0.085	0.077	0.018	0.037	0.01
Parathion-methyl	0.1	—	—	—	—		0.024
Pentachlorophenol	20	3.2	0.53	2.6	0.66		0.32
1,2,4-Trichlorobenzene	57	44	1.1	39	1.7		19
Trichloroethylene	2.8	3100	65	3,100	97		130
Trifluralin	0.5	—	—	—	—		0.2
Dichlobenil	5.0	—	—	—	—		7.8
Permethrin	0.023	—	—	—	—		0.066
Toxaphene	>1.5	0.003	<0.0001	0.003	<0.0001		0.0025

^a Species sensitivity distribution.

is no agreed way of testing this; and the ecology and physiology of the test organism may not be fully understood, making extrapolations to population effects problematic. Moreover, for many species, laboratory culturing procedures are not available and the test species will need to be collected from the field. This can mean that the age and quality of the organisms cannot be guaranteed.⁶² Where possible, organisms collected from the field should be taken from an uncontaminated site that has not been exposed to the test substance or chemicals with a similar toxic mode of action. In some instances, the test organism may not be available throughout the year. Therefore, when designing such studies, either species should be selected that are available all of the year or a wider range of possible test animals should be available so that a selection can be made at any time. The use of geographical information systems (GIS) to identify the proportion of a particular species and habitat exposed to a given pesticide could also assist in the identification of additional species for testing.⁴³

4 ORGANISM RECOVERY

Assessment of recovery is often a component incorporated into lower-tier toxicity tests for algae and the higher plant *Lemna* sp. An aliquot of previously exposed cells or fronds is placed in clean medium to establish whether cell division is reversibly or irreversibly retarded. However, the principle can be extended to other aquatic plants, invertebrates and fish. A few recovery studies have been reported for pesticides, including organophosphorus and carbamate pesticides, and for organisms including daphnids, chironomids and blackfly.^{63,64}

The potential for recovery will be determined by the length of time between exposures and the specific mode of action of the compound being considered. For example, studies into the effects of carbamate pesticides on chironomids indicated that toxic effects were reduced if animals had been allowed to recover for more than 6 h.⁶³ This observation was probably due to the reactivation of acetylcholinesterase during the recovery period—carbamate pesticides have been shown to be reversible inhibitors of AChE. No such recovery was observed for organisms exposed to organophosphorus insecticides, which are considered to be irreversible inhibitors.⁶³

5 SYSTEM RECOVERY

Once a system has been impacted by a contaminant, its rate of recovery will be determined by the persistence of the contaminant and the ecology, physiology and biochemistry of organisms in the system and in proximity to the system.

One approach to predict the likely recovery of a system is based on the dissipation half-life of the compound, the initial exposure concentration and the hazardous concentration for 5% of species.⁶⁵ If

recovery is required within a year after a single application, then the chemical's half-life should meet the condition:

$$T_{1/2} < \frac{\ln 2}{\ln \left\{ \frac{C_0}{HC_5} \right\}}$$

where $T_{1/2}$ is measured in years, C_0 is the initial concentration and HC_5 is the hazardous concentration for 5% of species. Although dissipation will be necessary for complete recovery, under some conditions it may not be sufficient and ecological recovery may lag behind the disappearance of the chemical. This lag will depend on a range of factors including accessibility of shelters, survival of resistant life stages and presence of untreated areas from which recolonisation can occur. In addition, ability to recolonise will depend on habitat selection and life history.

A number of experimental studies have been proposed for determining whether an impacted system is likely to be re-colonised. This is particularly relevant for mobile organisms such as fish, which may be resident in a section of a water body for a short time and may move from a toxic system. The study design consists of adding the test organism at the start of the study and at regular intervals thereafter. A similar approach can also be used with those invertebrate species with a high potential for re-colonisation, with the re-introduction of individuals into a previously treated system and monitoring of their subsequent survival and performance. For example, Crane *et al.*⁶⁶ investigated the toxicity of pirimiphos-methyl to *Gammarus pulex*. Beakers were spiked with the study compound at day 0 and toxicity to *Gammarus pulex* was assessed over 24 h at 1, 4, 8 and 12 days after application. Generally, mortality decreased with increasing time from spiking, probably as a result of pesticide degradation.

The factors that influence the recovery of a population after a significant perturbation are complex. One important factor is the life history of the individual species.⁶⁷ Aquatic organisms can vary widely in their reproductive and dispersal characteristics. For example some species may reproduce continuously, whereas others may reproduce at discrete times of the year. Generation times can vary from days (eg daphnids) to years (eg dragonflies). The ability for recolonisation can also vary—many aquatic insects have adult life stages with wings whereas other taxa (eg crustaceans and molluscs) will rely on recolonisation by more passive forms of dispersal (wind, flooding, transportation by birds).

6 SENSITIVE LIFE STAGE STUDIES

Sensitivity to toxicants is known to vary between species, but sensitivity can also vary between the different development stages of an organism.^{68–71} Standard ecotoxicity studies generally focus on neo-

nate or juvenile animals, which are likely to be the most sensitive life stage(s). However, in instances where it is known that a particular substance is likely to be more toxic to a life stage not studied in the standard tests (eg based on the results of studies with related compounds or based on the mode of action of the substance), it may be appropriate to test this additional life stage and, as a result, incorporate a smaller uncertainty factor into the risk-assessment process. For example, there are instances where older organisms have been shown to be more sensitive to a particular toxicant than younger ones. Studies with daphnids²⁰ have indicated that older organisms are more sensitive than neonates to chlorpyrifos. One possible reason for this is that the older animals have an increased filtration rate and receive a higher dose of chemical, as chlorpyrifos is likely to be associated with food. Berrill *et al*⁷² showed that 2-week-old tadpoles were more sensitive to endosulfan than newly hatched tadpoles because endosulfan affected the post-hatch development of the neuromuscular system. By understanding both the significance of different routes of uptake for different classes of chemical and the physiology of an organism, it may be possible to predict these types of difference in species sensitivity and hence identify the most appropriate life stage to test. The testing of sensitive life stages may be problematic due to the lack of test and culture methods for certain species.

7 POPULATION LEVEL STUDIES

As standard toxicity studies are generally performed on the most sensitive life stages, results may overestimate effects on populations. Life-cycle and population-level studies can be useful tools for assessing the likely impact of a pesticide on populations of aquatic organisms. The structure of a population at the time of toxicant exposure may be an important factor in determining toxicant impact.⁷³ Populations in nature consist of a mixture of life stages, yet toxicological studies usually only start with one life stage. Consequently it may be appropriate to consider age/stage structure when evaluating toxicant effects.

Both modelling and experimental approaches can be used to determine population level effects. Laboratory experimental studies have been performed using plant and invertebrate species⁹ and have ranged from simple studies evaluating effects on a limited number of life stages to complex multi-life-stage studies that simulate natural population dynamics.⁷⁴⁻⁷⁷ This type of test is normally restricted to invertebrates, plants and fish species that have relatively short life histories, and may not be possible with groups prone to cannibalism unless the individuals are held separately. Experiments with other fish may be possible under field conditions.^{78,79}

The data generated from population-level studies and from simple toxicity studies can be used to model the effects of compounds on populations in the field.⁸⁰⁻⁸³ Population models describe the dynamics

of a finite group of individuals through time and have been used extensively in ecology and fisheries management. These approaches are well developed for use on fish^{84,85} and invertebrates.⁸⁶⁻⁸⁹ By using models, the results of laboratory studies can be used to evaluate a range of scenarios⁹ and can assist in identifying short- and long-term changes in population structure.

Appropriate use of population models requires an understanding of the natural history of the species under consideration, as well as knowledge of how the toxicant influences its biology. Model inputs can include somatic growth rates, physiological rates, fecundity, survival rates of various classes within populations, and how these change in response to the study compound.⁹⁰ In addition, population-density effects may be important.

8 HIGHER-TIER LABORATORY STUDIES WITHIN ENVIRONMENTAL RISK ASSESSMENT FOR PESTICIDES

Higher-tier studies are generally carried out in response to indications of unacceptable risk from a lower-tier assessment. The exact nature of the study or studies to be performed will depend on a number of factors including the results of lower-tier studies and the physicochemical properties, environmental fate and use pattern of the compound of interest. A summary of the major higher-tier study types and where they fit within current procedures for risk assessment of pesticides is provided in Table 5. Generally the results from the approaches can be used in three ways to refine the lower-tier assessment: (1) the results can be used to justify the use of a lower uncertainty factor (eg species-sensitivity studies, organism-recovery studies; population-level studies); (2) the results can be used to justify an increase in the effects concentration used in the risk assessment (eg modified exposure studies); and/or (3) the results can be used to perform a probabilistic assessment (eg species-sensitivity studies). The combination of a number of the approaches (eg use of population studies coupled to pulsed exposure and dissipation studies) may also be appropriate.

Higher-tier laboratory approaches have a number of advantages over the use of standard ecotoxicity tests and/or mesocosm/field studies. These include: (1) the endpoints are more pertinent to actual environmental exposure; (2) they provide scope for the integration of 'realistic' measures of fate and exposure into the risk-assessment process; (3) they should provide more confidence when predicting actual effects in the environment; (4) they can be designed to determine effects on specific endpoints, eg mortality, growth, behaviour; (5) they can be used to identify more clearly those species that may be at risk, and assist in the targeting of multi-species tests; (6) the results may help explain observed effects (eg mode/specificity of action); (7) recovery can be assessed for both individuals and populations; (8) there are none of

Table 5. Higher-tier toxicity methods

<i>Test type</i>	<i>Rationale for test</i>	<i>Possible design of test</i>	<i>Data analysis</i>	<i>Output</i>	<i>Advantages/disadvantages</i>
Testing of additional species	To reduce uncertainty factors and/or develop species sensitivity distributions	Test a number of (8 may suffice) additional species:- From a range of groups or For compounds whose mode of action indicates that a particular group will be sensitive, tests should be performed on species from this group or If fish are likely to be sensitive, then only 5 additional species should be tested	Lower uncertainty factors by up to an order of magnitude or use statistical approaches to generate species-sensitivity distributions	Revised Toxicity Exposure Ratio (TER) or Hazardous Concentration for a specified percentage of species (HCx)	Identification of most sensitive species More relevance than standard species Refinement of TERs Allows probabilistic risk assessment There is disagreement on the Number of species that need to be tested No guidance available on uncertainty factors to use No guidance available on what level of population protection is acceptable Lack of standard test methods for additional species May need to obtain organisms from the field
Time-to-event analysis	To assess the toxic effects of exposure to pesticides over durations shorter than the standard test duration	Use data recorded over time for standard ecotoxicity tests	Compare time-to-event data with expected exposure duration	If duration < time to effect then reduce effect estimate	More realistic assessment of ecotoxicity Does not address 'latency'—ie effects brought on by earlier exposure Number of observations made during the standard test may not be adequate No guidance on how effects estimate can be reduced
Short-term exposure studies	To assess effects of short exposure durations on pesticide toxicity	Perform test over exposure duration predicted for the field	Use standard toxicity test statistical procedures	Revised effect measurement for use in risk characterisation	More realistic assessment of exposure Does not consider delayed effects
Pulsed-exposure studies	To simulate pulsed exposure conditions that are likely in the field	Static renewal or flow-through system, depending on detail required	Use standard toxicity test statistical procedures and/or toxico-kinetic models	Revised effect measurement for use in risk characterisation	More realistic assessment of exposure Includes potential for organisms to recover Results affected by a number of variables (including pulse duration, time between pulsed, organism recovery) so difficult to interpret
Dissipation studies	To assess the impact of dissipation processes on toxicity	Generally performed using sediment/water systems. Studies could also be performed to assess effects of photodegradation (by performing studies in the light), hydrolysis (by performing a static test rather than a flow-through study) or volatilisation.	Use standard toxicity test statistical procedures	Revised effect measurement for use in risk characterisation	More realistic assessment of exposure Can include effects of metabolites Guidelines not available on selection of test matrices Not suitable for all species (eg algae) Methods not available for assessing photodegradation and volatilisation
Studies using natural matrices	Account for differences between bioavailability in natural and laboratory systems	Perform toxicity studies using water samples collected from the field	Use standard toxicity test statistical procedures	Revised effect measurement for use in risk characterisation	Assessment of bioavailability under natural conditions No guidance available on selection of test matrix
Effects on sensitive life stages	To determine whether a particular life stage is sensitive or not	A range of approaches are available for a number of species.	Use standard toxicity test statistical procedures	Toxicity to sensitive life stages—possible reduction in uncertainty factor	Reduction of uncertainty factors
Population studies	Perform studies on a population of a particular species over a prolonged time period	Experimental and modelling approaches are available	Use population modelling approaches to interpret results	More ecologically relevant assessment of effects—possible reduction in uncertainty factor	Includes effects of recovery includes selection of tolerant organisms May be more ecologically representative
Recovery studies	To determine whether populations can recover after exposure to a pesticide	Expose organisms and transfer to clean media to determine time to recovery		Assessment of time required for organisms to recover	Difficult to perform on fish Disagreement over acceptable recovery period

the complications that are associated with the interpretation of data from multi-species studies; (9) relative to mesocosm studies, they can be performed with less regard to the season, the test organisms are usually readily available and the system is more controlled; and (10) sub-lethal effects can be determined.

However the approaches also have limitations: (1) they cannot provide information on species interactions; (2) results can be more difficult to interpret and compare than those from standard single-species tests; (3) the tests may be more costly than standard single-species studies; (4) standardised higher-tier procedures are not available; and (5) the quality of available organisms cannot always be guaranteed. Moreover, there is currently considerable debate and disagreement over the relevance of different test designs and outcomes. For example there is considerable disagreement over (1) the number of species that should be studied using the additional-species approach, recommended numbers range from less than 10 to 30–40; (2) the hazardous concentration level selected for use in probabilistic risk assessment (ranging from 5 to 10%); and (3) the acceptability of different recovery periods.

9 CONCLUSION

Higher-tier laboratory tests provide a useful intermediate between standard toxicity studies and semi-field/field tests. The review presented here has shown that there are a wide range of higher-tier studies available and that a significant body of work has been generated on most, but not all, of these methods. There is a need for flexible guidelines to be developed. However these guidelines should not be so flexible that it is impossible to compare results generated in different laboratories. The next 5 years are likely to see significant advances in the development of test methodologies and in the use of models in support of data analysis and risk assessment.

The most important constraint associated with higher-tier laboratory methods concerns their validation status. There is a need to validate the methods in a consistent and systematic way by comparing data and risk assessments generated from standard laboratory testing, higher-tier laboratory testing and mesocosms. This would allow evaluation of the advantages and limitations of laboratory higher-tier testing compared to standard studies and mesocosm studies.

Data sets on the ecotoxicity of chemicals to species should be further analysed on the basis of information on the mode of action of a compound. The development of lists of sensitive species associated with a particular mode of action would be highly beneficial when selecting organisms for testing. Many of the available test methods to support species-sensitivity analyses were developed in North America and additional tests may be required for application in other regions. Current methods for assessing effects on

communities from distributions of species sensitivity assume that all species are equally important. This is clearly not the case and consideration should be given to identifying important species in aquatic ecosystems. These species could include economically important organisms, keystone species or species that are aesthetically important in a particular water body.

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