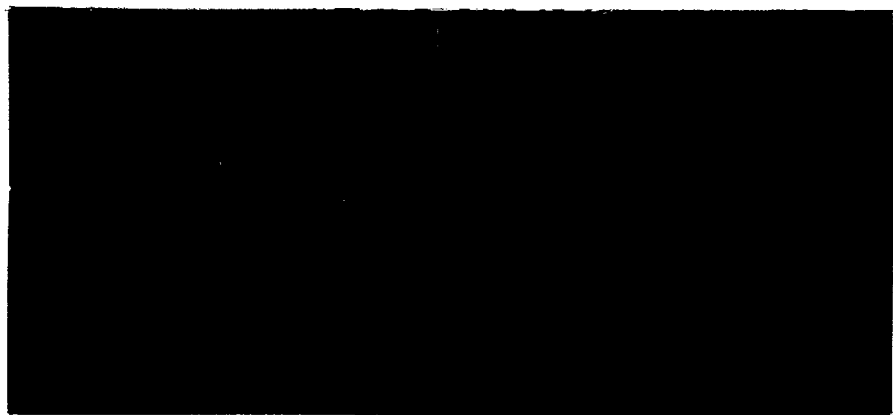7.5 fil

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First time round!

ER 1270-M

DETERMINATION OF THE TOXICITY OF SUBSTANCES TO AEROBIC BACTERIA BY MEASUREMENT OF GROWTH INHIBITION

For SCA . O.O.

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UNRESTRICTED

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SUMMARY

Using the results of tests such as the inhibition
of respiration of activated sludge (SCA 1982) to
predict the effects of a chemical in the
environment, eg on the activated sludge process,
can lead to wrong conclusions. The principal
reason for this is the greater effect some
chemicals have on growth than on metabolic
activity, such as oxygen uptake, of preformed
cells. Since it is not possible to predict which
chemicals fall into this category, it is necessary
to carry out a test to ascertain the effect of
chemicals on growth. (There are other reasons for
predictions of environmental behaviour not being
able to be made accurately from respiration tests -
adsorption onto solids, acclimatisation leading to
tolerance and/or biodegradation. For such
chemicals growth-inhibition tests are not
satisfactory and only simulation tests will decide
on their true behaviour.)

The Standing Committee of Analysts (UK) has devised
a test to assess inhibition of growth, based on
that described by Alsop et al (1980). The original
method, in which the turbidity of a medium
inoculated with settled sewage was measured, gave
inconsistencies and was not reproducible because of
the variable nature of sewage from day to day and
source to source. The present method greatly
reduces this variability by using an inoculum
containing a more constant number of cells which
are in the logarithmic phase of growth and which
contains less inert colloidal matter. This
inoculum was achieved by overnight incubation of
portions of medium containing various amounts of
settled sewage and selecting that culture which
gave a pre-determined optical density at the end of
16 h incubation.

A limited ring test of the UK method, which is soon
to be published, has shown that the reproducibility
is of the same order as that of the inhibition to
respiration method.

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INTRODUCTION

The determination of toxicity to aerobic
microorganisms is an essential element in the
evaluation of the effects of substances in sewage
treatment processes and in natural waters. This
method describes a procedure for determining
bacterial growth inhibition due to the presence of
toxic substances. Since the method utilises low
concentrations of microorganisms, it can be applied
as a toxicity screening test prior to ready
biodegradability assessment. The method augments
those toxicity methods described in a previous
publication (Standing Committee of Analysts 1982).

1. PERFORMANCE CHARACTERISTICS OF THE METHOD

1	Parameter determined	Toxicity (IC50) of soluble substances to aerobic micro-organisms.
2	Type of sample	Pure substances, mixtures or industrial waste-waters.
3	Basis of methods	Comparison of bacterial growth rate in the presence and absence of varying amounts of test substance or waste-water.
4	Range of application	0-100% inhibition
5	Standard deviation	?
6	Sources of error	Test substance colour, turbidity or tendency to precipitate in the test medium

| 7 | Time required for analysis | A total of 22 hours. Actual operator time: 6 hours over this period. |

2. PRINCIPLE

Shake flasks containing buffer, nutrients and growth substrate are inoculated with an overnight culture of microorganisms taken from domestic sewage and incubated at $22 \pm 2\ ^\circ C$ for up to 6 hours. The growth rate of this culture is determined by measurement of turbidity increase at a wavelength of 530 nm. Test substances are evaluated at a range of concentrations and their effect on the rate of bacterial growth determined. The toxicity of test substances is plotted as a function of concentration and the IC50 value obtained (concentration causing a 50% reduction in growth rate).

3. INTERFERENCES

Test substances that are coloured or hazy, or precipitate in the test medium may give rise to background optical densities at 530 nm. These can be corrected for by including controls. Flocculent growth of micro-organisms could interfere but does not usually occur; it has largely been eliminated by the use of a pre-grown culture as inoculum.

4. HAZARDS

4.1
Hygiene

Sewage may contain potentially pathogenic organisms, therefore suitable handling precautions should be taken to avoid infection.

4.2

Chemicals
Test substances may be toxic or their properties unknown, and therefore suitable handling precautions should be taken to avoid unnecessary contact.

5. REAGENTS

5.1
Good quality deionised or distilled water.

5.2

Phosphate buffer
Dissolve 8.5 g potassium dihydrogen phosphate, 21.75 g dipotassium hydrogen phosphate, 33.4 g disodium hydrogen phosphate dihydrate in about 500 ml distilled water and make up to 1 litre.

5.3

Nutrient broth/sodium acetate solution
Dissolve 8 $\pm$ 0.2 g Bacto nutrient broth and 6 $\pm$ 0.2 g sodium acetate in 1 litre distilled water.

5.4

pH adjustment reagents
Sodium hydroxide (1 M) and sulphuric acid (0.5 M).

5.5

Test substance stock solutions
Prepare a 1 g/l solution of the test substance in distilled water. The pH of this solution should be checked and adjusted to pH 7 $\pm$ 1 if necessary before making up to volume.

5.6

Reference substance stock solution
Prepare a 1 g/l solution of 3,5 dichlorophenol in distilled water.

5.7

"Seed" microorganisms
Supernatant from the primary settlement tank of a sewage works treating predominantly domestic sewage. This should be obtained freshly but may be stored at a temperature of 0-4 $^{\circ}$C for up to 5 days

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if necessary. Before use filter 200 ml through glass wool or glass fibre paper A to remove coarse material, discarding the first 180 ml and collecting the final 20 ml for use in the test.

6. APPARATUS

6.1   250 ml conical flasks with polyurethane foam bungs.

6.2   Incubator/shaker capable of maintaining a temperature of 22 ± 2 ^{o}C and a shaking speed of 150 rpm. Alternatively a shaker in a constant temperature room (22 ± 2 ^{o}C) may be used.

6.3   UV-visible spectrophotometer and matched cells (1 cm or 4 cm light path).

7. TEST PROCEDURE

Step procedure   Notes

Preculture

7.1 16 h prior to commencement of the test set up shake flasks containing 25 ml water (5.1), 4 ml buffer (5.2) and 10 ml nutrient broth/sodium acetate solution (5.3).

7.2 Inoculate three flasks with a range of volumes of sewage micro-organisms (5.7) eg 0.01, 0.1 and 1 ml and place in the incubator shaker (6.2) for 16 ± 1 h (note a).

(a) It is convenient to carry out this preculture stage overnight.

4

Setting up the test

7.3 After the preculture period take a sample from each flask and measure the optical density at a wavelength of 530 nm. Select the flask in which the culture is in the logarithmic phase of growth (note b).

(b) The optical density should be 1.0 ± 0.2 for 4 cm cells (or 0.3 for 1 cm cells) at 16 h to ensure that the culture is in the log phase of growth.

7.4 Label the test flasks and add the appropriate volumes of water, buffer, nutrients and test substance (note c).

(c) Suggested experimental designs are shown in Tables 1 and 2. Note that unseeded control flasks containing the test substance are set up.

7.5 Place all flasks in the incubator/ shaker and allow the contents to reach thermal equilibrium (note d).

(d) This step is advisable to minimise temperature shock on transfer.

7.6 Inoculate appropriate flasks with 1 ml of precultured inoculum (7.3) (note e)

(e) The volume of inoculum should be adjusted if necessary to ensure that the control flasks are still in log phase at the end of the 6 h measurement period.

7.7 At hourly, or other convenient intervals, remove 4 ml samples from each flask and measure the optical density as indicated in 7.3. During the sampling procedure, which should be as short as possible, leave the flasks in the incubator (note f).

(f) Aim to reduce sampling time to a minimum in order to maintain bacterial growth in the log phase.

7.8 Take the final reading at 6 h and treat the results as indicated below (Section 8).

8. CALCULATION AND INTERPRETATION OF RESULTS

8.1 Correct for any turbidity, colour or precipitation obtained in unseeded control flasks by subtracting the appropriate optical density readings from the corresponding values obtained in seeded flasks.

8.2 Plot $\log_{10}$ corrected optical density against time for each test substance concentration and the mean of seeded controls. From these plots the specific growth rate of the cultures can be calculated as 2.303 x slope of the line $(= \mu \ h^{-1})$.

8.3 Three types of curve may be obtained (Figure 1). The plot may be a straight line up to 6 h (A) after a short initial lag but in some cases there may be a departure from linearity before 6 h is reached (B); the inoculated controls always, and most test substances usually, fall into these two categories. A third type (C), observed in the presence of some toxic chemicals shows an initial lag followed by an increase in the rate of growth indicating a rapid acclimatisation or development by the test organisms of tolerance towards the test chemical.

8.4 Calculate % inhibition as

$$\frac{\mu_c - \mu_t}{\mu_c} \times 100\%$$

where μ_c = specific growth rate of the inoculated control

μ_t = specific growth rate of culture containing the test substance.

Alternatively, the optical density after 6 h (A), or at the latest time for which the line is straight (B), may be taken as a measure of the

biomass present at that time. Calculate the % inhibition for each concentration of test substance as

$$\frac{\text{control optical density} - \text{test optical density}}{\text{control optical density}} \times 100$$

It is important to note that these two methods do not give the same values; the % inhibition of biomass increases with time, while that for rate is constant. (The relationship between these two values is indicated in the Appendix.)

8.5 If curve is of type C and if an inflexion point is recognised, these facts should be reported.

Calculate the % inhibition from the growth rates both before and after the point of inflexion, if the data available justify this.

8.6 Lastly, plot % inhibition against the logarithm of the concentration of test substance and calculate or interpolate from the graph the EC50 as that concentration which inhibits the growth of the control by 50%.

If suitable data are available, the 95% confidence limit of the EC50, the slope of the curve and suitable values to mark the beginning of inhibition (for example, EC10 or EC20) and the end of the inhibition range (for example EC80 or EC90) can be calculated or interpolated.

In view of the variability often observed in the results it may in many cases be sufficient that the results be expressed in orders of magnitude for example EC50 <1 mg/l

     1 - 10 mg/l

    10 - 100 mg/l

     >100 mg/l

8.7   The results from this test can be used to select a concentration of substance for use in the ready biodegradability tests. A suitable concentration would be that at which <10% inhibition of growth occurs.

The results also give a more accurate indication of the likely effect of a test substance on sewage treatment processes than does the test for inhibition of the respiration of activated sludge (SCA, 1982). Even so, because of possible adsorption effects and possible reactions with other chemicals present in sewage, as well as biodegradation after a period of acclimatisation, a final judgement of the effects of chemicals on sewage treatment may not be made until a simulation test is carried out.

9. VALIDITY OF THE RESULTS

The sensitivity of the sewage microorganisms should be checked by means of a reference substance.

In a ring test, the EC50 of 3,5-dichlorophenol was found to lie in the range of X to Y mg/l.

If the EC50 of the reference substance does not lie in the expected range, the test should be repeated with sewage from another source.

REFERENCE

STANDING COMMITTEE OF ANALYSTS (1982) Methods for
assessing the treatability of chemicals and
industrial waste-waters and their toxicity to
sewage treatment processes. ISBN 011 751959 6, Her
Majesty's Stationery Office, London.

Table 1. Experimental design for determining the degree of microbial
          inhibition on a number of test substances.

Flask No	Contents (1)	Water (ml)	Phosphate buffer (ml)	Nutrient broth/sodium acetate solution (ml)	Inoculum (ml)	Test substance (2) (ml)	Concentration (mg/l)
1	Control	25	4	10	1	–	–
2,3	Test substance A	21	4	10	1	4	100
4,5	Test substance B	21	4	10	1	4	100
6,7	Test substance C	21	4	10	1	4	100
8,9	3,5 dichloro-phenol (3)	21 24.6	4 4	10 10	1 1	4 0.4	100 10
10	Control	25	4	10	1	–	–
11,12	Unseeded control	26	4	10	–	–	–
13	A (unseeded)	32	4	–	–	4	100
14	B (unseeded)	32	4	–	–	4	100
15	C (unseeded)	32	4	–	–	4	100

Notes (1) Total volume in each flask = 40 ml
      (2) Test substance stock solution = 1 g/l
      (3) Reference substance tested

Table 2. Experimental design for determining the IC50 of a single test substance.

Flask No	Contents (1)	Water (ml)	Phosphate buffer (ml)	Nutrient broth/sodium acetate solution (ml)	Inoculum (ml)	Test substance (2) (ml)
1	Control	25	4	10	1	–
2,3	1 mg/l test substance	24.6	4	10	1	0.4 (2)
4,5	3.2 mg/l	23.7	4	10	1	1.3 (2)
6,7	10 mg/l	21	4	10	1	4.0 (2)
8,9	32 mg/l	23.7	4	10	1	1.3 (3)
10,11	100 mg/l	21	4	10	1	4.0 (3)
12	Control	25	4	10	1	–
13	Unseeded control	26	4	10	–	–
14	3,5 dichloro-phenol 2.5 mg/l	25	4	10	1	0.1
15	10 mg/l	24.6	4	10	1	0.4
16	100 mg/l	21	4	10	1	4.0

Notes (1) Total volume in each flask = 40 ml
(2) Test substance stock solution = 100 mg/l
(3) Test substance stock solution = 1 g/l

APPENDIX

Let μ = specific growth rate of control culture
having an absorbance A_t at time t,

μ^1 = specific growth rate of inhibited culture
having an absorbance A_t^{1} at time t,

and A_o = absorbance of both cultures at time 0

then $\ln A_t = \ln A_o + \mu t$ \hfill (1)

and $\ln A_t^{1} = \ln A_o + \mu^1 t$ \hfill (2)

(This assumes no lag in and logarithmic growth over
the whole period.)

Subtracting (2) from (1):

$$\ln A_t - A_t^{1} = \mu t - \mu^1 t$$

Therefore $\mu^1 = \mu + \dfrac{1}{t} \ln \dfrac{A_t^{1}}{A_t}$

Now % inhibition of growth rate, I_g,

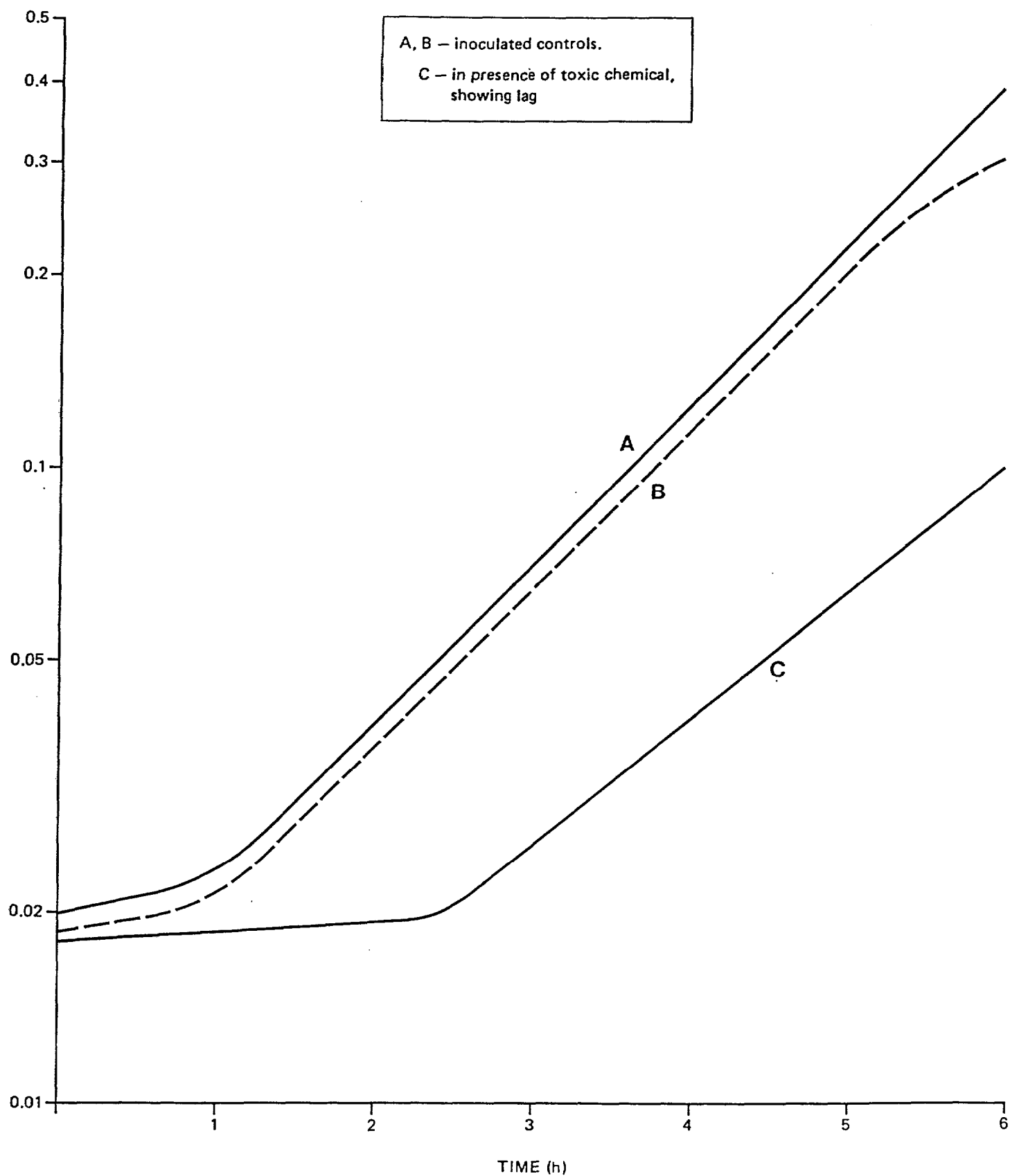$$= \left(1 - \frac{\mu^1}{\mu}\right) 100 = \left(1 - \frac{\mu - \frac{1}{t \ln A} A^1}{\mu}\right) 100$$

therefore $I_g = -\left(\dfrac{1}{\mu t} \ln \dfrac{A_t^{1}}{A_t}\right) 100$

The relationship between Ig and inhibition of
biomass (I_b) can thus be calculated, since

$$I_B = \left(1 - \frac{A_t^{1}}{A_t}\right) 100$$

If $A_o = 0.02$, it may be calculated that
for $t = 4h$ and $A_t = 0.3$, $\mu = 0.677$ h^{-1};
for $t = 6h$, $\mu = 0.451$ h^{-1}.

Figure 1. Observed types of growth curves

A, B — inoculated controls.

C — in presence of toxic chemical, showing lag

TIME (h)

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