C. 11. BIODEGRADATION

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST

1. METHOD

1.1. Introduction

The method described 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 purpose of this method 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.

This method is most readily applied to substances which, due to their water solubility and low volatility, are likely to remain in water.

For 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.

It is useful to have the following information to perform the test:

-water solubility,

-vapour pressure,

-structural formula,

-purity of the test substance.

Recommendation

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

1.2. Definitions and units

The respiration rate is the oxygen consumption of waste-water micro-organisms in aerobic sludge, expressed generally as mg O_2 per mg of sludge per hour.

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:

$$\left(1-\frac{2R_s}{R_{c1}+R_{c2}}\right)x100 = \text{per cent inhibition}$$

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.

EC_{50} in this method is the concentration of the test substance at which the respiration rate is 50% of that shown by the control under conditions described in this method.

1.3. 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.

1.4. 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 three 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.

1.5. Quality criteria

The test results are valid if:

-the two control respiration rates are within 15% of each other,

-the EC_{50} (30 minutes and/or three hours) of 3,5-dichlorophenol is in the accepted range 5 to 30 mg/litre.

1.6. Description of the test method

1.6.1. Reagents

1.6.1.1. 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 0,5 g/litre is appropriate if the procedure recommended below is followed.

1.6.1.2. Solution of control substance

A solution of 5,5-dichlorophenol can for example be prepared by dissolving 0,5 g 3,5-dichlorophenol in 10 ml of 1M NaOH, diluting to approximately 30 ml with distilled water, adding under stirring 0,5M H_2SO_4 to the point of incipient precipitation - approximately 8 ml of 0,5M 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.

1.6.1.3. Synthetic sewage

A synthetic sewage feed is made by dissolving the following amounts of substances in one 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_2HPO_4.

Note 1: 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 the addition of dipotassium hydrogen phosphate.

Note 2: If the prepared medium is not used immediately, it shall be stored in the dark at 0 to 4 °C, for no longer than one week, in conditions which do not produce any change in its composition. The medium may also be sterilized prior to storage, or the peptone and meat extract may be added shortly before carrying out the test. Before use, it shall be mixed thoroughly and the pH adjusted.

1.6.2. Apparatus

Measuring apparatus: The precise design is not critical. However, there should be head space and the probe should fit tightly in the neck of the measuring flask.

Normal laboratory equipment and especially the following is necessary:

-measuring apparatus,
-aeration device,
-pH-electrode and measuring equipment,
-O_2-electrode.

1.6.3. Preparation of the inoculum

Activated sludge from a sewage treatment plant treating predominantly domestic sewage is used as the mircobial inoculum for the test.

If necessary, on return to the larboratory, coarse particles may be removed by settling for a short period, e.g. 15 minutes, and decanting the upper layer of finer solids for use. Alternatively, the sludge may be mixed using a blender for a few seconds.

In addition, if it is thought that inhibitory material is present, the sludge should be washed with tap water or an isotonic solution. After centrifuging, the supernatant is decanted (this procedure is repeated three times).

A small amount of the 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 range between 2 and 4 g/litre. This level gives a concentration between 0,8 and 1,6 g/litre in the test medium if the procedure recommended below is followed.

If the sludge cannot be used on the day of collection, 50 ml of 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 adjusted, if necessary, to pH 6 to 8. The mixed liquor suspended solids should be determined as described in the preceding 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 per litre of sludge at the end of each working day.

1.6.4. Performance of the test

Duration/contact time:	30 minutes and/or three hours, during which aeration takes place
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
Oxygen meter:	Suitable oxygen electrode, with a recorder
Nutrient solution:	Synthetic sewage (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 three concentrations)
Controls:	Inoculated sample without test substance
Temperature:	20 ± 2 °C.

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

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

At least five concentrations, spaced by a constant factor preferably not exceeding 3,2, should be used.

At time '0', 16 ml of the synthetic sewage feed are made up to 300 ml with water. 200 ml of microbial inoculum are added and the total mixture (500 ml) poured into a first vessel (first control C_1).

The test vessels should be aerated continuously such as to ensure that the dissolved O_2 does not fall below 2,5 mg/litre and that, immediately before the measurement of the respiration rate, the O_2 concentration is about 6,5 mg/litre.

At time '15 minutes' (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 inoculum 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 is prepared (C_2).

After three hours the pH is recorded, and a well-mixed sample of the contents of the first vessel is poured into the measuring apparatus and the respiration rate is measured over a period of up to 10 minutes.

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 inoculum 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 mesurement of the chemical oxygen consumption is required, further vessels are prepared containing test substance, synthetic sewage feed and water, but no activated sludge. Oxygen consumption is measured and recorded after an aeration time of 30 minutes and/or three hours (contact time).

2. DATA AND EVALUATION

The respiration rate is calculated from the recorder trace between approximately 6,5 mg O_2/litre and 2,5 mg O_2/litre, 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.

If the respiration rates of the two controls are not within 15% of each other, or the EC_{50} (30 minutes and/or three hours) of the reference substance is not in the accepted range (5 to 30 mg/litre for 3,5-dichlorophenol), the test is invalid and must be repeated.

The per cent inhibition is calculated at each test concentration (see 1.2). The per cent inhibition is plotted against concentration on log-normal (or log-probability) paper, and an EC_{50} value derived.

95% confidence limits for the EC_{50} values can be determined using standard procedures.

3. REPORTING

3.1. Test report

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

-test substance: chemical identification data,

-test system: source, concentration and any pre-treatment of the activated sludge,

-test conditions:

-pH of the reaction mixture before the respiration measurement,

-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% confidence limits, EC_{20} and EC_{80},

-all observations and any deviations from this test method which could have influenced the result.

3.2. Interpretation of data

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 microorganisms, since the complex interactions occurring in the environment cannot be accurately simulated in a laboratory test. In addition, test substances which may have an inhibitory effect on ammonia oxidation may also produce atypical inhibition curves. Accordingly, such curves should be interpreted with caution.

4. REFERENCES

(1) International Standard ISO 8192-1986.

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

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

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

(5) Robra, B., Wasserl Abwasser 117, 1976, p. 80.

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

(7) OECD, Paris, 1981, Test Guideline 209, Decision of the Council C(81) 30 final.