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AQUATIC TOXICITY TESTS WITH SUBSTANCES THAT ARE POORLY SOLUBLE IN WATER AND CONSEQUENCES FOR ENVIRONMENTAL RISK ASSESSMENT

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Abstract—Aquatic toxicity tests with substances that are poorly soluble in water have been conducted using different methods, and estimates of toxicity have varied accordingly. The present study illustrates differences in toxicity values resulting from variation in test designs and solution preparation methods, and offers guidance on the best way to conduct these tests. Consequences for environmental risk assessment and classification are also discussed. The present study mainly considers active ingredients of plant protection products, but is also considered relevant to other chemicals. It is recommended that toxicity tests be conducted only up to the saturation limit, dispersants avoided, and solvents used only if necessary to support handling and speed of dissolution. Analytical measurements of exposure concentrations should reflect what organisms are exposed to. If acute toxicity testing at the saturation limit yields no adverse effects, further testing should not normally be required; the toxicity value of the endpoints should be considered as the saturation limit and adverse classification should not be required. Chronic testing, if required, should then be conducted at the practical saturation limit as this is the most realistic worst-case exposure scenario. If no adverse effects occur, the risk should be acceptable because higher aqueous exposure cannot occur. This could be substantiated by testing additional species. Assessment factors on no observed effect concentration (NOEC) values at the saturation limit require careful consideration in the risk assessment to avoid unnecessarily low regulatory acceptable concentrations. Environ. Toxicol. Chem. © 2012 SETAC

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INTRODUCTION

Aquatic ecotoxicological tests with substances that are poorly soluble in water are conducted in a variety of ways, and the resulting L(E)C-values (lethal [or other effect] concentrations) may differ considerably. The use of solvents or dispersants to aid the dissolution of a test substance in regulatory aquatic ecotoxicological tests is an area in which there has been conflicting guidance [1–4], further complicated because individual testing guidelines often provide their own recommendations. Yet, it is generally well known that the method by which a test solution is prepared and introduced into the test vessel, as well as the treatment of any undissolved test substance, can have a significant impact on the results and reliability of the test. Also, the expression of the effect data as analyzed concentration values, and how these are generated, needs to be considered carefully. The present study illustrates differences found between specific testing methodologies with substances that are poorly soluble in water (i.e., solution preparation and test conduct) and resulting toxicity values, using the herbicide diflufenican as an example, and offers harmonized guidance for the conduct and reporting of regulatory aquatic ecotoxicological tests, with particular relevance to plant protection products.

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BACKGROUND CONSIDERATIONS

The purpose of an aquatic ecotoxicological test is to determine the toxicity of a substance in aqueous solution, that is, truly dissolved in the test medium (note that substances or formulations specifically designed for release as a suspension or emulsion are beyond the scope of the present study). This is advocated because generally only the dissolved fraction of a substance is bioavailable for uptake and transfers across biological membranes, and hence is responsible for the true aquatic toxicity [5]. Undissolved material present in the test media has the potential to exert adverse (physical) effects on test organisms, which are unrelated to intrinsic substance toxicity [2,3]. Examples of this are blocking of fish gill membranes, encapsulation/entrapment of daphnids, or the reduction of light intensity in algal tests. Also, the oral uptake of undissolved particles may lead to release of substances in the digestive tract and subsequently cause toxicity. Although it is possible to consider effects caused from testing above the solubility limit in the interpretation of the results, in practice such effects cannot be easily distinguished from the intrinsic substance toxicity and may confound test results. Furthermore, test concentrations far above the water solubility of the test substance can contain better soluble impurities whose effects might also confuse the interpretation of true substance toxicity. As a consequence, for a given test substance, the outcome of aquatic tests containing excess undissolved substance can vary considerably based on the way the test media were prepared and sampled. This variability is often observed in the aquatic

toxicity testing of nanomaterials and is currently the subject of intensive research [6–8]. As effect concentrations from tests with undissolved material may lead to misinterpretation of the intrinsic substance toxicity, such tests might be inappropriate to base regulatory decisions on; therefore, undissolved material should be separated from the test solution prior to organism exposure. The exception is tests with substances of such low solubility that determining the dissolved concentration is not feasible and where confounding (physical) effects can be excluded.

Analytical measurements to confirm concentrations are often made during aquatic tests, particularly in regulatory tests, and the manner of sample collection and preparation can have large consequences for the magnitude and usefulness of the test endpoint estimate. For example, it is currently recommended [1–4] that solutions are filtered or centrifuged prior to chemical analysis, to remove undissolved fractions (including, for example, feces, exuviae from the test organisms, or whole test organisms, e.g., algae). However, this has not necessarily been done in the past, resulting in test concentrations above the solubility limit of the test substance, but still showing acceptable analytical results within a reasonable percentage of nominal concentrations.

In certain instances, the use of solvents can be justified to aid the preparation of a stock solution. Occasionally, the use of solvents may even be preferable, for example, to aid stability of the test substance or to avoid excessive sonication of the test substance or exposure system. However, because of the potential for interaction with the test substance or test system (test organism plus exposure system) that may result in an altered response in the test (e.g., Stratton [9]), their use should be restricted to situations where no other acceptable method of media preparation is available. Stock solutions prepared with water miscible solvents are more amenable to adding to the test media and mixing, and therefore help to accelerate the preparation of saturated solutions for substances of low water solubility. The addition of solvents does not increase the solubility in water per se; however, the saturation limit in the water/solvent solution may be to some extent greater than the solubility in pure water. Nevertheless, any amount above the saturation limit will precipitate when the stock is added to the test media and the solvent concentration consequently diluted, which has the potential to cause adverse effects on the test organisms (see above). Thus, precipitates should be removed prior to the addition of the test organisms. It must be noted that there can be further substance-specific analytical aspects to consider. For example, some hydrophobic substances have a tendency to adsorb to glassware, filters, and centrifuge tubes, making sample preparation difficult and liquid substances may not be amenable to either centrifugation or filtration. Increasing amounts of solvent above the maximum recommended level (0.1 ml/L) is not advisable due to potential effects on the test system, such as toxicity to the test organism [2,3]. Solvents can also provide a carbon source promoting microbial growth in the test vessels, which can deprive test organisms of dissolved oxygen, cause pH shifts, entrap organisms within the microbial film, or contribute to the degradation or adsorption of the test substance.

Solvents commonly used in aquatic ecotoxicology and their toxicity to aquatic organisms are listed in a European Centre for Ecotoxicology and Toxicology of Chemicals document [3]. All these solvents have low acute toxicity to aquatic organisms, though this is not the sole basis for their selection (see the *Suggestions* section below).

CASE STUDY ON THE IMPACT OF TESTING METHODOLOGY

To illustrate the potential problems associated with the use of solubilizing agents in the preparation of test solutions and with the presence of excess test substance, we selected data for the herbicide diflufenican from the European Union review process [10], with permission of the registration holder. The values of the aquatic toxicity endpoints for this substance are summarized in Table 1. The L(E)C50 values (50% lethality [or effect] concentration) were highest when there was no analysis of test concentrations (and 1% dispersant used), or when unfiltered samples were analyzed. The water solubility limit of diflufenican is approximately 50 μg/L, subject to slight variation with temperature, pH, test medium, and water hardness. The older tests (1984/1985) have reported L(E)C50 values greatly above the solubility limit, up to 105,000 μg/L, when a combination of dispersant/solvent was used. Even the newer tests (1997/1999) on fish and *Daphnia* report results above the limit of solubility: up to 109 μg/L for fish with dimethyl formamide as solvent; and in excess of 240 μg/L for *Daphnia* tested with a nonspecified solvent and reported as "in excess of visual limit of aqueous solubility [10]." These newer L(E)C-values were used in the risk assessment. Only the algal tests have values at or below the solubility limit because the substance is highly toxic to algae, as expected for an herbicide. In the consideration below, we use the term "saturation limit" rather than solubility limit to clarify that we are referring to the solubility limit under the specific test conditions and in the specific test medium, which may vary from the reported solubility limit in pure water.

The range of magnitude of the results with diflufenican may be explained by the solubilizing agent (solvent was used or not, or a solvent was used in combination with a dispersant); and the presence and treatment of undissolved test substance (either no excess material was present (testing below saturation limit), or excess material was present (testing above saturation limit) and concentrations were determined from samples containing undissolved test substance (i.e., without separation of the dissolved fraction of the test substance), or excess material was present, but the concentration of the test substance was analytically determined based solely on the dissolved fraction (i.e., recommendation of the Guidelines [1–4]).

With the exception of the final option (i.e., excess material present but analytical determination based solely on the dissolved fraction), all of the above-mentioned scenarios were used with the testing of diflufenican and directly contributed to the variability of the results in fish and *Daphnia* tests. By contrast, the algae test results were very consistent for each class of algae because the testing was conducted at or below the saturation limit; thus, the true toxicity of the dissolved substance was identified in these studies. This example illustrates how variations in testing methodology can result in completely different L(E)C-values when testing above the solubility limit. In the case of diflufenican, this is not critical because the algae endpoints drive the risk assessment; however, in other cases there is currently scope for the use of incorrect, erratic L(E)C values and misrepresenting toxicity with potentially critical consequences.

REGULATORY CONSEQUENCES OF TESTING AT SOLUBILITY LIMIT

A review of existing regulatory requirements for plant protection products raises the question of whether testing only up to the solubility limit (or in practical terms, the saturation

Table 1. Standard fish, *Daphnia,* and algae endpoints from the European Union review of the example substance, diflufenican

Species	Test type	Solvent used	Reported endpoint (μg/L)	Test year	Endpoint basis
Oncorhynchus mykiss (Rainbow trout)	96 h static	DMF, no concentration stated	LC50 109[a]	1998	Mean measured concentrations of unfiltered sample
Oncorhynchus mykiss (Rainbow trout)	96 h flow-through	1% Tween 80, 10 g DMF/L	LC50 75000	1984	No analysis of test concentrations; endpoint not used in EU risk assessment; use of solvent control not specified
Cyprinus carpio (Common carp)	96 h static	DMF, no concentration stated	LC50 >98.5[a]	1998	Mean measured concentrations of unfiltered sample
Cyprinus carpio (Common carp)	96 h flow-through	1% Tween 80, 10 g DMF/L	LC50 105000	1985	No analysis of test concentration; endpoint not used in EU risk assessment; use of solvent control not specified
Daphnia magna (Water flea)	48 h static	Solvent used, but not specified	EC50 > 240[a]	1999	Mean measured concentrations of unfiltered sample
Daphnia magna (Water flea)	48 h static	1% Tween 80 in 100 mg/L DMF	EC50 > 10000	1984	No analysis of test concentrations; endpoint not used in EU risk assessment
Scenedesmus subspicatus (Green alga)	72 h static	No solvent	E_bC50 0. 25	1997	Mean measured concentrations
Pseudokirchneriella subcapitata (Green alga)	72 h static	No solvent	E_bC50 0.27	1997	Mean measured concentrations
Microcystic aeroginosa (Blue-green alga)	72 h static	DMF, no concentration stated	E_bC50 51	1998	Mean measured concentrations
Anabaena flos-aquae (Blue-green alga)	72 h static	DMF, no concentration stated	E_bC50 51	1998	Mean measured concentrations
Navicula pelliculosa (Diatom)	72 h static	DMF, no concentration stated	E_bC50 3.5	1997	Mean measured concentrations

[a] Reported as above the visual limit of aqueous solubility.
DMF = dimethylformamide; LC50 = Concentration causing 50% lethality (mortality); EC50 = concentration causing 50% effect; E_bC50 = concentration causing 50% effect on biomass; EU = European Union.

limit under the specific test conditions) in some cases might leave registrants at a disadvantage, compared to (incorrectly) testing above the limit of solubility without expressing the result based on the concentration of the dissolved fraction. For example, a test may be listed as supplemental by the U.S. Environmental Protection Agency (U.S. EPA) [11], if the dose levels tested are less than 100 mg/L, but not high enough to produce an effect on the organisms or estimate a precise L(E)C50-value. This encourages the use of solvents and dispersants and forces the registrant to realize toxic effects above the saturation limit and more importantly, to report an L(E)C50 based on measured concentrations that include the nondissolved fraction. Contrary to this, following the U.S. EPA's own guidance for testing difficult substances [1], a test may be rejected if solubility was likely to have been a problem at the levels tested and precipitates were formed. In another national example, Australian and New Zealand Environment and Conservation Council/Agriculture and Resource Management Council of Australia and New Zealand guidelines [12] refer in general to Organisation for Economic Co-operation and Development (OECD) test guidelines for methodology, including allowing use of solvent up to 0.1 ml/L, but also reject data from tests with endpoint toxicity values well above the solubility limit (e.g., by factor > 2) in deriving endpoint toxicity lists for environmental assessment. According to the OECD Guidance Document [2], "exposure concentrations which exceed a 'reported' water solubility should not necessarily be considered invalid, but reviewed on a case-by-case basis, as it is often not possible to specify an upper exposure limit with a high degree of confidence," and further, "test treatments should be based on dissolved substance ... dissolved concentrations can be approximated by measurement following treatment to separate nondissolved material from the aqueous phase." Following these guidelines strictly would result in L(E)C50-values above the water solubility limit, or as "no toxic effects at saturation" [2]. Even if this value is not precise and is below the limit of 100 mg/L recommended as the highest test concentration, it means that the substance is not acutely toxic in water to the organisms tested.

CONSEQUENCES FOR AQUATIC RISK ASSESSMENT

What does this mean for the environmental risk assessment where exposure concentrations are calculated based on theoretical mass transport and dilution? To give an example, for a pesticide with a water solubility of 50 μg/L, if the L(E)C50-values for fish, *Daphnia,* and algae are all > 50 μg/L, and the calculated predicted environmental concentration (PEC) in nearby surface water is 120 μg/L (based on spray drift calculation, thus yielding an amount of substance entering the water irrespective of its solubility in water), then the short-term risk is normally (currently) considered unacceptable. This may lead to rejection of use, restrictions such as buffer zones, or trigger further higher-tier testing (with potential animal welfare implications). However, in this example, the calculated PEC is an artifact and unlikely to reflect any potential dissolved bioavailable concentration as it is above the solubility limit, and in reality, there were no unacceptable short-term effects at the saturation limit in the standard test species. Therefore, any indication of risk in the short-term risk assessment is misleading.

In practice, pesticide active ingredients are never applied alone but as formulations, a complex mixture of the active ingredient and other components (often including dispersants and/or solvents) designed to enhance the utilization of the

product. Usually, L(E)C50-values from short-term tests with the formulated product are available and more relevant to the risk assessment in this case. The OECD Guidance Document [2], also referred to by the European Commission Guideline [13], states that "studies on the formulated product might also be an appropriate way to deal with poorly soluble compounds especially if no effects occur at the solubility limit." If a substance exceeds its saturation limit in the environment, precipitated material will most likely become integrated with other particulate solids. Therefore, another option is to conduct tests in water-sediment systems [13]. Effects of adsorbed or precipitated substance can be evaluated by testing sediment-dwelling or bottom-feeding organisms. In either case, if additional testing with vertebrates is required, there are animal welfare issues to consider.

According to the OECD Guidance Document [2], "it is important to note that an absence of acute toxic effects at the saturation concentration cannot be used as the basis for predicting no chronic toxicity at saturation or at lower concentrations. Where chemicals are predicted to have no acute toxic effects at saturation, it is recommended to consult the regulatory agency. Some regulatory authorities may prefer to omit acute toxicity tests and proceed straight to chronic toxicity testing." The latter is also in line with the recommendation of the European Commission [13]. The European Centre for Ecotoxicology and Toxicology of Chemicals document [3] proposes the following conservative approach for an initial risk assessment: "The assessment factor usually applied to acute data should be applied to the highest measured soluble concentration (or solubility limit). If the risk assessment using the resulting value indicates an inadequate risk characterization ratio, then it may be necessary to determine the chronic toxicity of the substance." This would result in less animals being tested, compared to a conventional tiered testing approach. However, applying the usual assessment/safety factors to test endpoint values at the saturation limit might lead to very low regulatory acceptable exposure values, which are not based on any observed ecotoxicological effects [14]. Therefore, the use of assessment factors with endpoints from tests with substances that are poorly soluble in water should be considered carefully.

A similar approach is already proposed for classification and labeling. The European Centre for Ecotoxicology and Toxicology of Chemicals [3] recommends that if the substance is not toxic at the solubility limit, then it should not be classified. The OECD [15] clarifies this further by recommending that for substances not toxic at the solubility limit but with high bioaccumulation potential and failing the criteria for "readily biodegradability," a default precautionary classification should be given or a chronic test should be conducted at the solubility limit to check if there is any hazard.

However, it is notable that the European Water Framework Directive states regarding environmental quality standards (EQS): "... the EQS set up in this Annex are expressed as total concentrations in the whole water sample" [16]. The EQS are derived from aquatic ecotoxicological studies and, in general, the lower the endpoint estimates of toxicity, the lower the EQS (i.e., the lower the concentration allowed in monitored water bodies before concern is triggered). Therefore, once again, this could lead to a disadvantage in testing only up to the saturation limit with substances that are poorly soluble in water. We consider that this situation should be reevaluated because if the tests are conducted correctly below the saturation limit but the monitoring is conducted on total concentration, there is an unscientific comparison made between different measurement units. In this case, a justification may be possible to test above the saturation limit to use an equivalent exposure metric to derive the EQS, though again, this has implications for animal welfare if additional vertebrate tests are required.

SUGGESTIONS

The following suggestions are based on existing guidance [1–4], complemented with the experience of the authors and the acknowledged colleagues.

Solubility limit (saturation) under test conditions

Toxicity tests should be conducted only up to the maximum dissolved concentration under the test conditions (i.e., the saturation limit), or in case of a refined assessment for plant protection products, to the maximum environmental concentration (e.g., by testing organisms exposed to water transported from the field). The saturation concentration under test conditions of a substance that is poorly soluble in water can differ substantially from the reported water solubility (e.g., in double distilled water by OECD Guideline 105 [17]) depending upon the specific test conditions (e.g., pH, temperature, ionic strength, and chelating characteristics of the medium). Especially in marine test media, differences can be dramatic [18]. Analytical results at very low concentrations are sensitive to small perturbations of the exposure system and may vary considerably, or for extremely poorly soluble chemicals, current analytical techniques may be insufficient to experimentally determine water solubility or saturation concentrations. Differences in the saturation concentration between test designs should not necessarily be considered invalid as long as all reasonable efforts have been made to achieve the target concentration of dissolved test substance.

To determine the maximum dissolved concentration under test conditions for substances that are poorly soluble in water, or for substances of unknown physicochemical behavior in aqueous solutions, preliminary solubility trials need to be performed mimicking test conditions and the results of the experiment reported as suggested by the OECD [2]. The solubility trials should include separation steps (centrifugation or filtration) prior to analytical measurements to assure true test solutions containing dissolved concentrations.

Means of generating saturated test solutions

Solvents are the most common means to accelerate and facilitate the dispersal of the test substance into the test medium and may be essential for preparing stock solutions of hydrolytically unstable or viscous substances, although the use of a solvent does not increase solubility in the test medium per se. Other means of enhancing test substance dissolution in the test water are preferable; every effort should be made to avoid the use of a solvent or to eliminate the solvent prior to testing. For example, a stock solution of the test substance in a volatile solvent (e.g., acetone) directly added to the test vessel and evaporated to leave an evenly distributed thin layer of test substance [19] will maximize the interaction with water molecules and enhance the kinetics of dissolution. Other techniques include, for example, prolonged stirring or high-shear mixing, ultrasonication, temperature adjustment, pH adjustment, generator systems such as saturation columns, and new passive dosing techniques in which the test substance is added at excess quantity but in an inert carrier substrate (e.g., silica gel), from which it moves passively and continuously into the water according to the solubility limit [20,21]. The choice of technique

should be guided by the physicochemical properties of the test substance. With poorly soluble liquid substances, liquid-liquid saturation units can be useful for generating saturated solutions without excess undissolved or emulsified material [22]. In cases where using a solvent is unavoidable, a modified diluter system has been successful for generating solutions near the solubility limit [23]. All reasonable efforts should be made to achieve true test solutions containing dissolved concentrations of the test substance that are stable.

Concentrations based on measured dissolved substance

The results of toxicity tests ideally should be based on measured dissolved substance; otherwise the toxicity may be under- or overestimated. If precipitates occur in a stock solution at the "reported" water solubility limit, excess test substance should be separated (e.g., by centrifugation or filtration) prior to test organism exposure. However, dissolved substance may be lost via the separation technique, such as by adsorption to filters [24] or centrifuge tubes. Furthermore, when testing above the reported water solubility, it should not be assumed that a clear test medium indicates a true solution because crystals, aggregates, micelles, and so on, may not be easily detected by visual observation. In this regard, shining a laser light through the solution and observing the Tyndall effect [17,25] can be helpful. Ideally, a separation step for each test solution would help to ensure the removal of potentially undissolved material, but this is not always practicable, particularly with large volumes. Moreover, the removal of undissolved substances that form surface layers, emulsions, or micelles may be difficult. Possible modifications to the test design and appropriate reporting of the results should be discussed with the responsible authority prior to testing.

For substances with very low water solubility, direct analytical verification of the aqueous solution may not be feasible, and measurement of a stock solution prepared at a detectable concentration in solvent can be an option. However, if an experimentally determined water solubility value is not obtainable due to analytical limitations, that is, the water solubility is less than the detection limit, then there is no reference for an upper concentration in an aquatic toxicity test. In this case, a solvent stock solution is of little help since the target concentration is unknown. Water solubility calculators are available (e.g., www.vcclab.org) and can provide some guidance, but calculated solubility may differ considerably from an experimental saturation value [14]. The preferred option is the use of a saturation unit to generate a saturated solution under test conditions [22]. Without analytical verification, test results would need to be presented as a nominal value based on the percentage of a saturated solution and using the best available calculations and observations to estimate the saturation limit. If no toxicity is expected and the undissolved substance has no physical effects on the test organism, it may remain in the test vessel during the exposure, but the results must still be expressed based on the saturation limit estimated in the best available way, as above. In this latter case, in the absence of an analytical method, the presence of undissolved material could be used as evidence to demonstrate empirically that organisms were exposed to the saturation concentration. In some cases, it may be possible to separate the organisms from the undissolved test material using a physical barrier such as a mesh.

Solvents

Among the various solvents mentioned in the Guidelines, triethylene glycol and dimethyl formamide are recommended. Both solvents have a low volatility and a high ability to dissolve many organic substances, while reducing the problem of oxygen depletion commonly seen with ready biodegradable solvents like acetone, ethanol, or methanol [3]. This recommendation is based on the authors' practical experience and is supported by current guidance [3]. However, the final choice of solvent should be guided by the properties of the test substance and the toxicity of the solvent to the test organism. Toxicity values of some typical solvents are given in the European Centre for Ecotoxicology and Toxicology of Chemicals document [3] and in the review by Hutchinson et al. [26].

Solvent concentration

If a solvent must be used, the concentration must not exceed 0.1 ml/L for short-term tests or 0.02 ml/L in reproduction tests or tests with potential endocrine active compounds (solvents may impact reproduction or biomarkers of endocrine disruption at < 0.1 ml/L [26]). The concentration should be below 1/10th of the no observed effect concentration (NOEC) of the solvent depending on the test species and the length/type of toxicity test [2], although data for solvent toxicity is not available for all test species and test types. This is complicated by individual test guidelines, which provide differing limits. For example, the OECD acute fish guideline allows the use of up to 100 mg/L of a solubilizer in a test [27]. This is not the same as the 0.1 ml/L in the OECD fish early-life stage or *Daphnia* reproduction test guideline [28,29], or the OECD Guidance Document [2], as the associated mass of solvent depends on its density. Although solvent concentrations of 0.1 ml/L for acute tests and 0.02 ml/L in chronic tests represent the uppermost allowable limits, efforts should be made to keep the amount of solvent used in any test as far below these limits as possible.

Solvent control

When solvents are included in the test solutions, the concentration of solvent should be the same in all treatments, and in the solvent control. Every good test design should also include a negative (test medium) control without solvent, though the ethics of using additional animals must also be considered when testing vertebrates. The solvent control is the preferred basis to compare with treatment groups for calculating endpoint toxicity values because it is more similar to the treatment groups than the negative control. Alternatively, if there is clearly no statistically significant difference between the solvent and negative controls, both controls can be combined for greater statistical power [30]. However, it should be noted that this is in contradiction to a U.S. EPA memorandum [31], which requires the negative control to be used as the basis for comparing with treatment groups to calculate endpoint toxicity values and rejects the test if there is a significant difference between the solvent and negative control. Furthermore, a significant difference between negative control and solvent control is more common in studies with multiple endpoints than in studies with only one or two endpoints, as there is a higher chance for type II errors. In addition, a solvent control alone cannot distinguish potential synergistic or antagonistic interactions between the solvent and the test substance; thus, such tests may over- or underestimate true toxicity [32]. In any case, not incorporating solvent in the test solutions is the best way to avoid these problems.

Dispersants

The use of dispersants such as surfactants and emulsifiers should be avoided (e.g., Tween 80, Cremophor RH-40, meth-

ylcellulose 0.01%, HCO-40, recommended in some of the OECD Guidelines [33–37], whereas the general use of dispersants is allowed in others [27–29,38]). Dispersants, even if nontoxic, may have a profound effect on the physical form of the test substance in the test medium. They can interact with organic substances to increase the apparent water solubility at low concentrations. They may thereby directly or indirectly influence their bioavailability and hence, the apparent toxicity of the test substance. Also, they will lower the surface tension of aqueous media. For example, at surfactant concentrations below the critical micelle concentration, the apparent solubility of phenanthrene and pyrene were increased by a factor of three over the pure water solubility [39]. Thus, results from a test involving a dispersant may be specific for a defined substance/dispersant system, which may not be representative of the inherent toxicity of the test substance, and difficult to extrapolate to other exposure scenarios or conditions [3]. This does not apply to the testing of mixtures that may include a dispersant as a primary ingredient, such as with pesticide formulations. However, for pesticides, the toxicity of the active ingredient is normally known and can be compared to the toxicity of the formulated product.

Secondary difficult-to-test characteristics

Poorly water-soluble substances may have secondary characteristics that make them even more difficult to manage in aquatic tests (e.g., adsorption, volatility, hydrolytic instability, photodegradability, liquids, etc.). In these cases, additional modifications to standard test designs are required to properly test such substances. A complete discussion of these characteristics and potential mitigation measures is beyond the scope of the present study, but some problems with regard to adsorption are discussed below. The tendency to adsorb to available surfaces is often encountered with poorly soluble substances; at low concentrations, even a minor degree of adsorption can cause dramatic variations in analytically measured concentrations of dissolved substance. Adsorption can be exacerbated if test vessel surfaces become coated with a microbial film as a result of the use of solvents or from substances that form a substrate for such growth. Chemicals adsorbed to test containers are typically not available to test organisms, but chemicals adsorbed to food particles will be available to pelagic fish or invertebrates. In addition, when a chemical adsorbs to sediment it may be available to sediment-dwelling organisms, benthic fish, or bacteria. In many cases, adsorption can be reduced by equilibrating the test vessels with test solutions prior to exposure (i.e., conditioning) and/or by the use of flow-through systems [2]. Very strong adsorption may require testing in water-sediment systems with relevant benthic organisms or the use of a formulated product (for pesticides). In algal tests, the growing cell population provides an increasing surface area for adsorption. Algae are not amenable to flow-through testing; however, substance adsorbed to the algal biomass is still considered bioavailable and should be considered as part of the exposure concentration analyses [40]. The relevance of adsorptive loss must be considered in the context of biological exposure and analysis of a biological negative control (a test replicate without organisms) can be helpful in this regard. The testing approach for substances that are poorly soluble in water with secondary difficult-to-test characteristics should be decided on a case-by-case basis, possibly using preliminary tests of those characteristics, and, if applicable, in consensus with the appropriate regulatory authority [1–4].

Pragmatic testing approach

A pragmatic approach to establishing the toxicity of a substance that is poorly soluble in water, while considering animal welfare as well as logistic and financial constraints, would consist of some preliminary tests prior to definitive testing. A preliminary toxicity test (or range-finding test) at widely spaced concentrations should include one treatment of a saturated solution, which may also contain undissolved substance. In case dissolved concentrations are not measurable, a treatment containing undissolved substance provides some empirical evidence that saturation was maintained over the exposure period, although if toxicity is observed physical effects may be responsible. If insufficient data are available to predict solubility in the test medium, but a suitable analytical method is available to measure dissolved concentrations, the preliminary toxicity test can optionally be combined with a preliminary solubility trial including separation steps (e.g., centrifugation or filtration) prior to analytical measurements to approximate the maximum dissolved concentration (saturation limit) under test conditions.

Then, if no toxicity is observed in the preliminary test, perform the definitive test as a "limit test" with a saturated solution and base the test results on the measured dissolved fraction. In case dissolved concentrations are not measurable, conduct the test in the presence of undissolved material and state the results as no effects at the saturation limit, and estimate that saturation limit by the best available method of calculation and/or observation.

If toxicity was observed in the preliminary test, perform the definitive test using appropriate concentrations, but with any excess material removed (to exclude potential physical effects) and base the test results on the measured dissolved fraction. In case dissolved concentrations are not measurable, test a dilution series from a saturated stock, then state the test results as percentages of a saturated solution, and estimate the saturation limit by the best available method.

Risk assessment considerations

Short-term toxicity testing to the solubility limit should be given due consideration in the risk assessment. Where reported test endpoint values are above the limit of solubility and no adverse effects have been observed (e.g., LC50 or NOEC $>$ saturation limit), retesting should not be necessary, but the endpoint value should be considered as greater than the solubility limit under test conditions (the saturation limit). Further acute testing or adverse acute classification should not normally be required if there were no adverse acute effects at the saturation limit (i.e., the NOEC is equal to or above the saturation limit). For pesticides, if formulation data are available these can be used as part of the weight of evidence. Further, chronic testing for such compounds should only be required if there is a likelihood of prolonged exposure (e.g., in case of multiple applications for plant protection products, or when the DT50 in water is >2 d), if there is a potential for bioaccumulation (indicated by a $\log P_{ow} > 3$), or to substantiate the fact that the risk for substances not toxic at the saturation limit is acceptable. In the latter case, performing a chronic test only at the saturation limit (a "limit" test) should be an acceptable design and is justifiable in terms of animal welfare. Where substances are predicted to have no acute toxic effects at the saturation limit, it is recommended to consult the regulatory agency, as they may prefer to proceed straight to chronic toxicity testing. If there are no effects at the saturation limit

(or realistic water solubility limit in the field) in the tests with the three standard trophic levels (fish, invertebrate, algae), it may be suggested to test more taxonomic groups (preferably nonvertebrates) for reassurance, and lower the assessment factor to one so that the toxicity exposure ratio (TER) is acceptable (TER = 1) implying no risk at the water solubility limit.

A new assessment method worthy of consideration in the future is the general exposure threshold of no concern (ETNC) for related groups of substances [14]. A risk assessment can be performed by comparing the aquatic exposure threshold of no concern (ETNCaq) value with the aquatic exposure levels of substances that are poorly soluble in water (i.e., at most up to the saturation limit). Accordingly, the aquatic exposure levels of substances with water solubility below the ETNCaq will not exceed the ecotoxicological no-effect concentration; therefore, their risk can be assessed as being negligible.

CONCLUSIONS

Solvents have been used in tests with poorly soluble substances in a variety of ways over the years, and nondissolved (excess) material has also been dealt with in a variety of ways. This can result in variable and potentially erroneous endpoint toxicity values. Guidance is available on dealing with poorly soluble substances in regulatory aquatic ecotoxicology tests, but it is sometimes contradictory in individual test guidelines. Therefore, these test guidelines should refer to a general guidance document; preferably an updated version of the OECD guidance document [2]. In addition, the use of endpoint toxicity values from these tests in risk assessments is problematic. Hence, a clear and globally accepted guidance document covering both testing and appropriate endpoint selection for substances that are poorly soluble in water would improve aquatic testing strategy and risk assessment for plant protection products and help to reduce the number of animals tested. The present study gives suggestions, based on available regulations, guidelines, and experience, and therewith provides input for the development of consistent guidance. However, there will always be substances with a combination of characteristics making them difficult to test, requiring case-by-case approaches.

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