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Risk Assessment for Selected Xenobiotics by Bioassay Methods with Higher Plants

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ABSTRACT / Different bioassays with higher plants were approved for use in a bioassay procedure for testing of xenobiotics according to the German Chemicals Act. Selected environmental pollutants (atrazine, cadmium chloride, 2,6-dichlorobenzonitrile, pentachlorophenol, potassium dichromate, thiourea), all from a list of reference chemicals, were tested with these methods. Dose–response curves for growth of oats and turnips were evaluated in soil and vermiculite (nonsorptive substrate), and availability to plants was calculated by comparing the EC_{50} values for one chemical in both substrates. The most active chemical was atrazine, followed by 2,6-dichlorobenzonitrile, pentachlorophenol, potassium dichromate, cadmium chloride, and thiourea. The least available compound to plants was pentachlorophenol, tested with turnips (*Brassica rapa* var. *rapa*). The strongest inhibition of germination, demonstrated in an in vitro assay with garden cress (*Lepidium sativum*), was found with 2,6-dichlorobenzonitrile, the lowest with atrazine. The effect of an extended exposure of the plants to the chemicals was evaluated in a long-term bioassay with oats (*Avena sativa*) in hydroponic culture. Several dose–response curves during the growing period were derived. It was found that the EC_{50} values for atrazine and thiourea decreased markedly during the first four weeks; thereafter the changes were much smaller. As an overall conclusion, a bioassay procedure is proposed that can be included in the graduated plan recommended by the German Chemicals Act.

Environmental chemicals are substances produced or introduced into the environment by human activities [German Federal Society for the Advancement of Scientific Research (DFG 1979)] and are potentially hazardous to biota and humans. In this case, biota means all animals, plants, and microorganisms. Hazards can occur through acute or chronic exposure, synergism, or after accumulation or metabolism (Korte 1972).

In the Federal Republic of Germany, a graduated plan for hazard evaluation of chemicals is outlined in an additional decree to the Chemicals Act. Depending on the amount of the chemical that has been produced, different tests are required. If the tests indicate hazards, the next level can be advanced by the responsible agency.

At the basic level, chemical and physical data and the amount released to the environment are evaluated. These data give first indications of potential effects in the environment. Level 1 is recommended if the nationwide production of a chemical exceeds 100 tons per year or 500 tons in total since the beginning of production (Rudolph and Boje 1986). In this step a growth test with one higher plant species is recommended (Umweltbundesamt 1984, Rudolph and Boje 1986), in which a standard value (EC_{50}) is derived for the assessment.

KEY WORDS: Xenobiotics; Risk assessment; Bioassay; Higher plants

The tests recommended for level 2, which comes into force if production exceeds 1000 tons per years or 5000 tons at all, are not yet outlined. The assays described here were developed and combined for setting up these regulations and take several facts into account that were not yet covered in the guidelines in Germany (Rudolph and Boje 1986).

Pestemer and Auspurg (1986) suggested an additional estimation of a threshold concentration that damages the test plant to a small degree only. A suitable value could be damage of 5% or 10% compared with an untreated control, as already recorded according to the German Chemicals Act in bioassays with algae (Heidler 1987, Rudolph and Boje 1986, Umweltbundesamt 1984).

Another fact that should be taken into account is the availability of the chemicals to plants. Often only small percentages of the total amount of a chemical in soil are available to plants (e.g., Walker 1971, Pestemer 1983, Winteringham 1985). The aim of the work described here was to develop and evaluate different bioassay methods for use in the second level of assessment of chemicals according to the German Chemicals Act, especially on hazards to higher plants in the terrestrial compartment.

Reduction of germination and growth were tested in different assays: an in vitro test over three days with garden cress (*Lepidium sativum*) for inhibition of germination and seedling development, and a growth test

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Table 1. Chemicals tested, formulations used, and their solubility in water

Chemical	Formulation used[a]	Solubility in water (g/liter at 20°C)
Atrazine	Gesaprim 50 (50% a.i.)	0.047
Cadmium chloride	p.a. quality (Merck)	902.3
2,5-Dichlorobenzonitrile (dichlobenil)	Casoron 133 (47% a.i.)	0.021
Pentachlorophenol (PCP)	Na-PCP f. synth. (Merck)	22.4
Potassium dichromate	p.a. quality (Merck)	115.0
Thiourea	p.a. quality (Merck)	90.0

[a]a.i., active ingredient; f. synth., for synthesis; p.a., proanalysis.

with oats (*Avena sativa*) over 14 days and turnips (*Brassica rapa* var. *rapa*) over 10 days in soil and vermiculite (nonsorptive substrate). In the latter test, the availability to plants could also be determined. A long-term bioassay with oats in hydroponic culture was developed to determine the effect of an extended exposure (dose–response–time relationship). The plants were weighed regularly, so that growth data up to harvest were derived.

The assays described here were combined with other methods already recommended in the Federal Republic of Germany (Rudolph and Boje 1986) in a bioassay procedure which extends the graduated plan for testing of chemicals.

Materials and Methods

Chemicals

The chemicals tested in this study were selected from a list of reference chemicals that was proposed by the Federal Environmental Agency of the Federal Republic of Germany (Umweltbundesamt 1983) for the approval of test methods in ecotoxicology. All experiments were carried out with the formulations shown in Table 1. The concentrations (all quoted on the basis of active material) that were tested in all trials were obtained from previous experiments by Pestemer and Auspurg (1986).

Bioassay for Testing Inhibition of Germination (Dose–Response Relationship)

The influence of the chemicals on germination and seedling development of higher plants was tested using an in vitro test according to Moewus (1949) and Neururer (1975) with garden cress (*Lepidium sativum* Sperling's Mega Grossblättrige). These tests were carried out in four replicates with nine concentrations of the chemical, covering the dose–response relationship from no reaction to total inhibition of germination. Untreated controls with distilled water were included. Two sheets of filter paper, covering the bottom of a Petri dish, were moistened with 5 ml distilled water, containing the appropriate amount of the chemical. Twenty-five untreated cress seeds were placed on the paper, and the closed dishes were stored in dark at 25°C for 72 h. After that time, the radicle length of 20 randomly selected seeds per Petri dish was measured.

Bioassay for Testing Growth Inhibition (Dose–Response Relationship)

For assessment of growth inhibition oats (*Avena sativa* Fabian) and turnips (*Brassica rapa* var. *rapa* Runde Weisse Rotköpfige) were used as test plants to represent one monocotyledonous and one dicotyledonous species. Both plants have been found to be sensitive to the chemicals used in this study, fast growing, of low variation, and easy to handle (Pestemer and Auspurg 1986).

The plants were cultivated in self-watering pots with glass fiber wicks as described by Stalder and Pestemer (1980), containing 70 g (dry weight) of a sandy loam soil (pH 6.1, organic carbon 1.3%, sand 54.3%, silt 35.7%, clay 9.9%, maximum water-holding capacity 23.2 g/100 g soil) or 70 ml of a nonsorptive substrate (vermiculite, mesh size 1–3 mm). This provides the same volume of substrate and similar water content per pot in both substrates.

The chemicals were dissolved in water and mixed thoroughly into the soil, which then was stored overnight at 4°C to maintain a sorption equilibrium. The water-dissolved chemical was sucked into the vermiculite pots through the glass fiber wick of the self-watering system. In both substrates usually nine concentrations of each chemical and one untreated control were tested in four replicates.

Seven plants per pot were transplanted as 5 to 6-day-old seedlings. The greenhouse, where the pots were placed in a fully randomized design, had a temperature of 20–22°C during the day (16 h additional HQL lighting) and 15°C at night. Optimum nutrition was provided by watering the pots with a combination

of Penningsfeld-summer and Hoagland-A-Z solution or with a commercially available hydroponic fertilizer (Flory 9).

After 10 days (turnips) or 14 days (oats), the shoot fresh weight of all plants per pot was recorded.

Long-Term Bioassay for Testing Growth Reduction Influenced by Duration of Exposure (Dose–Response–Time Relationship)

To evaluate the influence of a long-term exposure at concentrations which showed slight or no effects in the bioassays for determining dose–response curves (see above), a method was developed to measure the growth of the plants over an extended period of exposure to the chemicals.

Oats were cultivated in Quickpot plates (2 cm diam., 4 × 5 pots), which were placed on 1-liter plastic boxes containing a nutrient–chemical solution (Figure 1). Six plants per box were transplanted, so that the distance between them was wide enough to avoid thinning until the end of the growth period. The solution was changed every week to maintain constant concentrations of chemicals and nutrients. The total fresh weight of all plants per pot (shoots and roots) was recorded weekly by weighing the whole system and calculating the plant weight by subtracting the weight of pots and nonplant material. The test was carried out with atrazine and thiourea at eight concentrations and six replicates over eight weeks.

Statistical Evaluation of Bioassay Data

The tests on germination and growth were evaluated by adaptation of logistic curves (nonlinear regression) to the data as described by Günther and others (1989). From these curves, all effective concentrations can be derived, e.g., EC_5, (5% growth reduction compared to the untreated control). Additionally the 95% confidence limits of each EC were derived.

For the long-term assays, a logistic curve was estimated for every week, and effective concentrations from these curves were compared.

Results and Discussion

For testing the ecological impact of a chemical, a median effective concentration (EC_{50}) is often estimated, because this is the most reliable point in a dose–response curve (Nyffeler and others 1982). In the investigations presented here the EC_{50} values and the EC_5 values for inhibition of germination and growth were calculated for all chemicals.

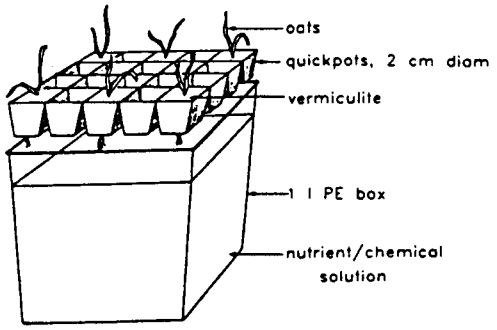

Figure 1. Bioassay pot for long-term assays.

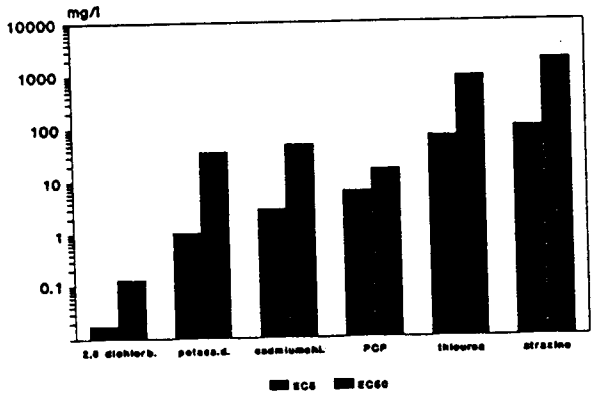

Figure 2. Effect of the chemicals tested on germination of *Lepidium sativum*.

Inhibition of Germination and Radicle Elongation

The concentrations causing 5% or 50% reduction of root length compared with the untreated control (EC_5 and EC_{50}) were calculated from the bioassay data to evaluate influence on germination and first growth stages.

As shown in Figure 2, 2,6-dichlorobenzonitrile, a typical germination inhibitor (Koopman and Daams 1960), was the most active of the chemicals in this test with an EC_5 of 0.018 mg/liter and an EC_{50} of 0.132 mg/liter. The other chemicals tested caused effects only at very high concentrations. For example atrazine, a photosynthesis inhibitor, showed effects at extremely high concentrations that exceeded the solubility in water.

The concentrations needed for inhibition of germination were not recorded, because in many tests the concentration was not high enough to reach 50% ungerminated seeds. This indicates that in many cases the reduction of radicle length is more sensitive than

Table 2. Mean environmental concentrations of tested chemicals

Chemical	Concentration (mg/kg soil)[a]	Substrate
Atrazine	0.0119	sediment
Cadmium chloride	up to 0.038 (only Cd)	sediment
2,6-Dichlorobenzonitrile	0.0045	sediment
Pentachlorophenol	up to 0.184	soil
Potassium dichromate	up to 1.0 (only Cr)	sediment
Thiourea	no results available	

[a]These concentrations were analyzed in different soils and sediments in several countries and collected by Rudolph and Boje (1986).

total inhibition of germination and therefore may be more suitable in this context.

Under normal conditions, excluding pollution by spills, the concentrations in soils and sediments are much lower than the measured values (Table 2), so that effects on germination are not likely to occur from the tested chemicals under field conditions.

Growth Inhibition

A general illustration of the growth inhibition data for the different chemicals tested is shown in Figure 3. EC_5 and EC_{50} values after 10 days (turnips) or 14 days (oats) of growth in soil are compared. The most active chemicals were atrazine (EC_{50} = 0.045, EC_5 = 0.021 mg/kg soil for turnips) and 2,6-dichlorobenzonitrile, both commercially available herbicides. The highest values were found with thiourea (EC_{50} = 339.4, EC_5 = 21.1 mg/kg soil) followed by cadmium chloride.

On average, turnip was the more sensitive test plant. Only for 2,6-dichlorobenzonitrile did oats show lower EC values than turnips. This shows that the use of only one test plant, as recommended for level 1 tests according to the German Chemicals Act, may not always give sufficient informations.

For pentachlorophenol, the EC_{50} was greater for turnips, the EC_5 was greater for oats. This indicates that the dose–response curves for the two test plants were not parallel for this chemical, which can be caused by different pathways of uptake, movement, and degradation in the plants (Sampford 1952, Streibig 1984).

Comparing the EC_5 values with the concentrations found in soils and sediments (Table 2), hazards from the tested chemicals for plant growth seem to be more probable than for germination.

The EC_{50} values for atrazine and 2,6-dichlorobenzonitrile remain considerable under the lowest con-

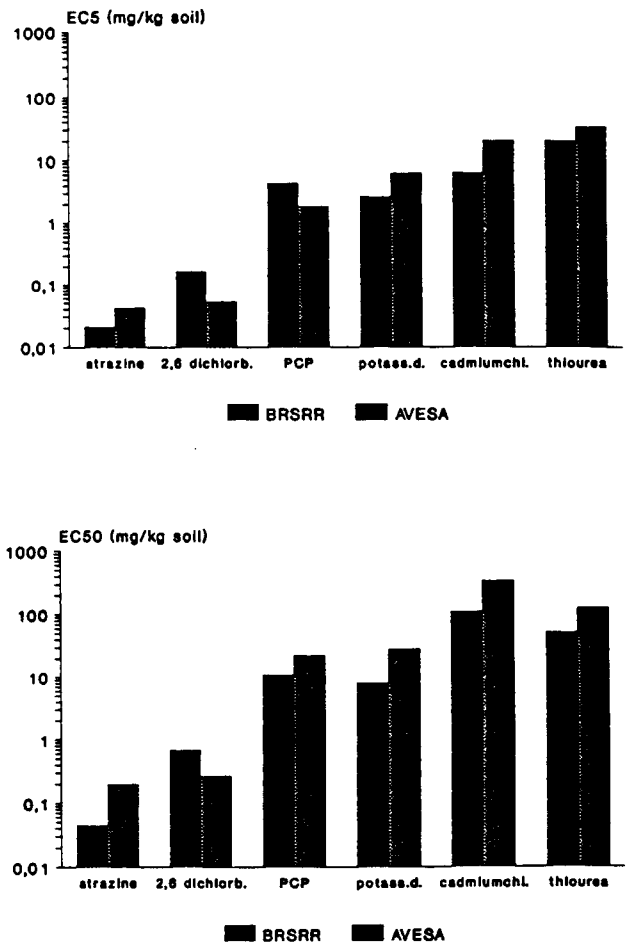

Figure 3. EC_5 and EC_{50} values for the chemicals tested in soil with *Brassica rapa* (BRSRR) and *Avena sativa* (AVESA).

centration (1 mg/kg soil), which has to be tested at level 1 according to the German Chemicals Act (Rudolph and Boje 1986). This shows that the growth test recommended there is useful only as a range-finding test, not to obtain dose–response curves. For this purpose more concentrations are necessary, adapted to the values found in a range-finding test.

Availability to Plants

In soil, it is mainly the amount of herbicide in solution that is available for the uptake by plants. It is important, therefore, to evaluate not only the total amount in soil, but also the proportion available to the plant. This was calculated by comparing the dose–response curves from soil and vermiculite for each chemical, as shown in Figure 4 for potassium dichromate. Table 3 shows the EC_{50} values and calculated

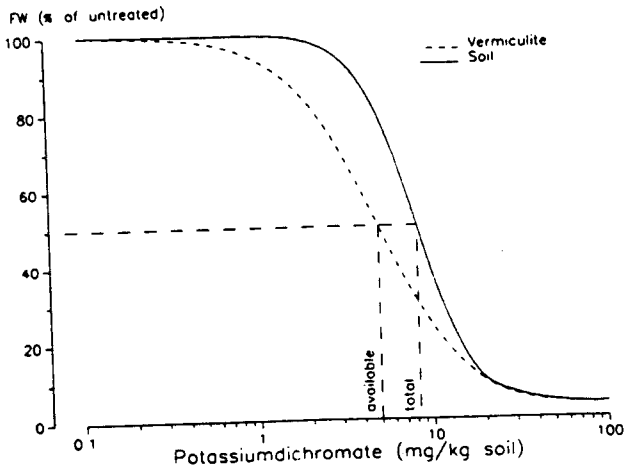

Figure 4. Calculation of availability to plants by comparison of dose–response curves from soil and vermiculite. The example shows data for potassium dichromate.

availability to plants for turnips, which are often used as the test plants for this purpose (Walker 1971, Stalder and Pestemer 1980, Pestemer 1983). Pentachlorophenol was the least available chemical (15.6%). For thiourea, availability was calculated to be about 100%. In an additional growth test with agar-agar (Pestemer, unpublished) and vermiculite no differences in phytotoxicity were found, so that adsorption of thiourea in vermiculite could be excluded. Additional evaluation of mineral nitrogen level (N_{min}) in the substrates showed a much higher degree of N_{min} in soil compared to agar-agar and vermiculite. This can signify a faster degradation of thiourea in soil, which can produce a metabolite of higher phytotoxicity. Because of the very low microbial activity in vermiculite and agar, degradation is not very probable.

The other chemicals were not tested this way because data from the literature indicated that little degradation should occur over the short time of the tests.

Heavy metals such as cadmium and chromium can only be removed from soil by leaching or uptake by plants. Pentachlorophenol is known to persist up to 12 months even in warm, moist soil (Harvey and Crafts 1952, in Bevenue and Beckman 1967) and adsorption in different soils was found to be extensive (Hilton and Yuen 1963).

2,6-Dichlorobenzonitrile is strongly adsorbed in soil (Massini 1961) and relatively persistent if incorporated (Barnsley and Rosher 1961), with a half-life of more than 50 days (Barnsley 1960).

Table 3. EC_{50} values and availability[a] to plants of tested chemicals

Chemical	EC_{50} Soil (mg/kg)	EC_{50} Vermiculite (mg/liter)	Availability (%)
Pentachlorophenol	11.32	1.76	15.7
2,6-Dichlorobenzonitrile	0.69	0.18	26.1
Potassium dichromate	8.25	4.96	60.1
Atrazine	0.045	0.031	68.9
Cadmium chloride	111.5	79.2	71.1
Thiourea	52.1	67.3	~100.0

[a]The availability was calculated by comparison of the EC_{50} values in soil and vermiculite and obtained from soil and vermiculite for *Brassica rapa*.

Influence of Time on Growth Reduction (Long-Term Bioassay)

In Figure 5 growth of the test plant oats, treated with different concentrations of thiourea, is shown over a period of eight weeks. During the first two weeks no reduction of fresh weight was observed, but leaf necrosis was seen with concentrations higher than 50 mg/liter. After the fourth week the plants treated with the two highest concentrations were dead. With these growth data, logistic curves were evaluated for each week and provided the EC_5 and EC_{50} values shown in Figure 6. The values for the second week were obtained from the short-term assay (14 days), because the adaptation of a curve failed with the data from the long-term assay.

It can be seen in Figure 6 that the EC_{50} values decreased from 170 to 30 mg/liter and the EC_5 from 22 to 12 mg/liter during the first four weeks. Thereafter the changes were much smaller. In the assay with atrazine the EC_{50} decreased from 0.18 to 0.03 mg/liter during the first four weeks and from 0.06 to 0.02 mg/liter for the EC_5.

This indicates that assays over two weeks, as recommended for level 1 according to the German Chemicals Act, may not always be sufficient to evaluate potentially hazardous concentrations of a chemical. More assays are needed to estimate the most suitable duration of long-term assays. In critical cases, growth tests over the whole growth period may be necessary to provide a full assessment, but it seems likely that this long time will not always be necessary.

Conclusions

According to the German Chemicals Act (level 1) phytotoxicity tests with higher plants are required (Rudolph and Boje 1986, Umweltbundesamt 1984). In

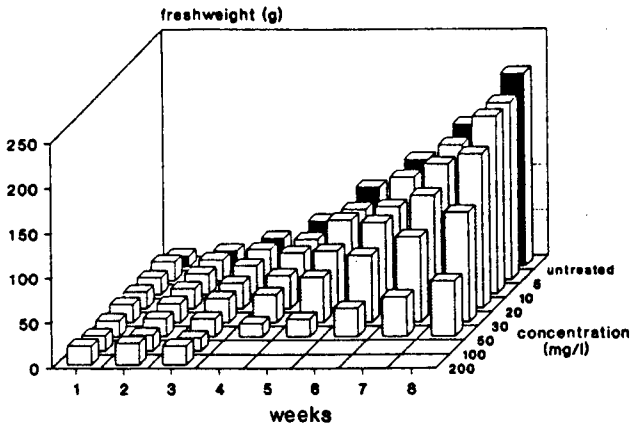

Figure 5. Growth of *Avena sativa* in a long-term assay with thiourea.

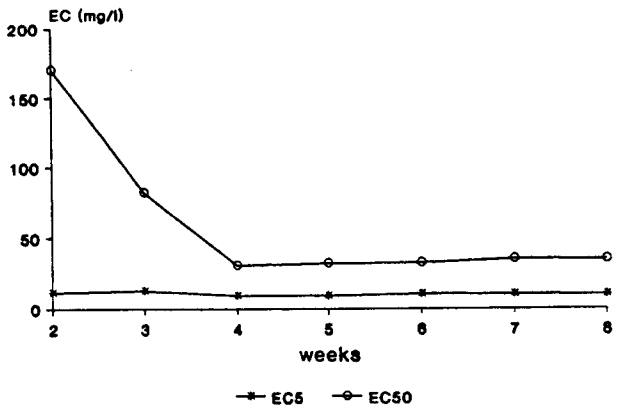

Figure 6. Development of EC_5 and EC_{50} values for thiourea during a long-term assay with *Avena sativa*.

the corresponding guideline a range-finding test is outlined from which the EC_{50} value is derived. These data give a first indication on a potential phytotoxic hazard but are not suitable for comparison of chemicals for ecotoxicological terms. For this purpose, the German Federal Society for the Advancement of Scientific Research (DFG 1979) proposed calculating the EC_{50} and the slope of the dose–response relationship. This calculation can be only an estimate, because it is not suitable to extrapolate from an effective concentration to concentrations where no effect will occur. It would be better to determine the lowest concentration where no damage can be recorded, the "no-observed-effect" level, with a specific test. Because of the small plant response and the large variation in plant material, this value can vary widely. For this reason, concentrations that damage the test organisms at a low level, e.g., EC_{10} (as used in algal assays) (Rudolph and Boje 1986) or EC_5, often are determined. In most cases there is no statistically significant difference from the untreated plants in this range because of the variation in growth of the control plants (± 10%). For the bioassays described here we suggest that the EC_5 value is the most suitable one, because the 95% confidence limits of the EC_{10} values reach up to 20% damage, while the upper confidence limit of the EC_5 usually lies in the range of the EC_{10}. If the data are interpreted this way, 10% growth reduction is the highest damage to be expected.

In the growth test according to level 1 (German Chemicals Act), the influence on germination is recorded by counting the number of emerged seeds in soil (Rudolph and Boje 1986). From these data, little information is given on the reaction of the seeds, because all plants that are not severely damaged will emerge. This gives no possibility of obtaining a dose–response relationship. For this reason, an assay that provides information specifically on germination should be included in the tests of level 2. The EC_5 value derived from the most sensitive test plant or test system should be used for comparison with the predicted concentrations in the environment.

Another important fact for risk assessment of chemicals is the availability to plants, which affects not only the activity on plants but also degradation and the potential duration of effects. Additionally, data on degradation rates and solubility in water, provided by the basic level investigations, must be taken into account.

The results of the long-term assays indicate that a test period of two weeks, as recommended for the level 1, is not sufficient to estimate the full extent of phytotoxic effects. The procedure for testing of chemicals and assessment of the results described here is summarized in Figure 7 and is based on a proposal from Rudolph and Boje (1986) for the assessment of aquatic tests in the basic and first level tests. The test method for the level 1 is already outlined in a guideline, the tests for level 2 should be determined according to the results from the basic and first levels (Umweltbundesamt 1984, Rudolph and Boje 1986). The methods for estimating the phytotoxicity in terrestrial compartments are not set yet, so the scheme proposed here may give an indication of the test procedure required in the future. The assessment of phytotoxic hazards in soil shown in this scheme should only be considered as a part of the overall concept of ecotoxicological risk assessment.

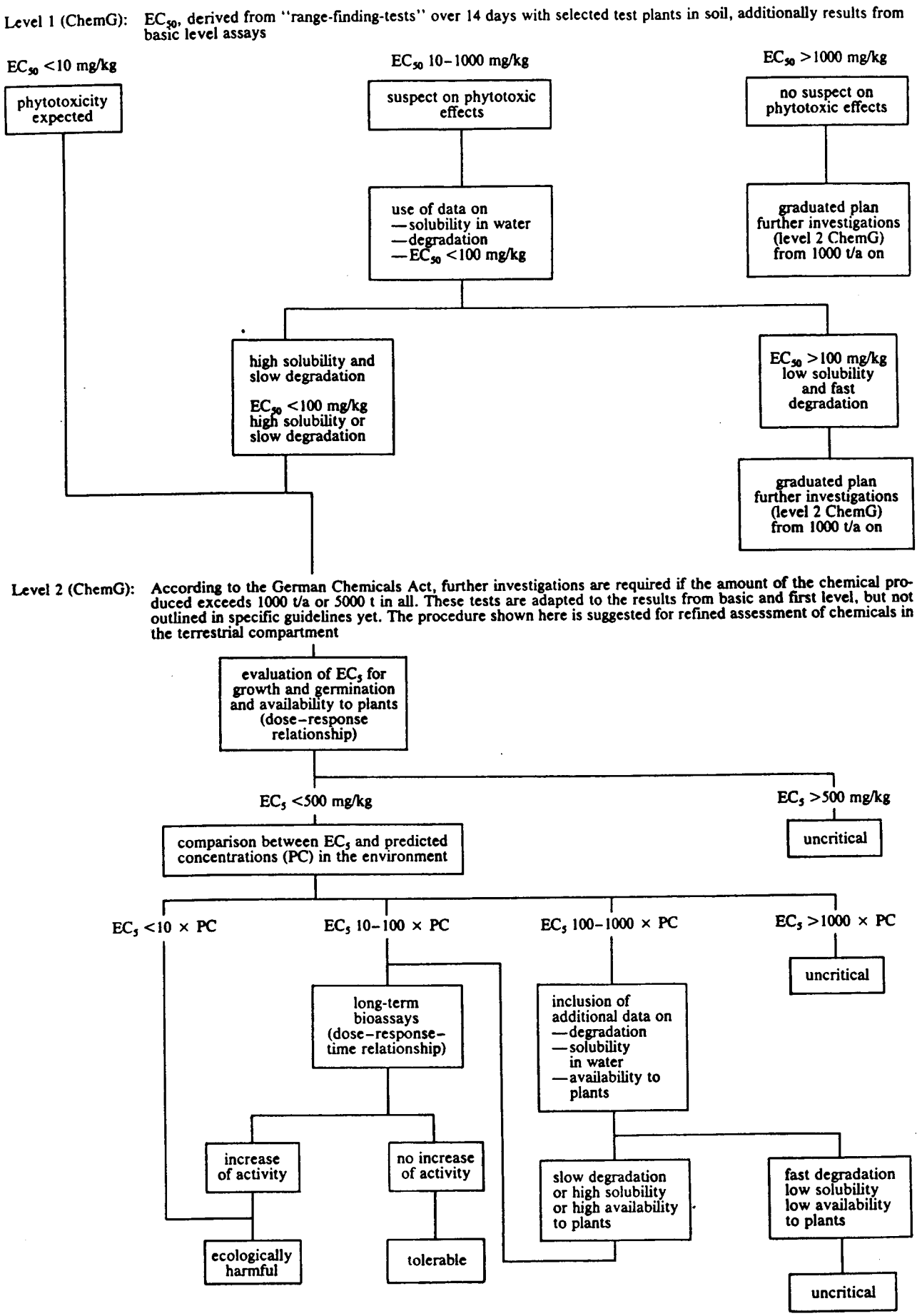

Figure 7. Proposed assessment procedure according to the German Chemicals Act (ChemG) to evaluate the risks of environmental pollutants to higher plants. t/a = tons per year.

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