209

Adopted:
4 April 1984

"Activated Sludge, Respiration Inhibition Test"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Water solubility
- Vapour pressure

• Guidance information

- Structural formula
- Purity of the test substance

• Qualifying statements

- This test guideline is most readily applied to substances which, due to their water solubility and low volatiliy, are likely to remain in water.

- For test substances with limited solubility in the test media, it may not be possible to determine the EC 50.

- Results based on oxygen uptake may lead to erroneous conclusions when the test substance has the propensity to uncouple oxidative phosphorylation.

• Recommendation

Activated sludge may contain potentially pathogenic organisms and should be handled with care.

• Standard documents

See Section 4, Literature.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

The method described in this test guideline assesses the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance. The method is based on that described by ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry), in which activated sludge obtained from a sewage treatment plant is used as the microbial source.

The purpose of this test guideline is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified and to indicate suitable non-inhibitory concentrations of test substances to be used in biodegradability tests.

A range-finding test may precede a definitive test. It provides information about the range of concentrations to be used in the main test.

Two controls without test substance are included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge should also be checked using a reference substance.

- Definitions

The respiration rate is the oxygen consumption of aerobic sludge or waste-water micro-organisms expressed generally as mg 0_2 per litre per hour.

EC 50 in this Test Guideline is the concentration of the test substance at which the respiration rate is 50 per cent of that shown by the control under conditions described in this guideline.

- Reference substances

It is recommended that 3,5-dichlorophenol as a known inhibitor of respiration be used as a reference substance and tested for EC 50 on each batch of activated sludge as a means of checking that the sensitivity of the sludge is not abnormal.

- Principle of the test method

The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or 3 hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC 50 value is calculated from determinations at different concentrations.

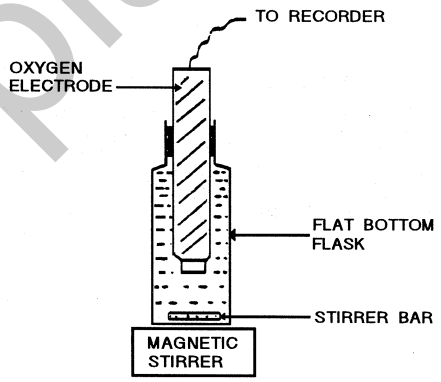
- Conditions for the validity of the test

The test results are valid if

– the two control respiration rates are within 15 per cent of each other ;

– the EC 50 (3 hours) of 3,5-dichlorophenol is in the accepted range 5 to 30 mg/l.

Figure 1 : Measuring Apparatus*



* The precise design is not critical. However, there should be no head space and the probe should fit tightly in the neck of the measuring flask.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Equipment

Normal laboratory equipment and especially the following is necessary :

- Measuring apparatus (see Figure 1)
- Aeration device
- pH-electrode and measuring equipment
- O_2-electrode.

Solutions of the test substance

Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of O.5 g/l is appropriate if the procedure recommended below is followed.

[Note : A solution of 3,5-dichlorophenol can be conveniently prepared by dissolving 0.5 g 3,5-dichlorophenol in 10 ml of 1N NaOH, diluting to approximately 30 ml with distilled water, adding under stirring 1N H_2SO_4 to the point of incipient precipitation — approximately 8 ml of 1N H_2SO_4 will be required — and finally diluting the mixture to one litre with distilled water. The pH should then be in the range 7 to 8].

Test concentrations

At least five concentrations, spaced by a constant factor preferably not exceeding 3.2, should be used.

Synthetic sewage feed

A synthedic sewage feed is made by dissolving the following amounts of substances in 1 litre of water :

- 16 g peptone
- 11 g meat extract
- 3 g urea
- 0.7 g NaCl

- 0.4. g $CaCl_2.2H_2O$
- 0.2 g $MgSO_4.7H_2O$
- 2.8 g $K_2 HPO_4$

[Note : This synthetic sewage is a 100 fold concentrate of that described in the OECD Technical Report "Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents" June 11, 1976, with moreover dipotassium hydrogen phosphate added.]

- Test system

Microbial innoculum

Activated sludge from a sewage treatment plant is normally used as the microbial innoculum for the test. Where possible, activated sludge should be obtained from a sewage work treating predominantly domestic sewage. If this is not possible, the activated sludge may be obtained from sewage works treating predominantly industrial waste water but used only following de-adaptation. Even so, results obtained with activated sludge from works treating industrial waste waters may be atypical.

On return to the laboratory the sludge is washed, if necessary, with tap water or an isotonic solution. After centrifuging the supernatant is decanted. This procedure is repeated three times. A small amount of the washed sludge is weighed and dried. From this result the amount of wet sludge can be calculated which must be suspended in water in order to obtain an activated sludge with a mixed liquor suspended solids level of 4 g/l (± 10 per cent). This level gives a concentration of 1.6 g/l in the test medium if the procedure recommended below is followed.

If the sludge cannot be used on the day of collection, 50 ml synthetic sewage is added to each litre of the activated sludge prepared as described above ; this is then aerated overnight at 20 ± 2°C. It is then kept aerated for use during the day. Before use the pH is checked and buffered, if necessary, to pH 6.0 to 8.0 using sodium bicarbonate solution. The mixed liquor suspended solids should be determined as described in the preceeding paragraph.

If the same batch of sludge is required to be used on subsequent days (maximum four days), a further 50 ml of synthetic sewage feed is added at the end of each working day.

- Test conditions

Duration/ contact time :	30 minutes and/or 3 hours, during which aeration takes place
Vessels :	Beakers are suitable
Water :	Drinking water (dechlorinated if necessary)
Air supply :	Clean, oil-free air. Air flow 0.5 to 1 litre/minute
Measuring apparatus :	Flat bottom flask such as a BOD-flask (see Figure 1)
Oxygen meter :	Polarographic oxygen electrode, connectable to a potentiometric recorder (200 mV range)
Nutrient solution :	Synthetic sewage feed (see above)
Test substance :	The test solution is freshly prepared at the start of the test
Reference substance :	e.g. 3,5-dichlorophenol (at least 3 concentrations)
Controls :	Innoculated sample without test substance
Temperature :	20 ± 2°C

- Performance of the test

A suggested experimental procedure which may be followed for both the test and reference substance for the 3-hour contact period is given below :

– Several vessels (e.g. 1-litre beakers) are used.

- At time "0", 16 ml of the synthetic sewage feed are made up to 300 ml with water. 200 ml of microbial innoculum are added and the total mixture (500 ml) poured into a first vesel (first control C_1). Aeration at 0.5 to 1 litre per minute is commenced using a Pasteur-pipette as aeration device.

- At time "15 min" (15 minutes is an arbitrary, but convenient, interval) the above is repeated, except that 100 ml of the test substance stock solution are added to the 16 ml of synthetic sewage before adding water to 300 ml and microbial innoculum to make a volume of 500 ml. This mixture is then poured into a second vessel and aerated as above. This process is repeated at 15-minute intervals with different volumes of the test substance stock solution to give a series of vessels containing different concentrations of the test substance. Finally, a second control (C_2) is prepared.

- After three hours the contents of the first vessel are poured into the measuring apparatus and the respiration rate is measured over a period of up to 10 minutes ; the measuring can also be carried out directly in the vessel.

- This determination is repeated on the contents of each vessel at 15-minute intervals, in such a way that the contact time in each vessel is three hours.

The reference substance is tested on each batch of microbial innoculum in the same way.

A different regime (e.g. more than one oxygen meter) will be necessary when measurements are to be made after 30 minutes of contact.

If measurement of the chemical oxygen consumption is required, further vessels are prepared containing test substance, synthetic sewage feed and water, but no activated sludge.

Observations

Oxygen consumption is measured and recorded after an aeration time of 30 minutes and/or 3 hours (contact time).

3. DATA AND REPORTING

• Treatment of results

The respiration rate is calculated from the recorder trace as mg O_2/l.h between approximately 6.5 mg O_2/l and 2.5 mg O_2/l, or over a 10 minute period when the respiration rate is low. The portion of the respiration curve over which the respiration rate is measured should be linear.

In order to calculate the inhibitory effect of a test substance at a particular concentration, the respiration rate is expressed as a percentage of the mean of the two control respiration rates :

$$1 - \frac{2R_s}{R_{c1} + R_{c2}} \cdot 100 = \textit{per cent inhibition}$$

where

R_s = oxygen-consumption rate at tested concentration of test substance

R_{c1} = oxygen-consumption rate, Control 1

R_{c2} = oxygen-consumption rate, Control 2

If the respiration rates of the two controls are not within 15 per cent of each other or the EC 50 (3 h) of the reference substance is not in the accepted range (5 to 30 mg/l for 3,5-dichlorophenol), the test is invalid and must be repeated.

The per cent inhibition is calculated at each test concentration as above. The per cent inhibition is plotted against concentration on log-normal (or log-probability) paper and an EC 50 value derived.

95 per cent confidence limits for the EC 50 values can be determined using standard procedures. In view of the variability often observed in the results, it is recommended that the results be expressed in orders of magnitude, e.g. less than 1, 1 to 10, 10 to 100, etc. (in mg/l).

- Interpretation of results

The EC 50 value should be regarded merely as a guide to the likely toxicity of the test substance either to activated sludge sewage treatment or to waste-water micro-organisms, since the complex interactions occuring in the environment cannot be accurately simulated in a laboratory test.

- Test report

The test report should include the following information :

Test substance : chemical identification data

Test system : source, concentration and any pretreatment of the activated sludge

Test conditions :

- test temperature
- test duration
- reference substance and its measured EC 50
- abiotic oxygen uptake (if any)

Results :

- all measured data
- inhibition curve and method for calculation of EC 50
- EC 50 and, if possible, 95 per cent confidence limits, EC 20 and EC 80
- all observations and any deviations from this test guideline which could have influenced the result

4. LITERATURE

1. International Standard ISO/TC 147/SC 5/WC 1, N53 No. D (June 1981).

2. B. Broecker and R. Zahn, *Water Research 11*, 165 (1977).

3. D. Brown, H.R. Hitz and L. Schaefer, *Chemosphere 10*, 245 (1981).

4. ETAD (Ecological and Toxicological Association of Dyestuffs Manufacturing Industries) Recommended Method No. 103, also described by :

5. B. Robra, *Wasser/Abwasser 117*, 80 (1976) and

6. W. Schefer, *Textilveredlung 6*, 247 (1977).