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Harmonisation of Ready Biodegradability Tests

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HARMONISATION OF READY BIODEGRADABILITY TESTS

European Chemical Industry
Ecology & Toxicology Centre

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

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A. INTRODUCTION

In a previous report (ECETOC, 1983) the applicability, limitations in use, reproducibility and significant technical weaknesses of the aquatic biodegradation test methods in the OECD guidelines (1981) and in Annex V of the 6th Amendment (EEC, 1984-a) were assessed. Recommendations were formulated for improving these tests. Subsequently a second ECETOC Task Force was set up :

i. To consider the various improvements to methods for assessing aquatic biodegradation as recommended in the recent report, and to identify those of high priority.

ii. To draw up a detailed programme of experimental work the aim of which is to enable the above high-priority improvements to be realised. It should be borne in mind that such work could be carried out jointly by industry and outside laboratories.

For abbreviations used in this report see Appendix 1.

B. BACKGROUND

According to the TF some of the problems enumerated in the former report (ECETOC, 1983) could be resolved by the harmonisation of existing protocols (especially those of ready biodegradability tests), others could be tackled by limited experimentation, while the remainder should be solved by basic scientific investigations, for example into biodegradation kinetics.

It is emphasised that the ready biodegradability tests (RBTs) are only fail-safe screens and do not "a fortiori" exclude further investigation when the result is positive. It is well known (Gerike and Fischer, 1979, 1981) that the stringency of the RBTs varies, decreasing generally in the order - Closed Bottle, MITI(I), Mod. OECD, AFNOR, Sturm test - and that this difference causes some concern. Whilst it is not the intention in this paper to strictly define all aspects of all RBT protocols, the harmonisation of certain test conditions is both rational and desirable. Proposals designed to achieve this are formulated together with recommendations for minimising the effect of nitrification which is known to cause variability in some test results.

The specific problems of testing poorly-soluble compounds, and the influence of the toxicity of the chemicals tested, are of high-priority and may be capable of

resolution in the short-term and with limited laboratory experimentation. They will be considered in subsequent reports.

More profound problems, commonly but not exclusively linked to inherent biodegradability tests, such as adaptation of the sludge, use of mixed feed, the influence of concentration and temperature, rate of introduction, and adsorption effects, will influence biodegradation kinetics. Further, the results obtained in sludge and natural-water simulation systems also depend on biodegradation kinetics, a better understanding of which will enable a more accurate prediction of the environmental fate of chemicals under a wide range of conditions. The study of biodegradation kinetics is seen as one of high priority, but is likely to be resolved only after longer-term laboratory experimentation, with close collaboration between experts already carrying out research in this field.

C. HARMONISATION OF READY BIODEGRADABILITY TESTS

1. Introduction

Several ready biodegradability test procedures have been proposed (e.g. OECD, 1981; EEC, 1984-a). As shown in our former report (ECETOC, 1983) the test conditions are neither fully optimised nor relevant for all environmental situations. The time for adaptation and biodegradation is restricted, and the pass levels are stringent. Chemicals that pass such tests are therefore believed to be so readily biodegradable that they will be easily degraded in most environmental aerobic fresh waters or in sewage treatment plants.

The choice of test procedures and conditions in these tests is necessarily a compromise between practicable and ideal procedures. Many of the differences between methods derive from historical or traditional factors,and the scientific justification for differences between test conditions is frequently unclear. Nevertheless, considerable experience with existing methods does indicate that for screening purposes they are effective. Table 1 summarises the most important test conditions for the existing methods. Five of these methods are included in the OECD guidelines and were tested in "Round Robin Tests" by a number of laboratories. The MITI (I) method was also recently accepted as a ready biodegradability test by the EEC (1984-a) (C7, in Annex V of 6th Amendment). A modified version of the MITI (I) test is under discussion and will be considered for inclusion (EEC, 1985). The ISO test can be regarded as an optimised OECD test, while the RDA test (EEC, 1984-b) was developed later to facilitate the testing of poorly-soluble

substances and to permit a more precise measurement of the biodegradation rate.

TABLE 1

Most Relevant Test Conditions for Ready Biodegradability Tests (OECD, 1981; ISO, 1983)

	AFNOR	OECD	ISO	RDA	MITI(1)	Sturm	Closed Bottle
1. Analytical Procedure	DOC*	DOC	DOC	DOC O_2	O_2	CO_2	O_2
2. Test Substance Concentration							
DOC (mg/l)	40	5-40	10-40	-	-	-	-
test substance (mg/l)	-	-	-	50	100	5-20	2-10
3. Inoculum							
effluent	x	x and/or	x and/or	-		-	x and/or
surf.waters	-	x and/or	x	-	-	-	-
soil extract mixture	-	x	-	-	-	-	x
sludge	-	-	-	x	x	-	-
sludge supernatant	-	-	-	-	-	x	-
cell density (cells/l)	$5x10^8$	-	-	-	-	10^7-10^8	10^6-10^8
suspended solids (mg/l)	-	-	30	30	30	-	-
4. Mineral composition							
Ammonia (mg N/l)	86	5.3	6.6.	4.0(1.5)	1.3	8.5	0.45
Phosphate (mg P/l)	242	11.6	116	116	29	19.3	11.6
Sodium (mg Na/l)	257	8.6	86	86	17.2	11.5	8.6
Potassium (mg K/l)	86	12.1	121	121	36.5	24	12.1
Magnesium (mg Mg/l)	5	2.2	2.2	2.2	6.6	2.2	2.2
Calcium (mg Ca/l)	14	9.9	9.9	9.9	29.7	9.9	9.9
Iron (mg Fe/l)	0.02	0.05	0.05	0.05	0.15	0.2	0.05
Trace elements	x	x	x	x	-	-	-
5. Nutrient vitamins							
yeast extract	x or	-	x or	x	-	-	-
vitamins solution	x	x	x	-	-	-	-
6. pH	7.5±0.1	7.2	7.2	7.2	7.2	-	7.2
7. Temperature °C	25±1	20-25	20-25	20±2	25±2	-	20±1

x = specified
- = not specified
* = DOC : dissolved organic carbon

Although these tests are performed according to standard protocols, the results obtained with each individual method for the same compound may, in fact, indicate a pass or fail on different occasions. Tables 2 and 3 show that the methods may be ranked in order of stringency when the results are expressed as pass or fail. The chance of a positive "biodegradability" score within a certain group of readily biodegradable chemicals varies from 10% in the Closed Bottle test to about 60% in the Sturm test. However, by harmonising certain common features of the test procedures and conditions it is expected that the range in variability between tests could be reduced and the intercomparability between laboratories improved without affecting the basic stringency of the tests as measures of ready biodegradability. To achieve this it is necessary to examine in depth the possible reasons for inconsistent results and identify those areas that can be harmonised without affecting the basic aims of the test.

The general principle of all ready biodegradability procedures is the incubation of a relatively small amount of inoculum containing a variety of aerobic microorganisms in a suitable medium (water and minerals), at neutral pH, with a concentration of dissolved oxygen above 2 mg/l and a temperature between 20 and 25°C. A limited amount of test chemical (2-100 mg/l) is added to the medium and serves as a source of carbon and energy.

Although the total number of bacteria present in an RBT inoculum may be up to 10^8 cells per litre, the number capable of degrading a specific chemical will depend on the structure of the chemical and the source of inoculum. When a readily biodegradable material is introduced, bacteria will grow rapidly and exponentially. Because the amount of test chemical is limited, the steep part of the exponential growth curve is normally restricted to a short time. The growth rate is then substrate-limited and slows down to zero. After depletion of the test chemical, cell death and mineralisation of the biomass become the determining processes at an exponentially-decreasing rate (about 30-60% per week). Where the substance is more resistant to degradation, or only a limited number of specific bacteria is present, then a "lag phase" of varying duration may occur. The length of this period may be determined by the slow generation-time of the specific bacteria present or by the period of adaptation necessary for the bacteria to develop enzyme systems which can degrade the chemical.

TABLE 2

The Percentage of Reported "Passes" in the Total Number of Test Runs(n)

1° EEC and OECD round-robin tests 1978 - 1979

Pass Levels*: >70% DOC removal at 19 days (ZW+Mod.OECD)
>60% BOD/ThOD at 14 days (MITI(I))
at 30 days (Cl.B)
>60% CO_2 removal/TOC at 14[a]+26 days;(Sturm)

Test Method / Test Subst.	Zahn Wellens (ZW)		Sturm[a]		Mod. OECD		Closed Bottle (Cl.B.)		MITI(I)	
	%	n	%	n	%	n	%	n	%	n
Aniline	100	22	90	16	97	35	75	16	35	31
D E G (1)	98	41	25	16	18	34	31	16	3	20
4 N P (2)	52	40	75	16	43	37	47	15	7	30
S A S (3)	50	24	31	16	12	33	27	15	7	29
T P B S (4)	10	40	0	16	3	37	6	17	0	39

2° OECD round-robin test 1979 - 1980

Pass levels*: > 70% DOC removal at 28 days
> 60% BOD/ThOD or CO_2/TOC

Test Method / Test Subst.	Modified Zahn Wellens		Sturm[b]		MITI II		MITI I		Mod. OECD		Closed Bottle	
	%	n	%	n	%	n	%	n	%	n	%	n
D E G (1)	100	6	60	5	88	8	34	9	45	19	29	7
4 N P (2)	100	7	80	5	56	9	38	8	47	18	29	7
T G A (5)	67	6	60	5	60	10	40	10	13	16	0	7
M P D (6)	100	5	60	5	50	8	14	7	69	16	17	6
D S T (7)	-	-	0	2	60	5	40	5	-	-	0	1
A T Q (8)	-	-	20	5	50	6	60	5	-	-	0	3
	92	24	52	27	61	46	36	44	43	69	16	31

* DOC : dissolved organic carbon
BOD : biochemical oxygen demand
ThOD : theoretical oxygen demand
TOC : total organic carbon

a With 14 day pre-adaptation
b Without pre-adaptation

(1) diethyleneglycol; (2) 4-nitrophenol; (3) sodium 4-acetylaminobenzene-sulfonate; (4) tetrapropylene benzene sulfonate; (5) thioglycollic acid; (6) 4-methyl-2,4-pentane-diol; (7) distearyl-thiodipropionate; (8) anthraquinone.

TABLE 3

Classification of the Test Methods on the Basis of Results on 29 Compounds[a]

(Gerike and Fischer, 1979, 1981)

Biodegradation Method (OECD, 1981)	% positives	% negatives
Zahn/Wellens	79	10
Coupled units	66	17
Sturm CO_2 evol.[b]	52	17
Mod. OECD	38	52
AFNOR	27	60
MITI[c]	25	75
Closed Bottle	14	76

[a] Positives > 70%, negatives < 30% elimination.

[b] With pre-adapted inoculum.

[c] With pre-acclimation of the inoculum on peptone.

The ready biodegradability tests include a non-specific analytical procedure for following the process. Soluble chemicals can be measured non-specifically by determining the Dissolved Organic Carbon (DOC). Biodegradation can also be measured indirectly by respirometric methods, e.g. the oxygen uptake in the system or the evolution of carbon dioxide. These methods, in principal, also allow the biodegradation of poorly-soluble chemicals to be measured. Respirometric methods will normally indicate less than 100% biodegradation compared to the theoretical total oxidation of the test compound because they do not take account of its consumption in the non-oxidative synthesis of new cells (commonly 30% of total test substance removal). Furthermore, the test period does not allow complete mineralisation of the biomass, which would result in more complete oxidation values. DOC measurement, however, may indicate nearly 100% elimination at a stage when about 30% of the carbon of the test substance is still present in the biomass resulting from growth. After 28 days this difference may be reduced to about 10%.

2. Possibilities for Harmonisation

There are two main causes of the variability of results obtained with the presently-accepted test protocols : those resulting from the analytical procedures chosen and those resulting from differences in biological criteria.

The various analytical procedures have to be used for practical reasons, but the biological criteria may be harmonised to some extent. However, it should be recognised that even when test procedures can be harmonised there will always remain some variability in the results due to the biological nature of the test systems. Although the analytical procedures should not directly influence the biological processes, the sensitivity of the analytical method may determine the lowest practicable test substance concentration, and the upper limit of biomass concentration in the inoculum. In particular, the lack of sensitivity of non-specific analytical methods limits the practicable concentration range to between 2 and 100 mg/l, the lower concentrations being used, when possible, to minimise toxic effects. This range, in combination with significant differences in the concentration and activity of bacteria from the different inocula, leads to widely different ratios between food (test chemical) and biomass - see theoretical calculations by Blok and Booy (1984). It has been demonstrated that this alone can lead to quite different biodegradation curves. Whilst the possibility of performing tests at a higher or lower concentration should be maintained so as to take into account purely analytical and toxicity limitations, the ratio between food and biomass from the inoculum could, however, be harmonised and would substantially eliminate this source of variation in results obtained with different protocols. In practice this leads to a proposal for two sets of test conditions for two different (high and low) test chemical concentrations. At present the analytical procedure in the Closed Bottle test is the only one sensitive enough to offer the possibility of measurement at low test substance concentrations. All the other methods have less sensitive test procedures and are of comparable sensitivity, although the MITI (I) procedure can be adapted to operate at low test substance concentrations.

Apart from the differences in analytical procedures, there are other test conditions in the various guidelines which differ unnecessarily. These could with advantage be harmonised, and a number of them are discussed in some detail below.

a) Mineral composition. From Table 1 it can be seen that in most protocols the mineral media are derived from the traditional BOD dilution water. In some cases, to adapt for higher test concentrations, these are multiplied by a particular factor. For example, the MITI (I) test has a factor 3 for Fe, Ca and Mg concentrations. The ISO and RDA tests have a factor 10 for

the phosphate buffer solution. The rationale for these differences does not seem to be scientifically based and there is every practical reason to simplify and standardise the mineral media used. For example, the function of the phosphates is primarily to buffer the pH, and levels used for this are considerably in excess of those required to promote cell growth in all of the test protocols. It is appreciated that changing the concentration of phosphate also changes the Na and K concentrations. With the exception of the AFNOR test (ratio Na/K: 3), the ratio in the other tests is about 0.7 . Concentrations and ratios of mineral constituents in synthetic fresh waters can be seen in Table 4. The Na/K ratios are quite different (3.5-12.4). The concentrations of inorganic ions in a raw sewage are listed in Table 5: the Na/K ratio is about 12.5. In Table 6 the mineral composition of an activated sludge grown on sewage from domestic origin is given: the Na/K ratio is 1.4. Although the relatively high concentration of K in the test media is very different from those given in Tables 4 to 6, the effect of this on the bacterial inoculum is expected to be low. However, as a precaution, at least some pre-acclimation of a fresh inoculum to a synthetic medium may be advisable.

TABLE 4

Mineral Concentrations (mg/litre) and Ratios in Several Synthetic Fresh Waters

	Alabaster and Abram (1965)		Freeman (1953)	Frear and Boyd (1967)	EPA (1975)	TNO (1980)
Na	11.3	113	17	54.7	13	27.4
K	-	-	3.4	11.7	1	7.8
Ca	5.9	59	28.5	81.6	6.9	54.4
Mg	1.5	15	7.0	-	6.1	17.5
Na/K			5	4.7	12.4	3.5
Ca/Mg	3.9	3.9	4.1		1.1	3.1

TABLE 5

Typical Composition of Ions in Domestic Sewage as % of Ash Content (McKinney,1962)

Na^+	25	Fe^{+++}	<1
K^+	2	HCO_3^-	40
NH_4^+	4	SO_4^{--}	10
Ca^{2+}	5	Cl	10
Mg^{2+}	1	PO_4^{---}	1

TABLE 6

Typical Composition of Activated Sludge Elements as % of Protoplasm Dry Mass (McKinney,1962)

C	49	Na	0.7
H	6	K	0.5
O	27	Ca	0.7
N	1.1	Mg	0.5
P	2.5	Fe	0.1
S	0.7		

Total hardness (concentration of Ca and Mg ions), and the ratio between Ca and Mg, are in general equal in the different protocols. Only in the MITI (I) test is there an increased concentration of these ions. The ratio and the levels are not too different from those in synthetic fresh waters.

b) Ammonium (NH_4^+) nitrogen. The most striking differences between the various protocols are seen in the concentrations of ammonium nitrogen which vary from 0.45 mg N/l in the Closed Bottle test to 86 mg N/l in the AFNOR test. Ammonium ion is added to the test media as a means of supplying essential nitrogen for biomass growth but the higher levels in the above range may be unnecessarily high for the following reasons.

i) Toxicity of nitrite. During the test, ammonium ion may be oxidised via nitrite to nitrate. For most species of bacteria a level of 10 mg NO_2^-/l, which is 1/10 of the EC_{50} of nitrite to bacteria at pH=6 (Blok, 1985), seems to be safe. In all methods, except the MITI (I) and Closed Bottle tests, there may be a risk that nitrite inhibits growth during the incubation. It has been demonstrated (EEC, 1985)

that temporary nitrite levels of up to 15 mg/l may occur after several weeks before the nitrite is further oxidised to the non-toxic nitrate.

ii) Toxicity of ammonia (NH_3). This is well known for higher organisms, but ammonia at the present test levels does not inhibit bacteria. Ammonium ions at the concentrations used in the tests are not known to be inhibitive.

iii) Ammonium ion as a nitrogen source for bacterial growth. During the biodegradation, 30-40% of the mass of the test chemical removed is converted into new cells. If about 5-10% of the biomass consists of nitrogen, the highest amount of ammonium nitrogen necessary for biodegradation of 100 mg/l of test chemical is 1.5 to 4 mg/l. Most tests, except the original MITI method, specify a concentration of ammonium which is unnecessarily high for the above purpose. In the RDA test the ammonium concentration is based on repeated addition of the test chemical (4x50 mg/l). With one initial addition of the test chemical (100 mg/l), the ammonium N added should be 4 mg/l, in line with the general principle of all other methods.

iv) Ammonium ion oxidation responsible for increased O_2 uptake. Respirometric methods with oxygen uptake as the analytical procedure may be influenced significantly by an extra oxygen uptake as a result of ammonium oxidation (nitrification). To date no completely satisfactory solution to this problem has been found. Nitrification inhibitors (e.g. allylthiourea) can be used for the 5-day BOD test but this does not guarantee the absence of nitrification over 28 days. Where the occurrence of nitrification is suspected either from the addition of nutrient ammonium ion or from the presence of nitrogen-containing test chemicals, it may be possible to quantitatively determine the contribution made to the total oxygen uptake by analysing the test solutions for ammonium nitrite and nitrate by colourimetric means. Such methods are not at present commonly practiced and their reliability and accuracy may require validation.

An elegant solution to the problem would be the substitution of ammonium by nitrate as a nitrogen source. However, preliminary investigations (Gerike, 1984) in this direction have not been encouraging, perhaps because the nitrate was also utilised as an oxygen source. Further experimentation is, however, necessary.

In view of the above comments, a first step in controlling the nitrification problem would be to limit nitrogen in the nutrient solution to the lowest amount necessary to supply the needs of cell growth. Clearly, evaluation of the biodegradability of compounds with a high N content may still require an analytical balance of ammonium, nitrite and nitrate, to distinguish the oxidation of carbon from nitrification.

c) Trace nutrients. The methods are very inconsistent in this respect, the addition of nutrients being based on general microbiological experience. When activated sludge at 30 mg suspended solids per litre is used as inoculum the necessary nutrients are supplied by the sludge itself. The Closed Bottle test and the Sturm test may perhaps suffer from a trace nutrient shortage. Variations in trace nutrient content may give rise to a greater than normal variability in results, even within the same method, when lower concentrations of inoculum are used.

d) pH and temperature. Most test media are initially at pH 7-7.5 In poorly-buffered media there is a serious risk of pH variation during the test period. Closed systems, as in the MITI (I), Sturm and RDA tests, are less likely to develop a balanced carbonate buffer and are therefore more at risk. In the MITI (I) and Sturm tests this may lead to a more alkaline medium whereas an increase in acidity is more likely in the Closed Bottle and RDA tests. Harmonisation by increasing the phosphate buffer level will certainly contribute to a better comparability of the test results.

Different temperatures of incubation are prescribed in the various test protocols. The values, as well as the ranges, are different (20° ± 1°C; 25° ± 1°C, or 20° ± 2.5°C). In the extreme case a difference of 7 degrees could theoretically cause differences in the growth curves, and a difference in lag time of one week may result. The effect of temperature on the bacteria in inocula taken under natural winter conditions and

transferred to the laboratory where the temperature could be, e.g. 15°C higher, should also be considered. Pre-acclimation of the inocula to the higher temperature and a more harmonised restriction of the temperature range is therefore advisable to obtain results of better comparability. Standardisation to 22 ± 2°C is therefore suggested.

Because of the dependence of dissolved oxygen saturation on temperature, tests in which the dissolved oxygen is measured, or pressure- dependent respirometric tests, may require a more critical temperature control than that based on the biological requirements discussed above.

e) Test substance concentration. Most methods except the Closed Bottle test prescribe concentrations of between 5-40 mg DOC/l corresponding to about 12-100 mg of test substance/l. As outlined above, variations within this range, combined with variations in inoculum concentrations, may influence the shape of the growth curve. For this reason a narrower range of test substance concentration is advisable. For those chemicals expected to have inhibitory effects, lower concentrations, as in the Closed Bottle test, are advisable.

For harmonisation, the possibility of using two test concentrations (low and high) combined with a constant food-to-biomass ratio should be considered.

f) Inoculum.

i) Origin. The definition of the source and composition are not uniform in the present test protocols: soil extracts, river waters, secondary sludge or effluents from sewage treatment plants may be used. Although the use of inoculum from such different sources may favour the environmental relevance of the tests it is certainly unfavourable for harmonisation. Variety in the mixture of bacterial species seems to be the principle criterion for the above range of choices, but there is no easy way to measure or control this. The preparation of inocula from bacterial cultures with a simple synthetic feed, as performed in the original MITI test, is in contradiction to this criterion. On the other hand, it is not justified to choose an inoculum derived from a source with specific adaptation to the xenobiotic to be tested (e.g. sludge from an

industrial sewage plant already exposed to the chemical or a related chemical).

There is some evidence from comparative tests (NNI,1982) that secondary sludges from sewage treatment plants offer the most suitable source of a diverse and active inoculum. These may be preferable for several other reasons :

- Sewage treatment plants are widely distributed throughout the community and thus offer a ready source of inocula.
- Although sewage treatment plants differ in several aspects there are many general features that make sludges from them more comparable than those from other sources, i.e. the use of domestic waste, the mean growth rate in the system or the sludge age, floc biocoenosis, adaptation to concentrations of chemicals which are nearer to those in the tests, pH, temperature, the aerobic character, the hardness and the ammonium ion concentration.
- The concentrations of living organisms per gram of solids lie within a factor of 5 for most secondary sludges.
- As a sludge contains all necessary trace nutrients, results become less dependent on differences in this respect.

A disadvantage in using activated sludge as inoculum is its poorer microbial variety compared to that in certain rivers or soils.

ii) Quantity. Although the preparation of a specific inoculum is sometimes well defined within narrow margins, the differences between the final cell concentration in the test media may be large. The description of optional alternatives within one protocol makes this situation even worse. A total cell count by agar plating techniques may give some indications of concentration but it is a laborious procedure and it is known that only a low percentage of the species will grow on agar media. Although subject to some limitation, the easiest way to define the inoculum concentration is by defining the amount of total suspended solids of a secondary sewage sludge.