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Evaluation of the Toxicity of Substances to be Assessed for Biodegradability

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EVALUATION OF THE TOXICITY OF SUBSTANCES TO BE ASSESSED FOR BIODEGRADABILITY

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European Chemical Industry
Ecology & Toxicology Centre

Avenue Louise 250, B. 63
B - 1050 Brussels, Belgium

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A. SUMMARY

The ability to distinguish between true non-biodegradability, and inhibition of biodegradability due to the toxic effects of the compound tested present problems. It results in possible false identification of substances as non-biodegradable.

The following chemicals :

cetyl trimethyl ammonium bromide,
benzyl dimethyldodecyl ammonium chloride,
o-chloroaniline,
o-chlorocresol,
chloramine-T,
4-nitrophenol,

were evaluated in a variety of tests to assess their toxicity to aerobic sludge organisms. The tests employed were : BOD5 and Closed Bottle inhibition tests, inhibition of respiration of activated sludge, growth inhibition of activated sludge, light emission from Photobacterium phosphoreum (MICROTOX test) and the repetitive die-away test (RDA). Results from these tests were compared with results obtained from a number of ready biodegradability tests using the compounds listed above at anticipated non-toxic and toxic concentrations.

No test method evaluated consistently forecast toxicity due to the chemicals tested. The MICROTOX and nitrification inhibition tests were too sensitive. A combination of sludge respiration rate and/or growth tests seem most appropriate. To avoid toxicity to the sludge, biodegradability testing should be made at 10% of the EC_{50} value. Compounds with an EC_{50} value greater than 300 mg/l are unlikely to be toxic in ready biodegradability tests. Compounds with an EC_{50} value of less than 20 mg/l may pose problems necessitating the use of the stringent closed bottle test, or the use of C^{14} labelled test materials. Compounds with an intermediate EC_{50} that is between 20-300 mg/l, need to be evaluated at a range of concentrations in biodegradability tests, or may need to be evaluated carefully to define the precise no-effect level. Procedure recommendations to distinguish between the inhibition of biodegradability and inertness of the test substance are made.

B. INTRODUCTION

In 1983 we reviewed the current status of biodegradation testing, drew attention to a number of problems associated with current test methods, and suggested areas for improvement, and where further studies were required (ECETOC[+], 1983). One such area concerned the problem of distinguishing between true non-biodegradability and inhibited biodegradability as a result of toxic effects of the substances tested. In some instances, where high concentrations of test substances are required in order to achieve analytical precision, a toxic but biodegradable substance may be falsely identified as non-biodegradable, due to its toxicity to the microorganisms employed in the test system.

Where the biodegradability of chemicals is being assessed, especially to meet the requirement of notification schemes (e.g. EEC[+], 1984-a), the test concentrations used are normally far higher than any predicted environmental concentration. Where these high concentrations are toxic to the test system then a negative result in a biodegradability test is not reliable. To avoid misleading results it is essential that biodegradability tests are selected and conducted so that non-toxic levels of test substance are used. In those cases where toxicity is suspected, it is prudent to assess the toxicity of the test substance to the test micro-organisms at or about the concentrations to be used in the biodegradability test. Where toxicity is demonstrated, the test procedure should be modified or a different test should be selected, so that non-inhibitory concentrations of the substance can be used.

A variety of microbial tests are used to assess the toxicity of chemicals, employing suppression of growth, substrate utilisation, enzyme activity, or oxygen uptake, as a measure of toxicity, but at present no firm guidance has been given on which test or tests are most appropriate for biodegradability testing. ECETOC initiated a limited study aimed at comparing a number of the most common techniques used to define the toxicity of chemicals. A range of chemicals was selected which were known to be either toxic or apparently resistant to biodegradation, and to have given variable results in biodegradation studies.

[+] See Appendix 1 for standard abbreviations.

In addition to the toxicity test assessment, a number of ready biodegradability tests were conducted so that a judgment could be made on the value of the toxicity tests in determining non-inhibitory levels of chemicals for ready biodegradability testing.

This report summarises the study findings. The meaning of the abbreviations used in this report is given in Appendix I.

C. CURRENT SITUATION

It is questionable whether the test conditions in the variety of methods available to determine toxicity truly reflect the conditions in any of the ready biodegradable test procedures. For example, the OECD sludge respiration inhibition test (OECD, 209, 1984) is performed at high bacterial concentrations and over a short time. It measures the effect on respiration and not on growth. The turbidity test (Anon, 1985) on the other hand, is performed at low bacterial concentrations and probably measures growth of the least sensitive species.

The BOD inhibition (BOD/I) and Closed Bottle inhibition tests (HMSO,1982) measure the effect of a test substance on the degradation of glucose or other appropriate substrates* but this may not reflect the effects of the test substance on those species responsible for degrading it. Test methods using single species, or specific activity (e.g. nitrification) may also have an inappropriate sensitivity for toxicity screening purposes.

Although in the AFNOR (OECD, 301A, 1981) and RDA (Blok, 1979) tests, a toxicity control experiment is included, this uses an additional substrate and a shorter period of incubation and the degree of inhibition may not represent the degree of suppression of the biodegradation potential of the inoculum. For easily degradable substrates such as glucose and acetate the initial number of active micro-organisms

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* Substrate : in the context of toxicity testing, chemical(s) added as a carbon source to the nutrient media allowing normal sustained growth of the microbial inoculum.

in the inoculum will not be critical. Even if a large proportion of the inoculum is killed, the remaining organisms will proliferate and degrade the substrate, still giving a high degradation rate with no indication of the toxic effect of the test substance added.

When a chemical is slowly degraded, the initial number of active microorganisms in the inoculum is of great influence on the biodegradation rate and will thus determine the biodegradation level obtained after 28 days. This effect has been described by Blok and Booy (1984). As a toxic effect of a test substance may effectively reduce the number of viable cells present in the inoculum, comparable effects as with lower inoculum concentrations can be expected.

In general, the toxic action of the test substrate will increase the "lag phase" of biodegradation as has been clearly demonstrated by Nyholm et al.(1984) for 4-nitrophenol. For this reason, in the current study on the effectiveness of the toxicity test procedures, the results of the biodegradation tests have been presented as biodegradation curves in addition to biodegradation values after fixed time periods. This permits a more precise identification of the toxicity characteristics of the test chemical.

D. METHODS AND MATERIALS

1. Choice of Test Chemicals.

   Based on the experience of the participants (cf. Appendix K), and published reports, chemicals were selected which were known to be :

   i) toxic to micro-organisms and
   ii) biodegradable, but giving variable results and
   iii) soluble in water at the concentrations used in the tests.

   The following were selected :

Cetyl trimethylammonium bromide	$C_6H_{33}(CH_3)_3NBr$	(CTAB)
Benzyl dimethyldodecylammonium chloride	$C_6H_5CH_2N\ (CH_2)_{11}CH_3\ (CH_3)_2Cl$	(BDMDAC)
o-Chloroaniline	$C_6H_4(NH_2)Cl$	(CA)
o-Chlorocresol (2-chloro-5-methyl phenol)	$C_6H_3CH_3(OH)Cl$	(CC)
Chloramine T	$C_7H_7ClNNaOS.3H_2O$	(CT)
4-Nitrophenol	$C_6H_4(OH)NO_2$	(NP)

The test chemicals CT, CTAB, BDMDAC and CC were distributed to all participants from one of the participating laboratories. Samples of CA and NP were obtained locally by the testing laboratories. All chemicals were of "pro analysi" specification.

2. Choice of Toxicity Test Methods

The toxicity tests used in the study by each of the participating laboratories are given in Table 1.

2.1. BOD5 - and Closed Bottle Inhibition Tests

The BOD5 inhibition test was based on a UK protocol (HMSO, 1983) and the Closed Bottle inhibition test on the standard method given in the OECD Guideline 301D, (1981). They establish toxicity values by measuring the inhibitory effect of chemicals on the oxygen uptake resulting from the degradation of a readily degradable substrate, in this case either glucose/glutamic acid (G/Gl) or a fatty alcohol ethoxylate (FA/8EO).

When a test chemical is non-toxic and biodegradable, an identical or higher oxygen uptake than the controls can be expected.

The BOD5 inhibition toxicity test will be referred to as BOD/I in the report to differentiate it from the normal BOD5 biodegradation test.

2.2. Activated Sludge - Inhibition of Respiration

The activated sludge respiration-inhibition test described in the OECD Guideline 209, (1984) measures the inhibitory effect of the test chemical on oxygen uptake of a respiring sludge while it is degrading a standard substrate at high concentrations, thus giving a high respiration rate.

During the present study one laboratory examined the effect of test chemicals on a pure nitrifying culture in which inhibition of ammonia oxidation was a measure of toxicity. These cultures are in some cases more sensitive to toxic chemicals than the normal sludges.

2.3. Growth Inhibition

This test is being examined as a possible addition to other test methods in the UK (Anon, 1985) and has been used in the USA (Alsop et al.,1980). In principle, an inoculum of sewage micro-organisms is grown in a nutrient broth, and rate of growth is measured by the increase in turbidity of the solution with time. Addition of a toxic chemical to the culture medium inhibits the cell growth, and the turbidity of the test solution is thus lower than that of the control.

2.4. MICROTOX

The MICROTOX method (Dutka and Kwan, 1981; Bulich, 1982; Slooff et al.,1983; De Zwart and Slooff, 1983) is one in which a marine luminescent bacterial species (Photobacterium phosphoreum) is cultured in the presence or absence of the test chemical, under saline conditions. The light emission of the bacteria is reduced by toxic compounds, and the light intensity is the measure of toxicity.

This test method has been widely used in the USA, Canada and France, predominantly to correlate MICROTOX inhibition with toxicity to fish or Daphnia in the hope that this quick and relatively easy method could in some cases supplement more expensive biomonitoring tests for industrial effluents.

2.5. Repetitive Die-Away Test (RDA)

The RDA test has been described by Blok (1979) and is proposed for inclusion in the EEC guidelines as a ready biodegradability test. In the present study the test was used in a similar manner to the BOD/I and Closed Bottle inhibition tests. The oxygen uptake was measured after one week incubation with the sodium acetate or FA/8EO and combinations with the test substances. Toxicity is demonstrated as the reduced oxygen uptake compared to that with the acetate or FA/8EO alone.

2.6. General Remarks

The BOD/I, Closed Bottle and Activated Sludge Inhibition tests are the most widely used toxicity tests and represent two ends of the test spectrum with low micro-organism levels in the first two tests (10^5 cells/litre) and very much higher levels in the Activated Sludge test (10^9-10^{10} cells/litre), a factor that might be anticipated to produce different results.

3. Choice of Ready Biodegradability Test Methods

With the limited time and resources available, it was not possible for all participating laboratories to assess all test chemicals and test methods. As a consequence, each laboratory selected a limited number of chemicals and tests. The studies conducted are shown in Table 2.

The tests selected were selected to cover both high and low inoculum regimes, since the effect of toxic chemicals at different inoculum levels was thought to be a major concern in both toxicity and degradability testing. Details of the tests used are as follows:

3.1. OECD 301B - Sturm Test

In this test for ready biodegradability, accepted in a modified form by the EEC (1984-a, method C5), carbon dioxide evolution is measured as a test parameter. The laboratory using this technique also measured residual DOC's at the end of the test (24 days) and compared these with the initial DOC values. The inoculum used in the test was collected from a local sewage works which treats predominantly domestic effluents. The inoculum at a concentration of 15 mg/l suspended solids (SS) was pre-aerated for eight days at 23° in the mineral solution before addition of the test substance.

3.2. OECD 301C - Modified MITI Test

This test, described in the OECD guidelines and included in modified form as an EEC method (1984-a, method C7) uses oxygen uptake as the indicator of biodegradability. The two laboratories employing this technique used "HACH" respirometers in which CO_2 is absorbed in an alkaline absorbent, and the BOD is read on a mercury manometer. The tests were conducted at a temperature of 22 ± 3°C and the sludges for preparing inoculum were obtained from domestic sewage treatment works. The inoculum was preconditioned for one week as recommended by Blok et al.(1985) and 30 mg SS of activated sludge was added per l of medium. Test chemical was added to the appropriate volume of medium plus inoculum in the bottles after the preconditioning period. The bottles were incubated for 28 days, with daily oxygen uptake measurements.

3.3. OECD 301D - Closed Bottle Test

This test, included in the EEC methods (1984-a, method C6) for ready biodegradability assessment, was used by one laboratory. The inoculum used was one drop/l of filtered effluent from sewage works treating predominantly domestic sewage. The tests were conducted at 20 ± 1°C and BOD determinations were made after 5, 15 and 28 days.

3.4. OECD 301E - Screening Test

This test used inocula derived from sewage treatment plants treating predominantly domestic sewage. There was a deviation from the OECD guideline in that each Erlenmeyer flask containing 15 mg/l SS of inoculum was shaken for 8 days in the prescribed mineral solution prior to the addition of the test substance to precondition the sludge, as recommended by Blok et al.(1985). DOC measurements were made at intervals during the 28 day study.

3.5. RDA Test-Single Addition

The RDA test was performed as described by the EEC (1984-b) in one laboratory. The following modifications were made. The sludge at 30 mg/l SS was preconditioned in the medium for 14 days without addition of substrate. Nitrification was inhibited in all tests by the addition of up to 1 mg/l of allyl thiourea. Two concentrations of test substance, about 8-9 mg ThOC and about 38-43 mg ThOC per litre medium, were tested.

E. RESULTS

1. Toxicity Values

To facilitate a comparison of the test results, they were (with the exception of the Closed Bottle test) expressed as EC_{50} values, the concentrations of test substances causing a 50% inhibition in the test. Results are given in Table 3.

Table 3 demonstrates that there was a wide variation in the toxicity test results, not only between tests but also between laboratories using the same test. The following conclusions were drawn.

1.1. Test methods

1.1.1. MICROTOX. With the exception of the nitrifying culture inhibition, MICROTOX gave the most sensitive response to toxic substances. From a knowledge of the degradability of some of the test compounds it could be concluded that this method is too sensitive to be recommended for use as an indicator of toxicity before biodegradability testing. It would grossly overestimate the toxicity of many chemicals.

1.1.2. Nitrifying culture. As expected, this test proved to be extremely sensitive to some substances (CA and CC). However, this sensitivity was not consistent with all chemicals tested. In the case of quaternary salts (BDMAC, CTAP) the test results were comparable with those of the other methods. These results are in accord with those obtained by Blok (1976 and 1981). The variable sensitivity of this test makes it unsuitable as a toxicity screen before biodegradation testing. It may however be suitable in specific situations e.g. examining the effect on nitrification in treatment works.

1.1.3. Growth inhibition. The sensitivity of this method seems to be similar to or slightly higher than that of the BOD/I and Sludge Respiration methods. There was an acceptable correlation between the results obtained by the two laboratories who used this test method.

1.1.4. BOD/I. This is a logical test to use before the BOD5 and Closed Bottle tests for biodegradation. The use of FA/8EO or G/Gl compared with the use of acetate or glucose did not show a consistent difference in results.

1.1.5. RDA test. From the limited number of results it appears that this method gives similar results as the other toxicity tests. The sensitivity is similar or slightly lower.

1.1.6. Inhibition of sludge respiration. This type of test gave reasonably consistent results and may be of value for biodegradation tests using high inoculum concentrations. Because the sludge concentrations are high compared to those in the biodegradability tests, this method may be less sensitive for some compounds (quaternaries, CT).

1.2. Compound toxicity

In view of the extreme sensitivity of the Microtox and nitrifying culture methods, the results obtained with them are excluded from the following comments on compound toxicity.

1.2.1. CTAB. This quaternary bacteriostat has been shown to be readily detoxified by adsorption onto solids (Larson and Vashon, 1983). Results obtained during this study support this view, those tests using low biomass solids (Growth Inhibition and BOD/I) giving the lowest EC_{50} values and those with high biomass solids (Sludge Respiration) giving the highest. The spread of overall results was high (factor 40) but the spread between different laboratories for one method was lower (factor 6-7). It should be noted that biodegradation of CTAB was observed at 1 and 3.2 mg/l in one BOD/I series of toxicity tests.

1.2.2. BDMDAC. A clear pattern of response was obtained: Growth Inhibition is the most sensitive test, followed by Sludge Respiration. The BOD/I test is surprisingly the least sensitive. This is probably due to the biodegradable nature of BDMDAC in the relatively long test time and the presence of an alternative carbon source. The general spread of results for BDMDAC was similar to those of the other quaternary compound CTAB (factor 13) while correlation in results between laboratories for one method is acceptable (factor 2-3). Biodegradation was noted in the BOD/I toxicity test at 1 and 3.2 mg/l (Lab. 5).

1.2.3. CA. The results for this compound vary by a factor of 13. Poor correlation was obtained for Sludge Respiration, Growth Inhibition and BOD/I toxicity tests in different laboratories. These findings are contrary to those previously published (Gerike and Fischer, 1981).

1.2.4. CC. The spread of results was relatively small (factor 4) and the methods show no consistent differences, some Growth Inhibition and BOD/I tests giving higher results, and some lower, than the Sludge Respiration test.

1.2.5. CT. The EC_{50} varied by a factor 40. As CT is a chlorine release agent, the BOD/I test results were most affected, due to the toxicity of chlorine in situations where there is little organic matter for it to react with. Growth-test results were less affected because the high organic content of the growth medium will lead, by reaction, to a reduction in the amount of active chlorine and thus in the number of bacteria killed. The high sludge concentration in the Sludge Respiration test has a similar effect. The spread of results between laboratories for one test method is acceptable (factor 2-3).

1.2.6. NP. The limited results for NP show a moderate spread (factor 3-5). The BOD/I test appeared the least responsive. Growth and Activated Sludge Respiration tests gave similar results.

2. Biodegradation Results

Table 4 and Figures 1-6 summarise the results of the biodegradability trials. The percentage biodegradation as a function of time is given, as well as the test chemical concentrations and the number of the laboratory. An estimate of the critical toxicity ranges is given in Figures 1-6, and a broad categorisation of the extent and rate of biodegradation is given in Table 4.

All chemicals selected proved to be readily biodegradable in one or all of the tests, but there appeared to be marked differences in the toxic effects of the chemicals tested. This is generally shown either by the percentage biodegradation finally achieved and/or the presence of variable lag phases, with rapid degradation once breakdown starts.

F. DISCUSSION

It is emphasised that the selection of test chemicals was based on their known toxicity and variable response under conditions of ready biodegradability testing. The present study confirms the frequently erratic nature of the results of toxicity testing. When all results are considered, it is clear that all of the chemicals were readily biodegradable under appropriate test conditions and that the use of a high and toxic level of test chemical in ready biodegradability testing will result in a false indication of non-biodegradability.

Comparison of the EC_{50} values in Table 3 and the concentration ranges of test chemicals which proved to be toxic under biodegradation testing (Figures 1-6) indicates that no single toxicity test can give EC_{50} values that consistently relate to toxicity under all biodegradation test conditions.

A more detailed comparison of results is presented in Table 5 in which the maximum concentration of test substance which gave uninhibited biodegradation in specific biodegradation tests is related to the most appropriate toxicity test result. From these limited results it is apparent that for CA and CC none of the tests adequately predicted the toxicity of these compounds, whilst for the other test chemicals, inhibition of Sludge Respiration proved most appropriate for the tests with high inoculum concentrations (15 to 30 mg SS/l). No method consistently predicted the toxic effects of the chemicals in the Closed Bottle test.

Not unexpectedly, in most cases, the EC_{50} concentration were shown to be inhibitory in the biodegradation tests. The EC_{50} value can be used to estimate a no-effect level. Where determined, the no-effect concentrations (EC_o) of the test chemicals were 1.5 to 10 times lower than the corresponding EC_{50} values (Table 6). In practice, it would thus be prudent to take a value of one tenth the EC_{50} as a probable non-inhibitory level and apply this to biodegradation testing.

If a concentration of 1/10 of the EC_{50} were employed, then an EC_{50} of greater than 300 mg/l obtained in any of the toxicity tests would indicate, with a reasonable degree of certainty, that toxicity would be unlikely to cause low results if biodegradation tests were carried out at a concentration of less than 30 mg/l. The use of a test concentration of up to 30 mg/l would also permit the use of those ready biodegradability test methods where addition of ca. 20 mg/l DOC is required to give an acceptable degree of analytical precision.