C. 9. BIODEGRADATION

ZAHN -WELLENS TEST

1. METHOD

1.1. Introduction

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms in a static test.

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

The substances to be studied are used in concentrations corresponding to DOC-values in the range of 50 to 400 mg/litre or COD-values in the range of 100 to 1000 mg/litre (DOC = dissolved organic carbon; COD = chemical oxygen demand). These relatively high concentrations have the advantage of analytical reliability. Compounds with toxic properties may delay or inhibit the degradation process.

In this method, the measure of the concentration of dissolved organic carbon or the chemical oxygen demand is used to assess the ultimate biodegradability of the test substance.

A simultaneous use of a specific analytical method may allow the assessment of the primary biodegradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

-are soluble in water under the test conditions,

-have negligible vapour pressure under the test conditions,

-are not inhibitory to bacteria,

-are adsorbed within the test system only to a limited extent,

-are not lost by foaming from the test solution.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of low results and in the selection of appropriate test concentrations.

1.2. Definitions and units

The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn - Wellens test':

$$D_t = \left[1 - \frac{(C_T - C_B)}{(C_A - C_{BA})}\right] x100$$

where:

D_T = biodegradation (%) at time T,
C_A = DOC (or COD) values in the test mixture measured three hours after the beginning of the test (mg/l) (DOC = Dissolved Organic Carbon, COD = Chemical Oxygen Demand),
C_T = DOC or COD values in the test mixture at time of sampling (mg/l),
C_B = DOC or COD value of the blank at time of sampling (mg/l),
C_{BA} = DOC or COD value of the blank, measured three hours after the beginning of the test (mg/l).

The extent of degradation is rounded to the nearest full percent.

Percentage degradation is stated as the percentage DOC (or COD) removal of the tested substance.

The difference between the measured value after three hours and the calculated or preferably measured initial value may provide useful information on the elimination of the substance (see 3.2, Interpretation of results).

1.3. Reference substances

In some cases when investigating new substances reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. Principle of the test method

Activated sludge, mineral nutrients and the test material as the sole carbon source in an aqueous solution are placed together in a one to four litre glass vessel equipped with an agitator and an aerator. The mixture is agitated and aerated at 20 to 25 °C under diffuse illumination or in a dark room for up to 28 days. The degradation process is monitored by determination of the DOC (or COD) values in the filtered solution at daily or other appropriate regular time intervals. The ratio of eliminated DOC (or COD) after each interval to the value three hours after the start is expressed as percentage biodegradation and serves as the measure of the extent of degradation at this time. The result is plotted versus time to give the biodegradation curve.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. Quality criteria

The reproducibility of this test has been proven to be satisfactory in a ring test.

The sensitivity of the method is largely determined by the variability of the blank and, to a lesser extent, by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor.

1.6. Description of the test procedure

1.6.1. Preparations

1.6.1.1. Reagents

Test water: drinking water with an organic carbon content < 5 mg/litre. The concentration of calcium and magnesium ions together must not exceed 2,7 mmole/litre; otherwise adequate dilution with deionized or distilled water is required.

Sulphuric acid, analytical reagent (A.R.):	50 g/l.
Sodium hydroxide solution A.R.:	40 g/l.
Mineral nutrient solution: dissolve in one litre deionized water:	
ammonium chloride, NH_4Cl, A.R.:	38,5 g,
sodium dihydrogenphosphate, $NaH_2PO_4.2H_2O$, A.R.:	33,4 g,
potassium dihydrogenphosphate, KH_2PO_4, A.R.:	8,5 g,
di-potassium mono-hydrogenphosphate, K_2HPO_4, A.R.:	21,75 g.

The mixture serves both as a nutrient and as buffering system.

1.6.1.2. Apparatus

Glass vessels with a volume of one to four litres (e.g. cylindrical vessels).

Agitator with a glass or metal stirrer on a suitable shaft (the stirrer should rotate about 5 to 10 cm above the bottom of the vessel). A magnetic stirrer with a 7 to 10 cm long rod can be used instead.

Glass tube of 2 to 4 mm inner diameter to introduce air. The opening of the tube should be about 1 cm above the bottom of the vessel.

Centrifuge (about 3550 g).

pH-meter.

Dissolved-oxygen meter.

Paper filters.

Membrane filtration apparatus.

Membrane filters, pore size 0,45 µm. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step.

Analytical equipment for determining organic carbon content and chemical oxygen demand.

1.6.1.3. Preparation of the inoculum

Activated sludge from a biological treatment plant is washed by (repeatedly) centrifuging or settling with test water (above).

The activated sludge must be in an appropriate condition. Such sludge is available from a properly working waste-water treatment plant. To get as many different species or strains of bacteria as possible, it may be preferred to mix inocula from different sources (e.g. different treatment plants, soil extracts, river waters, etc.). The mixture is to be treated as described above.

For checking the activity of the activated sludge see 'Functional control', below.

1.6.1.4. Preparation of the test solutions

To the test vessel add 500 ml of test water, 2,5 ml/litre mineral nutrient solution and activated sludge in an amount corresponding to 0,2 to 1,0 g/litre dry matter in the final mixture. Add sufficient stock solution of the substance to be tested so that a DOC concentration of 50 to 400 mg/litre results in the final mixture. The corresponding COD-values are 100 to 1000 mg/litre. Make up with test water to a total volume of one to four litres. The total volume to be chosen is dependent on the number of samples to be taken for DOC or COD determinations and the volumes necessary for the analytical procedure.

Normally a volume of two litres can be regarded as satisfactory. At least one control vessel (blank) is set up to run in parallel with each test series; it contains only activated sludge and mineral nutrient solution made up with test water to the same total volume as in the test vessels.

1.6.2. Performance of the test

The test vessels are agitated with magnetic stirrers or screw propellers under diffuse illumination or in a dark room at 20 to 25 °C. Aeration is accomplished by compressed air cleaned by a cotton-wool strainer and a wash bottle if necessary. It must be ensured that the sludge does not settle and the oxygen concentration does not fall below 2 mg/litre.

The pH-value must be checked at regular intervals (e.g. daily) and adjusted to pH 7 to 8, if necessary.

Losses from evaporation are made up just before each sampling with deionized or distilled water in the required amounts. A good procedure is to mark the liquid level on the vessel before starting the test. New marks are made after each sampling (without aeration arid stirring). The first samples are always taken three hours after the start of the test in order to detect adsorption of t est material by the activated sludge.

The elimination of the test material is followed by DOC or COD determinations made daily or at some other regular interval. The samples from the test vessel and the blank are filtered through a carefully washed paper filter. The first 5 ml of test solution filtrate are discarded. Sludges difficult to filter may be removed previously by centrifugation for 10 minutes. DOC and COD determinations are made at least in duplicate. The test is run for up to 28 days.

Note: Samples remaining turbid are filtered through membrane filters. The membrane filters must not release or adsorb any organic material.

Functional control of activated sludge

A vessel containing a known substance should be run in parallel with each test series in order to check the functional capacity of the activated sludge. Diethyleneglycol has been found useful for this purpose.

Adaptation

If analyses are carried out at relatively short intervals (e.g. daily), adaptation can be clearly recognized from the degradation curve (see Figure 2). The test should therefore not be started immediately before the weekend.

If the adaptation occurs in the end of the period, the test can be prolonged until the degradation is finished.

Note: If a broader knowledge of the behaviour of the adapted sludge is needed, the same activated sludge is exposed once again to the same test material in accordance with the following procedure:

Switch of the agitator and the aerator and allow the activated sludge to settle. Draw off the supernatant liquid, fill up to two litres with test water, stir for 15 minutes and allow to settle again. After the supernatant liquid is drawn off again, use the remaining sludge to repeat the test with the same material in accordance with 1.6.1.4 and 1.6.2, above. The activated sludge can also be isolated by centrifuging instead of settling.

The adapted sludge may be mixed with fresh sludge to a concentration of 0,2 to 1 g dry weight/litre.

Analytical means

Normally samples are filtered through a carefully washed paper filter (for washing use deionized water).

Samples which remain turbid are filtered through membrane filters (0,45 µm).

The DOC concentration is determined in duplicate in the sample filtrates (the first 5 ml are discarded) by means of the TOC instrument. If the filtrate cannot be analysed on the same day, it must be stored in the refrigerator until the next day. Longer storage cannot be recommended.

The COD concentration: is determined in the sample filtrates with a COD analytical set-up by the procedure described in reference (2), below.

2. DATA AND EVALUATION

DOC and/or COD concentrations are determined at least in duplicate in the samples according to 1.6.2, above. The degradation at time T is calculated according to the formula (with definitions) given unter 1.2, above.

The extent of degradation is rounded to the nearest full percent. The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn -Wellens test'.

Note: If complete degradation is attained before the test time is over and this result is confirmed by a second analysis on the next day, the test can be concluded.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

-the initial concentration of the substance,

-all other information and the experimental results concerning the tested substance, the reference substance if used, and the blank,

-the concentration after three hours,

-biodegradation: curve with description,

-date and location where test organisms were sampled, status of adaptation, concentration used, etc.,

-scientific reasons for any changes of test procedure.

3.2. Interpretation of results

Removal of DOC (COD) which takes place gradually over days or weeks indicates that the test substance is being biodegraded.

However, physico-chemical adsorption can, in some cases, play a role and this is indicated when there is complete or partial removal from the outset, within the first three hours, and the difference between control and test supernatant liquors remains at an unexpectedly low level.

Futher tests are necessary if a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant or sludge as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC (COD) in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation. Low, or zero removals of DOC (COD) may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a compound-specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 302 B, Decision of the Council C(81) 30 final.

(2) Annex V C.9 Degradation: Chemical Oxygen Demand, Commission Directive 84/449/EEC, Official Journal of the European Communities, No L 251,19.9.1984.

Appendix

EVALUATION EXAMPLE

Organic compound:	4-Ethoxybenzoic acid
Theoretical test concentration:	600 mg/1
Theoretical DOC:	390 mg/l
Inoculum	Sewage Treatment plant of. ..
Concentration:	1 gram dry material/litre
Adaptation status:	not adapted
Analysis:	DOC-derermination
Amount of sample:	3 ml
Control substance:	Diethyleneglycol
Toxicity of compound:	No toxic effects below 1000 mg/l
	Test used: Fermentation tubes test

Test time	Control substance				Test substance		
	Blank DOC (1) mg/l	DOC (1) mg/l	DOC net mg/l	Degradation %	DOC (1) mg/l	DOC net mg/l	Degradation %
0	—	—	300,0	—	—	390,0	—
3 hours	4,0	298,0	294,0	2	371,6	367,6	6
1 day	6,1	288,3	282,2	6	373,3	367,2	6
2 days	5,0	281,2	276,2	8	360,0	355,0	9
5 days	6,3	270,5	264,2	12	193,8	187,5	52
6 days	7,4	253,3	245,9	18	143,9	136,5	65
7 days	11,3	212,5	201,2	33	104,5	93,2	76
8 days	7,8	142,5	134,7	55	58,9	51,1	87
9 days	7,0	35,0	28,0	91	18,1	11,1	97
10 days	18,0	37,0	19,0	94	20,0	2,0	99

(1) Mean values of triplicate determinations.

Figure 1

Examples of biodegradation curves

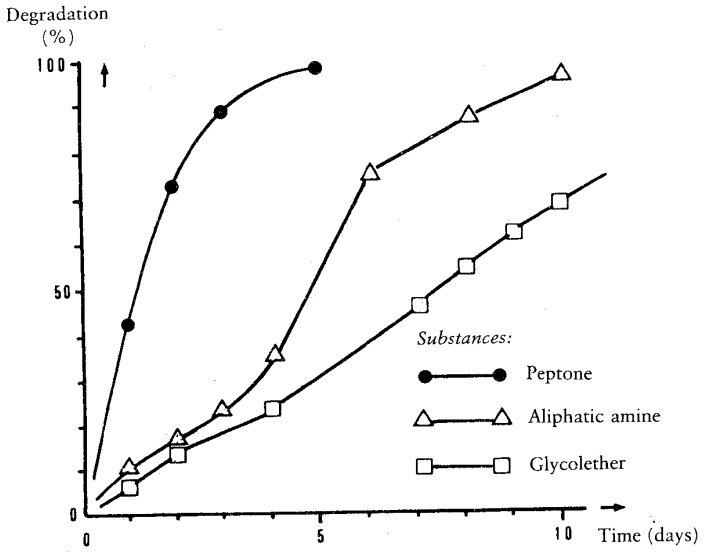

Figure 2

Examples of sludge adaptation