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Poorly water soluble organic compounds: Guidance for the interpretation of results from aquatic ecotoxicity tests and a proposal for a modified classification and risk evaluation.

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SUMMARY

Circa 30% of the new substances that have to be notified within the framework of the EC and the Dutch Chemical Substances Act for an evaluation of the potential hazards/risks have a poor water solubility (<1 mg/l). A problem is recognized in the interpretation of results of aquatic ecotoxicity tests carried out with these compounds since the current OECD and EC guidelines give only little guidance to the testing of these compounds.
This document intends to give guidance for the interpretation of results obtained from toxicity tests with poorly water soluble organic compounds. A characterization of the preparation and the composition of the test media is necessary for a proper interpretation. It is suggested to use the terms 'water-soluble fraction medium', 'water-accommodated fraction medium' and 'whole medium' for this characterization. Several factors that influence the dissolved fraction of organic compounds are discussed. Special attention is paid to the interpretation of results from tests with (effect) concentrations above the water solubility limit.
A modification of the current EC classification of compounds on the basis of environmental effects for the aquatic environment is proposed. In this proposal the classification is based on the toxicity of a compound rather than on its water solubility. Some recommendations for the ecotoxicological risk evaluation of compounds with a poor water solubility are presented.

SAMENVATTING

Ongeveer 30% van de stoffen die aangemeld worden in het kader van EEG richtlijnen en de Wet Milieugevaarlijke Stoffen zijn slecht oplosbaar in water (<1 mg/l). Het interpreteren van de resultaten van aquatische toxiciteitstesten met deze stoffen vormt een probleem aangezien in de bestaande OESO en EEG test-richtlijnen slechts beperkte aanwijzingen worden gegeven voor het uitvoeren van testen met slecht oplosbare stoffen.
In dit rapport worden richtlijnen gegeven voor het beoordelen van de resultaten van toxiciteitstesten met slecht water oplosbare stoffen. Hierbij is het noodzakelijk dat de bereiding en samenstelling van het test-medium gekarakteriseerd wordt. Hiertoe worden een aantal termen geïntroduceerd. Een aantal factoren die van invloed zijn op de opgeloste fractie van de stof worden bediscussieerd. Vooral aan de interpretatie van effecten boven de wateroplosbaarheid wordt aandacht besteed.
Tevens wordt in dit rapport een voorstel gedaan voor een aanpassing van het classificatieschema van de EEG voor effecten op aquatische organismen. Hierbij wordt als uitgangspunt de toxiciteit gehanteerd en niet, zoals in het huidige schema, de wateroplosbaarheid.
Als laatste worden enkele aanbevelingen gedaan voor het uitvoeren van een ecotoxicologische risico-evaluatie voor slecht in water oplosbare stoffen.

LIST OF DEFINITIONS

Dispersion
A disperse phase (i.e. the dissolved or suspended substance in a colloidal solution or suspension) suspended in a disperse medium (Uvarov and Isaacs, 1986).

Dispersion medium
A medium in which a substance in the colloidal state is dispersed; the solvent in a colloidal solution (Uvarov and Isaacs, 1986).

Emulsion
A colloidal solution in which the disperse phase consists of minute droplets of liquid (Uvarov and Isaacs, 1986).

Physical effects
Effects observed above the water solubility limit caused by the undissolved fraction of the compound in the test medium.

Poorly water soluble compound
Compound with a water solubility limit < 1 mg/l (EC, 1991).

Toxic effects
Effects observed related to the concentration of the solute fraction of the compound in the test medium.

Suspension
A two-phase system consisting of very small solid or liquid particles distributed in a fluid dispersion medium (Uvarov and Isaacs, 1986).

Water-soluble fractional medium
A medium consisting of only the solute fraction of a compound. Can be obtained by separating the undissolved and dissolved fraction by centrifugation or filtration (Girling, 1989).

Water-accommodated fractional medium
A medium consisting of the dissolved fraction of the compound and a stable suspension of undissolved particles or droplets without continuous energy input to keep the suspension (Girling, 1989).

Whole medium
A medium consisting of both the dissolved and undissolved fraction of the compound distributed uniformly through the test medium. Continuous energy input is necessary to keep the undissolved particles dispersed uniformly through the medium (Girling, 1989).

1. INTRODUCTION

Within the framework of the European Community (EC) Directive 79/831/EEC and the ensuing Dutch Chemical Substances Act all new substances have to be notified to the competent authorities. New substances are defined as those which were not on the market within the EC at any time within the 10 years prior to 18 september 1981 and which, therefore, do not appear in the European Inventory of Existing Chemical Substances (EINECS; EC, 1987). The EC Directive 79/831/EEC (EC, 1979) requires an evaluation of the potential hazards/risks of notified substances to man as well as the environment. Circa 30% of these new compounds have a poor water solubility (< 1 mg/l)[1]. A problem is recognized in the interpretation of results of aquatic toxicity tests carried out with these compounds since the current OECD and EC guidelines give only little guidance to the testing of these compounds.
Recently the problem has gained more attention. At present an OECD review on aquatic toxicity tests is being prepared in which the testing of poorly water soluble compounds will be addressed. In the United Kingdom the Ad Hoc Toxicity Group has formulated guidance for the analytical chemistry requirements in aquatic testing and some proposals for the testing of these compounds (Arnold et al., 1992). Whitehouse and Mallet (1992) provided guidance on the testing of 'difficult' substances like poorly water soluble compounds.

Since current guidelines give only little guidance for the testing of substances of poor water solubility expert judgement will be a considerable part of the evaluation of the results obtained from tests with these type of substances. Therefore, this document intends to give guidance for the interpretation of test results of poorly water soluble organic compounds which are to be used in the ecotoxicological risk evaluation or classification of new substances.
A meaningful interpretation of these tests can only be obtained when special attention is paid to the characterization, the preparation and description of the test medium. Therefore, several methods of preparing aqueous media are presented and factors are discussed which are known to influence the dissolved fraction of the compound. Direct information on test methods with compounds of poor water solubility is hardly available in open literature. An on-line search of the literature resulted in only a few references. However, the subject of test methods in the risk-assessment of oil products has gained more attention in recent publications. Because of the partially comparable difficulties that are being faced when dealing with these compounds some information presented here is obtained from this literature.

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[1] This figure is based on new substances notified to the Chemical Substance Bureau of the Ministry of Housing, Physical Planning and the Environment of The Netherlands in 1992.

The EC method of classifying compounds on the basis of environmental effects for the aquatic environment uses the water solubility as a starting point (EC, 1991). For compounds with a water solubility less than 1 mg/l only physico-chemical criteria like degradability, the octanol/water partition coefficient and the bioconcentration factor are used to classify their potential risk to the aquatic environment. Toxicological criteria are not used in contrary to the classification of compounds with a higher solubility. Thus, the classification of a compound depends on its solubility rather than on its toxicity. This leads to the undesirable situation that compounds which differ in solubility but are comparable in toxicity at identical exposure levels are classified differently.
In this document we propose a modification in the classification of these compounds.

In The Netherlands a hazard and risk evaluation for the aquatic environment is carried out according to the Dutch Risk Assessment System for New Substances (Toet et al., 1991 and Vermeire et al., 1992). This system uses the quotient of the PEC (predicted environmental concentration) and the NEC-eco (no effect concentration) as a basis for this evaluation. The PEC is calculated as follows:

- emissions of new substances are estimated using the expert system PECker (Van de Poel, 1991). All emissions are considered to originate from point sources, which produce a continuous load towards water, air and soil.
- all water emissions are assumed to be collected and passed through a WWTP (waste water treatment plant). Using a WWTP simulation model the concentration in the effluent is calculated (Struijs et al., 1991).
- the concentration in surface water is calculated using dilution factors for the effluent (De Greef and de Nijs, 1990).

The NEC-eco is extrapolated from single species toxicity data making use of the modified EPA method (Slooff, 1992).
Usually only data from acute toxicity tests are available. One of the problems with poorly water soluble substances occurs when no effects in these tests are observed at the limit of the water solubility. In this case an estimation of the NEC-eco is not possible. Another problem occurs when effects are detected above the water solubility, especially when they are concentration-related. The relevance of these effects with respect to the extrapolation to the real environment is unknown. Two remarks can be made about this situation. First, it is not clear whether concentrations above the water solubility limit occur in the real environment. Second, it is not clear whether these effects are caused by toxicological effects or by physical damage. In this report only the second remark is dealt with. The first remark lies outside the scope of this report.

In Chapter 2 guidance is presented for the interpretation of results obtained from aquatic ecotoxicity tests. In Chapter 3 a modification of the current classification of compounds in the EC is proposed. Recommendations for the risk evaluation of poorly water soluble substances are presented in Chapter 4.

The test methods and test results discussed in this document particularly address acute and chronic aquatic toxicity tests with algae, *Daphnia magna* and fish as test organisms, with organic compounds, according to EC directives (EC, 1989).

2 INTERPRETATION OF TEST RESULTS OBTAINED WITH POORLY WATER SOLUBLE COMPOUNDS

2.1 Physico-chemical information

Before results obtained from tests with poorly water soluble compounds (< 1 mg/l) can be interpreted, information on the following aspects is needed for a proper estimation of the stability of the compound in the solution or the condition of the test concentration during the experiment:

- boiling point
- water solubility
- vapour pressure
- octanol/water partition coefficient
- degradability
- hydrolytic stability
- critical micelle concentration (CMC, in case of e.g. detergents)

The determination of the water solubility of compounds with a solubility below 1 mg/l depends more on the method of determination or the conditions of measurement, compared to compounds with a higher water solubility. Therefore, estimates of the water solubility as result of these determinations can be variable (Kenaga and Goring, 1980).

2.2 Preparation and characterization of test media

In the case of poorly water soluble compounds, the preparation of test solutions solely by direct addition of the test compound to the water is not always possible. With the aid of solvent carriers, sonication, generator columns etc. the compound may be solubilized. However, the preparation of the test solution strongly influences the composition of the medium and consequently the results of the toxicity test. Depending on the method of preparation the solution might consist of a true solution of the compound or a solution containing also non-dissolved components besides the dissolved fraction.

Like Girling (1989, 1992), we suggest to use the terms 'water-soluble fraction (wsf)', 'water-accommodated fraction (waf)' and 'whole media (wm)' to characterize the composition of the test solution. This characterization of the preparation of the medium is of importance because it strongly determines the composition of the test medium. The dissolved fraction is the fraction responsible for the observed toxicity whereas non-dissolved particles, which might occur when a test is carried out above the solubility limit, can interfere with the condition of the test organism causing physical damage. Sometimes a concentration-related effect is observed above the water solubility limit. It is not clarified yet if this is caused by toxic or physical effects on the organism.
Recognising the composition of the solution would improve the interpretation of the test results.

Two methods of media preparation can be distinguished (according to Girling, 1989):

- Fractional media:
  Water-soluble fraction and water-accommodated fraction media are indicative of a fractional medium, an aqueous medium which contains only the fraction of the compound that can be retained after mixing and settlement. Fractional media can be prepared in two ways.
  Water-soluble fractions can be produced by mixing the compound and the water followed by settlement for some time and successively separation of the dissolved and non-dissolved fraction by filtration or centrifugation. Filtration offers the risk of the possible solution of impurities from the filter. Also, a generator column can be used. Water is passed over granular material coated with the product to obtain a fully saturated solution. Principles and methods are discussed by e.g. Billington (1988).
  A water-accommodated fraction medium consists of the aqueous phase with a dissolved fraction of the compound as well as an undissolved fraction of the compound in stable dispersion. A water-accommodated fraction medium can be obtained by high energy mixing (e.g. ultrasonication) followed by settlement for some time and some form of separation of the dissolved and undissolved phases leaving a stable dispersion of undissolved compound.

  Both these media consist of the compound in the aqueous phase in line with its water solubility and can be retained without continuous energy input.

- Whole media:
  Whole media contain a dissolved and undissolved quantity of the compound. By continuous energy input the product is kept dispersed uniformly through the aqueous medium.
  A dispersion can be obtained by e.g. shaking, stirring, blending, homogenizing or ultrasonication. If the mixing is not continued the dispersion will change into a medium with separated phases of the compound, i.e. a suspension, e.g. with coalescent droplets, or undissolved particles floating in the solution, on the surface or sinking to the bottom of the test vessel. The properties of the test medium are highly dependent upon the mixing conditions. The amount of compound incorporated in the aqueous phase depends on the mixing energy and mixing duration (Girling, 1989). The proper mixing duration to obtain an optimal incorporation can be determined by analytical measurements (Bennet, 1990). A chemically dispersed medium is obtained by using dispersants or emulsifying agents.
  Only by continuous mixing an uniform concentration of the test compound through the medium can be obtained. Obviously, continuously mixing is a potential for harm to small, fragile organisms like daphnids.
  In case of compounds that have the potential to form micelles (like detergents) the critical micelle

concentration (CMC) might indicate the possibility of the presence of micelles in the solution.
In whole media both a dissolved and an undissolved fraction of the compound are present. The presence of undissolved components of the compound is a potential risk for physical damage to the test organism or might enhance toxicity. Special attention should be paid to the description of the test medium and the method of analyses to decide whether results of the test might have been partially caused by physical fouling of the test organism.

All these approaches do not enhance the solubility in the test solutions. They only should have been used to obtain the desired test concentrations.

2.3 Aspects which influence the dissolved fraction

The true dissolved fraction of the compound in the aqueous phase is also influenced by several factors other than the method of preparation. These factors can change the amount of bioavailable compound and have an effect on the result of the toxicity test. The most important factors are:

- Dissolved Organic Material (DOM)
  It is recognized (Chiou, 1986, Kristensen, 1991, Jaffé, 1991) that the amount of organic material in the medium is directly related to the amount of true dissolved compound. An increase of e.g. humic acids enhances the apparent water solubility but decreases the bioavailable fraction of the compound. This can be significant for low soluble compounds, like e.g. organic compounds with a log Kow > 4 (Kristensen, 1991). Underestimation of toxicity of compounds with preference for organic material may occur when analyses are not accounted for DOM.
  Also, faecal particles and the presence of food in chronic toxicity tests might lower the bioavailable test concentration (Jaffé, 1991).
- Temperature
  The water solubility of the compound is directly related to the temperature. An increase of the temperature with 10 degrees can raise the solubility with a factor 2.
- Solvent usage
  Often solvents are used to derive the required test concentration. According to OECD and EC guidelines the amount of solvent should not exceed 0.1 ml per litre medium (or 100 mg/l). As a rule both a control and a solvent control must have been tested. The solvent control should contain as much solvent as the highest tested concentration.
  Herzel and Murty (1984) and Whitehouse and Mallet (1992) discussed that solvents do not enhance the solubility of the test compound in the aqueous medium. They can be used to facilitate the preparation of test concentrations close to the water solubility limit or as a dispersant to produce stable dispersions of a test compound. Therefore, test concentrations far above the water solubility limit

obtained with a solvent are likely to consist of a water-accommodated dispersion of the dissolved and undissolved fraction of the test compound rather than a true solution.

- Feeding
  In long term experiments uptake of the compound by food can add to the observed toxic effect. Compounds with a log Kow > 6 do have a preference for adsorption to organic materials and possibly effect the organism by means of oral uptake of the compound (Bruggeman, 1984).
- Sorption to glass
  When the compound shows a tendency to sorb to the surface of the vessel (e.g. strongly lipophilic substances) or when very low concentrations of the compound are tested special care should have been taken to prevent a significant loss of the compound from the solution. Silanisation of the vessel or pretreatment of the vessel with a solution of the test compound prior to testing will reduce losses to the surfaces of the vessel.

2.4 Analysis of test compound concentrations in the test medium

When results of tests are interpreted special attention should be paid to the method used for the analysis of test concentrations. Analyses are primarily performed to measure the stability of test concentration levels during the test. Occasionally, low test compound concentrations will cause greater variability in analytical results than would be expected at higher concentrations. The occurrence of problems in the analysis of low concentrations depends on the characteristics of the test compound and the analytical technique being used.
The measured amount of the compound depends on the treatment of the medium. In case of filtration or centrifugation of a whole medium the undissolved fraction is removed and the analysis only concerns the dissolved fraction of the medium. Untreated samples possibly contain non-bioavailable fractions of the compound that may be included in the analysis. If the purpose of the test intends to reflect a situation in which both toxic and physical effects might occur the inclusion of the particulate fraction in the analysis might be more relevant than the analysis without this fraction.
In case the DOM is determining the bioavailable part of the compound and analysis is performed without special treatment to remove the DOM, the analyzed amount is an overestimation of the true solute fraction of the compound in the medium and thus an underestimation of toxicity.
If stable test concentrations are hard to achieve, even with measurements like e.g. pretreated glass surface, covered test vessels, shorter periods between medium renewals, analyses should have been carried out more frequently then usual. In spite of this actual test concentrations may appear to be less than 80 % of the original test concentration. If this is not due to a low recovery of the compound in the chemical analysis the effect concentration should be calculated as the geometric mean of the nominal and actual test concentrations during the experimental period.

2.5 Test and effect concentrations above the water solubility limit

In some cases testing above the water solubility limit is necessary to obtain a saturated solution especially when the solubility of the test compound is not defined well. Only in certain cases it might be informative to use data obtained from test concentrations above the water solubility limit, especially when no effects are seen below the water solubility limit. It is proposed to draw the line at 10 times the water solubility limit. Aspects as mentioned before in this Chapter should be considered first to judge if these data are appropriate, otherwise they should be excluded from the classification or risk evaluation. In case of an homogenous suspension of the dissolved and undissolved fraction of the compound the observed effects above the water solubility might have been caused by a contribution of the undissolved particles to the apparent toxicity due to physical damage to e.g. the respiratory apparatus of invertebrates or the gills of fish. Whitehouse and Mallet (1992) presume the possibility that undissolved particles could make up the loss of compound from the solution that might be significant with low test concentrations of compounds that have a low solubility and a high log Kow. This would result in a higher body-burden of the organism and thus a higher apparent toxicity. They suppose that this is a speculative suggestion which requires further investigation to be validated.

A special problem occurs with algae toxicity tests where the number of cells is often estimated spectrophotometrically. Besides the algae cells also undissolved particles originating from the tested compound might be included in the measurements. Since there is no clear method of separating these different type of components it is preferred to use direct cell counting methods for estimating the biomass of the algae culture, to use chlorophyll-a content as a parameter or to add an additional control of the test concentration without algae. Besides, the undissolved particles might reduce the light intensity for algal photosynthesis.

3 CLASSIFICATION

In the current EC classification of compounds on the basis of environmental effects for the aquatic environment compounds with a water solubility limit less than 1 mg/l are not classified based on their intrinsic toxicity in contrary to compounds with a higher water solubility. Only physical-chemical characteristics like the degradability, the octanol-water partition coefficient and the bioconcentration factor are used as classifying criteria (see Figure 1). Thus, the toxic potentials of a low soluble compound are assessed differently from better soluble compounds, although they might cause comparable toxic effects at identical exposure levels.
It is proposed here to use also the toxicity data of compounds of low solubility in order to obtain a classification of compounds that is as much as possible based on toxicity data. Figure 2 shows the proposed modification of the current classification. In contrast to the current classification this proposal uses toxicity as a starting point. Compounds that have a toxicity > 1 mg/l and a water solubility limit < 1 mg/l are classified on physical-chemical properties and additional evidence from chronic toxicity data.

If no effects are observed to the water solubility (for compounds with a water solubility limit of < 1 mg/l as well as > 1 mg/l) the toxicity is presumed to be at least at the level of the solubility.

4 RISK EVALUATION

The ecotoxicological risk evaluation can be based on acute or chronic toxicity data. For the risk evaluation the following situations can be distinguished, if only acute data are available:

1. tested under the water solubility and effects observed,
2. tested above the water solubility and no effects observed,
3. tested to the water solubility and no effects observed.
4. tested above the water solubility and effects observed.

ad 1. In this case a NEC-eco can be extrapolated and a risk evaluation can be made.
ad 2. In this case no effects on aquatic organisms were observed at concentrations exceeding the water solubility limit. Therefore no NEC-eco can be extrapolated and no immediate adverse acute effects on aquatic organisms may be expected.
If no long-term toxicity data are available, no evaluation of long-term risks is possible.
ad 3. In this case the PEC is assumed to be <= 10 times the water solubility. No acute effects on aquatic organisms were observed at concentrations in the same order of magnitude as the PEC. Therefore, no immediate adverse effects on aquatic organisms may be expected. If no long-term toxicity data are available no evaluation of long-term risks is possible.
ad 4. In this case concentration-related effects are observed above the water solubility limit. The risk evaluation is carried out according to ad 1.

Recommendations

In ad 2 and 3 a (semi)chronic test should be carried out if the substance is not biodegradable. Test concentrations should not exceed the water solubility.

For chronic data two situations can be distinguished:
1. Effects are observed. In this case a NEC-eco can be extrapolated and a risk evaluation can be made.
2. No effects are observed. In this case no NEC-eco can be extrapolated. Therefore, it can be stated that no adverse direct effects on the aquatic environment will be expected. However, other aspects, e.g. bioaccumulation or biomagnification still have to be taken into account.
It should be noted that at present only in some cases chronic aquatic toxicity tests can be asked at the base-set level.

5 SUGGESTIONS

From fish studies it is known that sediment-sorbed compounds can add to the toxic effect in case sediment particles are used as a food source (Schrapp, 1991). This is likely to occur with compounds that have a log Kow > 6 (Jaffé, 1991). When the environmental fate of a compound suggests accumulation in the sediment, which will be often seen in case of poorly water soluble compounds, it seems more relevant to use tests with sediment fed fish or with sediment dwelling organisms like oligochaetes.
Current EC and OECD guidelines are not quite suited for 'difficult compounds' like the poorly water soluble compounds. It would be more appropriate to develop tests like those described above, for a better estimation of the risks of these compounds.

Sometimes a concentration related effect is observed above the water solubility. Yet, only speculations can be made whether these effects are caused by physical fouling of the organism or this is due to an enhanced solution of undissolved particles in the test medium because of bioaccumulation of the compound and thus a loss of the compound from the solution (see section 2.5 and Whitehouse and Mallet, 1992). For a full understanding of these effects and a proper interpretation of test results more research should be carried out. This research should comprise the screening of toxicological effects, accumulated internal concentrations as well as physical effects by means of histological investigations at concentrations above the water solubility of a compound to determine the origin of the effects as discussed above.

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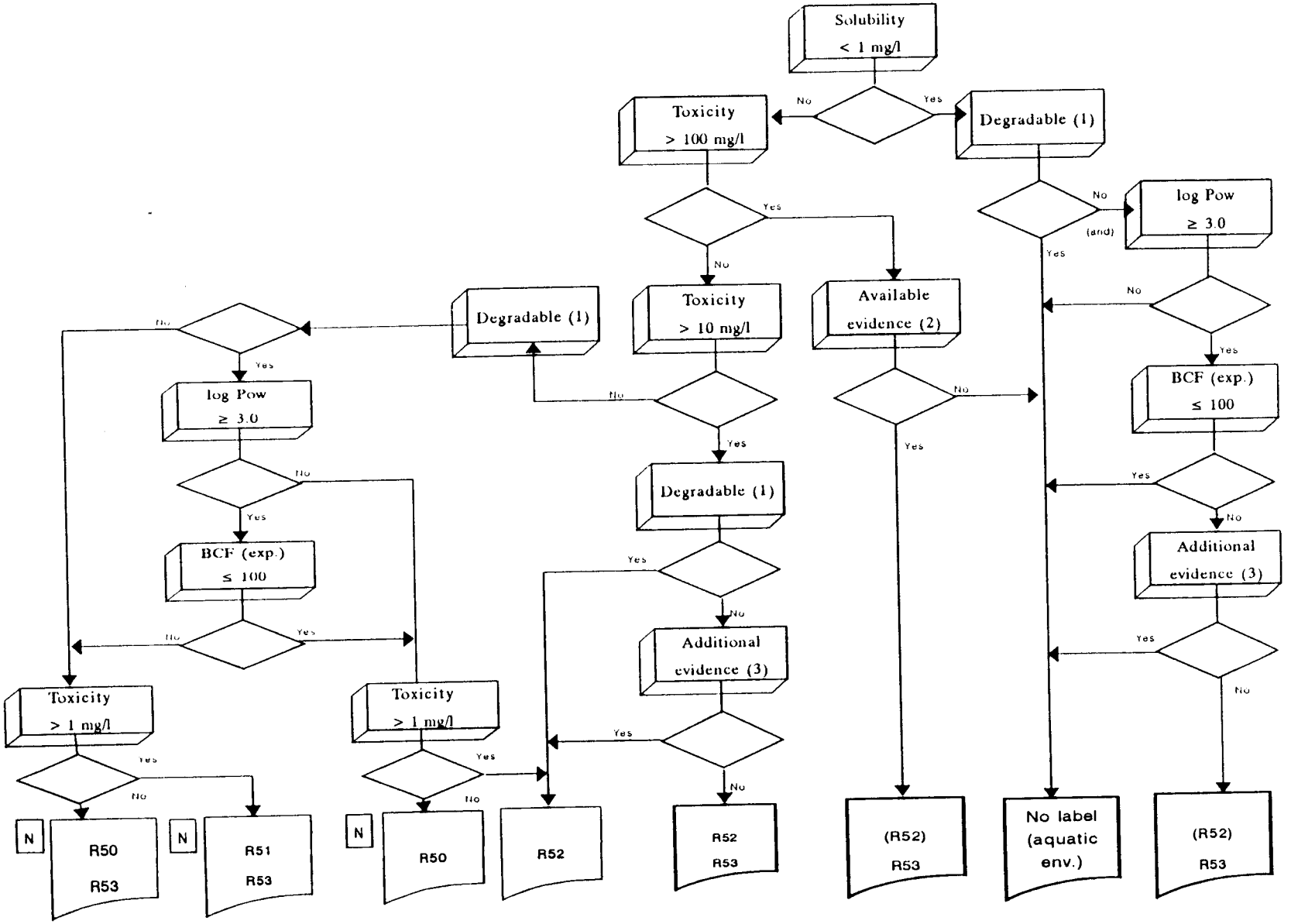

Figure 1. Classification on the basis of environmental effects (aquatic environment). Choices of risk phrases according to Annex VI (EC, 1991).

Notes Figure 1:

(1) "Substances are considered readily degradable if the following criteria hold true:

(A) If in 28 day Biodegradation studies the following levels of degradation are achieved:
- in tests based upon dissolved organic carbon : 70%
- in tests based upon oxygen depletion or carbon dioxide generation : 60% of the theoretical maxima

These levels of biodegradation must be achieved within 10 days of the start of degradation, which point is taken as the time when 10% of the substance has been degraded.

OR

(B) If in those cases where only COD and BOD data are available when the ratio BOD5/COD is greater then or equal to 0.5

OR

(C) If other convincing scientific evidence is available to demonstrate that the substance can be degraded (biotically and/or abiotically) in the aquatic environment to a level of > 70 % within a 28 day period."

(2) "Substances which on the basis of the available evidence concerning their toxicity, persistence, potential to accumulate and predicted, or observed, environmental fate and behaviour may nevertheless present a danger immediate or long-term and/or delayed to the structure and/or functioning of aquatic ecosystems."

(3) Such additional scientific evidence should normally be based on the studies required at level 1 (Annex VIII), or studies of equivalent value, and could include :

i) a proven potential to degrade rapidly in the aquatic environment;

ii) an absence of chronic toxicity effects at the solubility limit (or 1 mg/l) e.g. a no-observed effect concentration of greater than a solubility limit (or 1 mg/l) determined in a prolonged toxicity study with fish or Daphnia.

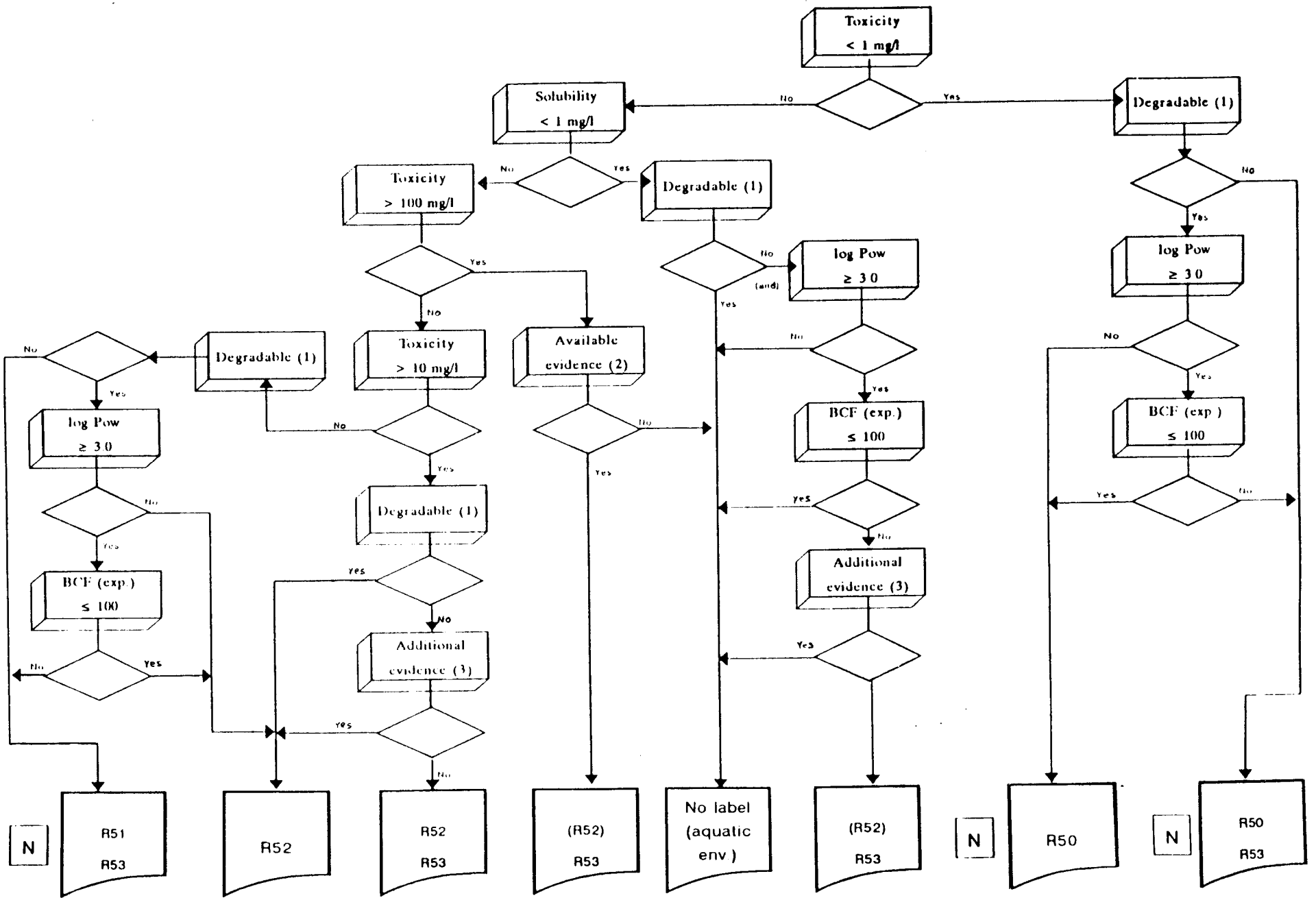

Figure 2. Proposal for a modified classification on the basis of environmental effects (aquatic environment). Notes: see Figure 1.