OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Zahn-Wellens/EMPA[1] Test

INTRODUCTION

1. The original Zahn-Wellens test (1) was adopted in 1981 as OECD Guideline 302 B for determining inherent biodegradability. Later proposals were made by Switzerland and Germany to modify this guideline by merging it with elements contained in a test developed by EMPA (2), hence the change in name of the test. The merged version of the test was further changed in respect to the mineral medium used. The medium retained is identical with that which is used in the DOC Die-Away, CO_2 Evolution, Mamometric Respirometry and Modified OECD Screening methods of Guideline 301 (adopted 1992) for determining ready biodegradability.

PRINCIPLE OF THE TEST

2. A mixture containing the test substance, mineral nutrients and a relatively large amount of activated sludge in aqueous medium is agitated and aerated at 20-25°C in the dark or in diffuse light for up to 28 days. Blank controls, containing activated sludge and mineral nutrients but no test substance, are run in parallel. The biodegradation process is monitored by determination of DOC (or COD[2]) in filtered samples taken at daily or other time intervals. The ratio of eliminated DOC (or COD), corrected for the blank, after each time interval, to the initial DOC value is expressed as the percentage biodegradation at the sampling time. The percentage biodegradation is plotted against time to give the biodegradation curve.

3. Specific analysis of the test substance may be useful in cases where molecular changes, caused by biochemical reactions (primary biodegradation) are to be detected.

INFORMATION ON THE TEST SUBSTANCE

4. It is necessary to know the water solubility and vapour pressure of the test substance and it is also advisable to know its foaming properties. The chemical structure should be known if the measured values of DOC or COD are to be checked. Information on the toxicity of the test substance to bacteria is useful for selecting appropriate test concentrations and in interpreting results showing poor biodegradability (3). The test is usually performed only after failure to pass a test for ready biodegradability. Thus, the physical and inhibitory properties may have already been ascertained.

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(1) EMPA: Swiss Federal Laboratories for Materials Testing and Research.

(2) DOC: Dissolved Organic Carbon
COD: Chemical Oxygen Demand

APPLICABILITY OF THE METHOD

5. Chemicals which are non-volatile and are soluble in water to at least 50 mg DOC/l may be assessed by this method, provided also that they do not significantly adsorb, are not lost by foaming and do not inhibit bacteria at the concentration tested.

SENSITIVITY

6. The limits of sensitivity are given by the sensitivity of the DOC determination (normally 0.5-1 mg C/l) or the COD determination (15 mg O_2/l) and also by the variability of the blank. The relatively high concentration of test substance (50-400 mg DOC/l) gives the advantage of greater analytical reliability.

REFERENCE COMPOUNDS

7. In order to check the functional capability of the activated sludge, a test using a reference compound of known biodegradability should be run in parallel with each series. For this purpose, ethylene glycol, diethylene glycol, lauryl sulfonate and aniline are recommended. Biodegradation of these compounds must reach at least 70% (DOC or COD) within 14d.

REPRODUCIBILITY

8. The test has been shown to have good reproducibility in ring tests.

DESCRIPTION OF THE METHOD

Apparatus

9. (a) Cylindrical glass vessels with a volume of 1-5 litre, each equipped with a stirrer of inert material rotating about 5 to 10 cm above the bottom of the vessel (a magnetic stirrer with a 7-10 cm long rod can also be used) and a glass tube of 2-4 mm inner diameter to introduce air at about 1 cm above the bottom of the vessel, or vessels of the same size equipped with a glass frit at the bottom, permitting aeration and agitation.

(b) A supply of compressed air passed through a cotton wool strainer and a wash-bottle containing water, or from an aeration pump delivering air free from dust, oil and organic impurities.

(c) Normal laboratory equipment, especially a centrifuge (capable of at least 1000 g), pH meter, dissolved oxygen measuring apparatus and membrane filters (pore size 0.2-0.45 µm).

(d) Analytical equipment for determining DOC (4) or COD (5).

Reagents

10. Use analytical grade reagents throughout.

Water

11. Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It should contain only minimal amounts of organic carbon so that high blank values are eliminated. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed materials from bacteria and algae. For each test series use only one batch of water, previously checked by DOC analysis.

Stock solutions for mineral medium

12. Prepare the following stock solutions:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 . . . . . 8.5 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 . . . . . 21.75 g
Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4.2H_20$ . . . . 33.4 g
Ammonium chloride, NH_4Cl . . . . . 0.5 g

Dissolve in water and make up to 1 litre.
The pH of the solution should be 7.4.

(b) Calcium chloride, anhydrous, $CaCl_2$ . . . . . 27.5 g
or Calcium chloride dihydrate, $CaCl_2.2H_20$ . . . . . 36.4 g

Dissolve in water and make up to 1 litre.

(c) Magnesium sulphate heptahydrate, $MgS0_4.7H_20$ . . . . . 22.5 g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeCl_3.6H_2O$ . . . . . 0.25 g

Dissolve in water and make up to 1 litre.

NOTE. In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HC1 or add 0.4 g ethylenediaminetetra-acetic acid (EDTA, disodium salt) per litre.

If a precipitate forms in a stock solution, replace with a freshly made solution.

Preparation of mineral medium

13. Mix 10 ml of solution (a) with 800 ml water, add 1 ml of solutions (b), (c) and (d) and make up to 1 litre.

Inoculum

14. Collect a fresh sample of activated sludge from a sewage treatment works (BOD_5 of effluent should be < 25 mg/l) and wash twice with mineral medium or tap water. Separate the sludge by centrifuging for 3-5 minutes at about 1000 g or by settlement. In special cases, to get as many different species and strains as possible, mix samples from different sources (e.g. other treatment plants, soil extracts, river water, etc) and treat the mixture as above. Use the sludge within 6 h of sampling, otherwise disperse it in mineral medium and aerate until required. Check the activity of the sludge with the procedural control using a reference compound, as described later (paragraph 18).

Preparation of vessels

15. Before starting the test, make certain with appropriate methods that no inhibition of sludge occurs at the chosen concentration of test substance if this is not already known (6)(7). If an inhibitory effect is found, reduce the concentration of test substance to a level which is unlikely to be inhibitory.

16. To an appropriate number of test vessels introduce 500 ml mineral medium and the appropriate amounts of test substance and inoculum to reach respectively between 50 and 400 mg DOC/l (between 100 and 1000 mg COD/l) and 0.2-1.0 g dry matter/l in the final volume. Ensure that the ratio between inoculum and test compound (as DOC) lies between 2.5:1 and 4:1. Make up to the required volume with mineral medium. The final volume, between 1 and 5 litres, depends on the number of samples to be taken for DOC or COD determinations and the volumes necessary for the analytical procedures; normally a volume of 2 litres is satisfactory.

17. Set up one or two blank vessels in parallel to contain only activated sludge and mineral medium with volumes identical to those of the test suspensions.

18. Also, set up one vessel in parallel with each test series as a procedural control, using one of the reference compounds in place of the test substance. If information on abiotic degradation is required, a sterile uninoculated solution of the test chemical can be prepared.

Number of vessels

19. The following vessels are used in a typical run:

- 1 or 2 containing test substance and inoculum (test suspension);
- 1 or 2 containing inoculum alone (inoculum blank); and
- 1 containing reference compound and inoculum (procedure control).

It is mandatory to follow DOC in the test suspension and inoculum blanks in parallel. It is advisable to follow DOC in the other vessel in parallel as well but this may not always be possible.

PROCEDURE

20. For practical reasons, do not start the test immediately before a week-end. Run the test, normally for up to 28d, in the dark or in diffuse light at 20-25°C. Aerate the suspensions with purified, humidified air and, if necessary, stir to ensure that sludge does not settle and that the concentration of dissolved oxygen does not fall below 1 mg/l. Check the pH value at regular intervals (e.g. on each day of sampling) and adjust to pH 6.5-8.0 with NaOH (40 g/l) or H_2S0_4 (50 g/l) if necessary.

Sampling

21. Follow the biodegradation of the test substance by determining the DOC or COD in samples of suspension taken:

- 3h $\pm$ 30 mins after addition of the test substance in order to estimate any adsorption of it by the activated sludge (see example in Figure 1);
- on at least 4 occasions in the interval between the 1st and 27th day;
- on the 27th and 28th days, or, if the plateau is attained in less than 28d, on the last two days of the test run.

The volume of sample taken depends on the type of carbon analyser. Additional sampling may be necessary in order to describe the reaching of the plateau or if adaptation is to be followed.

22. Immediately prior to each sampling, replace losses due to evaporation.

Adaptation

23. If adaptation (see curve 1, Figure 2) is to be followed, carry out analyses for DOC or COD at relatively short intervals (e.g. daily). Prolong the test beyond 28d if adaptation occurs in the final days of the test period.

24. If more detailed knowledge of the behaviour of the adapted sludge is needed, re-expose the same activated sludge to the test substance. To do this, stop aeration and agitation and allow the sludge to settle. Draw off the supernatant liquid, re-fill the vessel to the original volume with mineral medium, stir for 15 minutes and repeat this operation once more. Alternatively, isolate the sludge by centrifuging (paragraph 14). Repeat the test using the recovered sludge, which may be augmented with fresh sludge if insufficient recovered sludge is available to yield 0.2-1 g dry matter/l.

Analytical methods

25. Filter the samples of sludge suspensions (test, blank and procedure control) as soon as they are taken, discarding the first 5 ml of filtrate. Use either carefully washed paper filters or membrane filters, which are suitable if they neither release nor adsorb organic compounds. Otherwise wash the membranes three times in deionised or distilled water at about 60°C, and store them in water. Separate sludges which are difficult to filter by centrifugation or by other suitable separation techniques.

26. Determine the DOC or COD in duplicate in the filtered or centrifuged samples by any suitable methods e.g. (4) (5). If primary biodegradation is to be followed, use specific analyses, e.g. UV spectroscopy, in addition to DOC or COD. If the filtrates cannot be analysed on the day of sampling, store at 2-4°C for a maximum of 48h, or at -18°C for longer periods. Storage for long periods however is not recommended.

DATA AND REPORTING

Treatment of Results

27. Calculate the percentage degradation at time t from

$$D_t = \left[1 - \frac{C_t - C_B}{C_A - C_{BA}}\right] \times 100$$

where:

D_t = percentage degradation at time t;
C_A = concentration (mg/l) of DOC or COD in the test suspension measured after 3h ± 30 min of incubation;
C_t = mean concentration (mg/l) of DOC or COD in the test suspension at time t;
C_{BA} = mean concentration (mg/l) of DOC or COD in the blanks measured after 3h ± 30 min of incubation;
C_B = mean concentration (mg/l) of DOC or COD in the blanks at time t.

Carry out the same calculation for the reference compound.

Display the course of biodegradation graphically (as in Figures 1 and 2) and record all results on data sheets.

Validity and interpretation

28. The test is considered valid if the procedural control shows the removal of the reference compound by at least 70% within 14d and if the removal of DOC (or COD) in the test suspension took place relatively gradually over days or weeks, since this indicates biodegradation.

29. However, physico-chemical adsorption can, in some cases, play a role and this is indicated when there is complete or substantial removal in the first 3h and the difference between blanks and test solutions remains at an unexpected low value. In such cases additional information is obtained from a comparison between the 3h value, the expected initial value calculated from the amount of test substance added and the value measured before the inoculum is added. If a more precise distinction between biodegradation (or partial degradation) and adsorption is to be drawn, carry out further tests, preferably a respirometric test for ready biodegradation, using the supernatant of the acclimatised sludge as inoculum.

30. Low and zero values of removal of the test substance may be due to its inhibition of bacteria; eliminate this possibility by testing for inhibition at the concentration used if this has not already been done (paragraph 15).

Test report

31. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Inoculum:

- source, concentration, status of adaptation.

Test conditions:

- analytical methods used;
- procedure control and compound used in the control.

Results:

- biodegradation curve;
- toxicity evaluations;
- the degree of biodegradation attained at the end of the test after 28d, or earlier if complete degradation is attained in less than 28d, as "inherent biodegradability in the static test after x days";
- any significant difference between the DOC (or COD) in the first sample at 3h after starting the test and the value calculated from the amount of test compound added as "adsorbed by the activated sludge";
- the adaptation phase (days), the biodegradation phase (days) and the endpoint of biodegradation reached after x days as identified from the biodegradation curve.

Discussion of the results.

LITERATURE

(1) Zahn R. und Wellens H. (1974). Ein einfaches Verfahren zur Prufung der biologischen Abbaubarkeit von Produkten und Abwasserinhaltsstoffen. Chemiker Zeitung 98, 228-232.

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(3) Reynolds, L. et al (1987). Evaluation of the toxicity of substances to be assessed for biodegradability. Chemosphere 16, 2259.

(4) DIN 38409, Teil 3 (1983). Bestimmung des gelösten organischen Kohlenstoffgehaltes (DOC).

(5) ISO Standard 6060 (1986). Water Quality-Determination of Chemical Oxygen Demand.

(6) OECD (1984). Test Guideline 209, Paris.

(7) ISO Standard 8192 (1986). Water Quality-Test for inhibition of oxygen consumption by activated sludge.

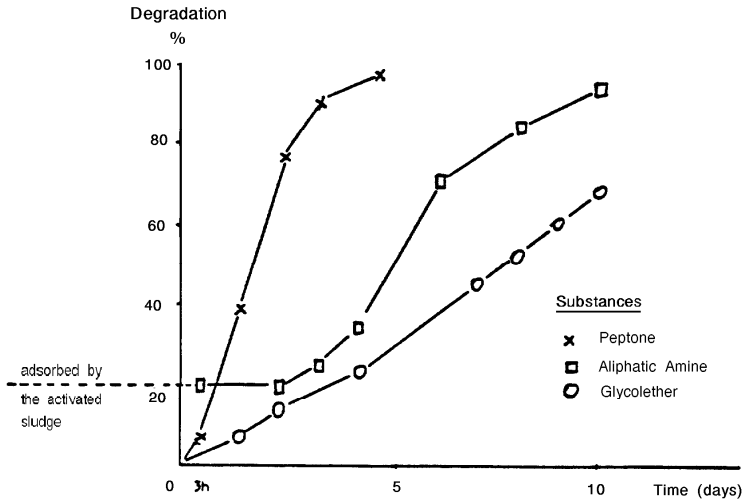

Figure 1: Examples of Biodegradation Curves

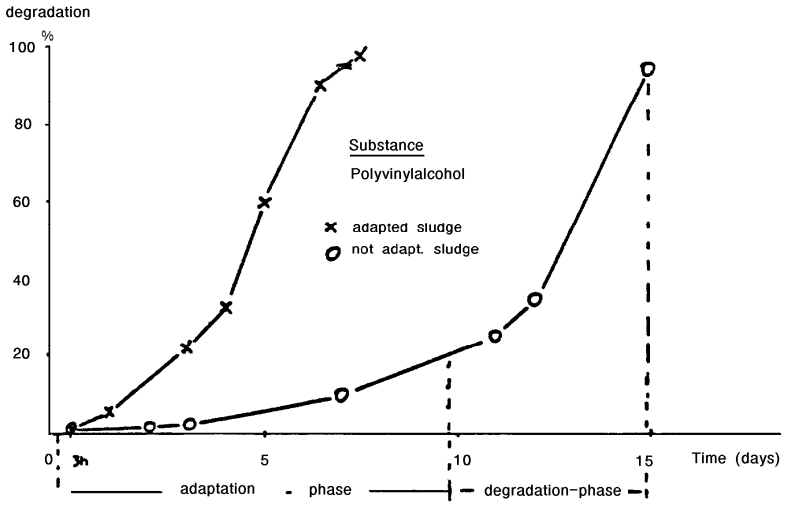

Figure 2: Example of Sludge-adaptation