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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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.
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

<table>
<thead>
<tr>
<th>Parameter determined</th>
<th>Toxicity (IC50) of soluble substances to aerobic microorganisms.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of sample</td>
<td>Pure substances, mixtures or industrial waste-waters.</td>
</tr>
<tr>
<td>Basis of methods</td>
<td>Comparison of bacterial growth rate in the presence and absence of varying amounts of test substance or waste-water.</td>
</tr>
<tr>
<td>Range of application</td>
<td>0-100% inhibition</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>?</td>
</tr>
<tr>
<td>Sources of error</td>
<td>Test substance colour, turbidity or tendency to precipitate in the test medium</td>
</tr>
</tbody>
</table>
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 ± 2 °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 ± 0.2 g Bacto nutrient broth and 6 ± 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 ± 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 °C for up to 5 days.
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 °C and a shaking speed of 150 rpm. Alternatively a shaker in a constant temperature room (22 ± 2 °C) may be used.

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

7. TEST PROCEDURE

<table>
<thead>
<tr>
<th>Step procedure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preculture</td>
<td></td>
</tr>
</tbody>
</table>

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).
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). Transfer.

(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

$$2.303 \times \text{slope of the line} = \mu \text{ h}^{-1}.$$

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 $\mu_c$ = specific growth rate of the inoculated control

$\mu_t$ = 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.)

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.

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

\[
\begin{align*}
&<1 \text{ mg/l} \\
&1 - 10 \text{ mg/l} \\
&10 - 100 \text{ mg/l} \\
&>100 \text{ mg/l}
\end{align*}
\]
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

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

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

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.

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

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
Let $\mu$ = specific growth rate of control culture having an absorbance $A_t$ at time $t$, then $\frac{1}{\mu} = $ specific growth rate of inhibited culture having an absorbance $A_t^1$ at time $t$, and $A_0$ = absorbance of both cultures at time 0

\[
\ln A_t = \ln A_0 + \mu t \tag{1}
\]

and \[
\ln A_t^1 = \ln A_0 + \frac{1}{\mu} t \tag{2}
\]

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

Subtracting (2) from (1):

\[
\ln A_t - \ln A_t^1 = \mu t - \frac{1}{\mu} t
\]

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

Now % inhibition of growth rate, $I_g$,

\[
= \left(1 - \frac{1}{\mu}\right) \times 100 = \left(1 - \mu - \frac{1}{\mu} \ln \frac{A_t^1}{A_t}\right) \times 100
\]

therefore $I_g = -\left(1 \ln \frac{A_t^1}{A_t} \right) \times 100$

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

\[
I_B = \left(1 - \frac{A_t^1}{A_t}\right) \times 100
\]

A1
If $A_o = 0.02$, it may be calculated that for $t = 4h$ and $A_t = 0.3$, $\mu = 0.677 \text{ h}^{-1}$; for $t = 6h$, $\mu = 0.451 \text{ h}^{-1}$. 
Figure 1. Observed types of growth curves

A, B – inoculated controls.
C – in presence of toxic chemical, showing lag
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