DEVELOPMENT AND EVALUATION OF A GROWTH INHIBITION TEST WITH SEWAGE BACTERIA FOR ASSESSING BACTERIAL TOXICITY OF CHEMICAL COMPOUNDS

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Abstract

To estimate the toxicity of chemical compounds to growing activated sludge bacteria a growth inhibition test with sewage bacteria has been developed. The growth of bacteria is estimated by determining the optical density at 530 nm (OD530). The calculation of toxicity is based on the estimation of biomass growth inhibition. A detailed test procedure was developed to provide reliable and reproducible results. The test system was evaluated with different phenolic compounds as reference substances. It was demonstrated that the substrate sensitivity of the test system was different from that of the respiration inhibition test. Furthermore, the results obtained proved to be in good agreement with literature data from other bacterial toxicity tests.

Keywords

Activated sludge, growth inhibition, respiration activity, substituted phenols, bacterial toxicity

1. Introduction

The estimation of the toxicity of chemicals to microorganisms is an important criterion for evaluating the environmental risk of chemicals in wastewater. Therefore, rapid and sensitive methods for the assessment of toxicity are of importance. In the last few years several test methods for assessing the ecotoxicological effects of chemicals have been developed. Currently, measurement of the respiration activity is the most widely accepted parameter and finds application in on-line toxicity monitoring systems and in laboratory toxicity screening assays (Dutka et
al., 1983; Broecker and Zahn, 1977; Brown et al., 1981; King, 1984; King and Painter, 1986; Green et al., 1975; Thibault and Tracy, 1978; Summers and Slon, 1981; Anderson et al., 1988, Pagga and Günther, 1981). Other bacterial test systems include the measurement of dehydrogenase activity (Strotmann et al., 1993a), β-galactosidase activity (Katayama-Hirayama, 1986), intracellular ATP level (Pill et al., 1991), uptake of glucose (Larson and Schaeffer, 1982) and activity of exoenzymes (Tubbing and Admiraal, 1991). Also the Microtox test (measurement of the luminescence of Photobacterium phosphoreum) (DIN 38412 part 34; also proposed as an International Standard) and the motility test with Spirillum volutans are used to estimate bacterial toxicity (Dutka et al., 1983; Dutka et al., 1978).

Methods based on the measurement of the growth inhibition of defined species have been described for Pseudomonas putida (Bringmann and Kühn, 1975), Bacillus thuringensis (Süssmuth et al., 1992) and Escherichia coli (Nendza et al., 1990).

The present method is based on a suggestion by Painter for an ISO standard (1987, personal communication). The objectives of this investigation were (I) to establish a test method for estimating growth inhibition by chemicals of a mixed culture of sewage bacteria, (II) to optimize test conditions in order to obtain reproducible and reliable results and (III) to evaluate the proposed test method with a variety of test chemicals. The data obtained in this test system can be used both for estimating the effect of chemicals on the growth of mixed bacterial cultures in an aquatic environment and for assessing non-inhibitory concentrations of chemicals for subsequent biodegradability tests.

2. Materials and Methods

Chemicals
The chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany), Riedel-de-Häen (Seelze, Germany) and Fluka (Neu-Ulm, Germany).

Respiration activity
Short-term respiration tests were performed according to OECD guideline 209 (OECD, 1984). Prior to testing, the pH of the activated sludge was adjusted to 7.0. Samples were incubated at room temperature for 30 minutes unless otherwise indicated. The respiration rate is defined as mg O$_{2}$ consumed l$^{-1}$ h$^{-1}$ and the specific respiration rate is the respiration rate per g of mixed liquor suspended solids (MLSS).

Inoculum
Municipal activated sludge was grown in a laboratory-scale wastewater treatment plant using municipal wastewater from the town of Frankenthal, Germany. The operational parameters of the treatment plant have already been described (Strotmann et al., 1993b). Industrial activated sludge was taken from BASF’s own wastewater treatment plant in Ludwigshafen.
Growth inhibition tests

Growth medium

The growth medium consisted of a phosphate buffer, a nutrient solution, a magnesium solution and a trace element supplementation. The phosphate buffer contained (per litre of deionized water) 8.5 g KH$_2$PO$_4$, 21.75 g K$_2$HPO$_4$ and 33.4 g Na$_2$HPO$_4$·2H$_2$O. The nutrient solution consisted of 8 g nutrient broth (Bacto) and 6 g sodium acetate (CH$_3$COONa) per litre. The magnesium stock solution contained (per litre) 20 g MgSO$_4$·7H$_2$O. The trace element solution according to Janssen et al. (1984) consisted of (per litre) 700 mg CaCl$_2$·2H$_2$O, 200 mg FeSO$_4$·7H$_2$O, 10 mg ZnSO$_4$·7H$_2$O, 10 mg H$_3$BO$_3$, 10 mg CoCl$_2$·6H$_2$O, 3 mg MnSO$_4$·H$_2$O, 3 mg Na$_2$MoO$_4$·2H$_2$O and 2 mg NiCl$_2$·6H$_2$O. The medium was prepared by mixing 635 ml water, 100 ml phosphate buffer, 250 ml nutrient solution, 10 ml magnesium solution and 5 ml trace element solution. The pH was finally adjusted to 7.0 ± 0.2 with 1 M HCl or 1 M NaOH.

Preparation of a preculture

A preculture was prepared by inoculating 20 ml of the growth medium with 0.5 ml of the supernatant of an activated sludge suspension which had settled for 5 to 15 minutes. The preculture was cultivated in 100 ml Erlenmeyer flasks with a baffle at the bottom. The flasks were filled with 20 ml of medium at most, and closed with cotton plugs. Incubation took place at 20 to 25°C on a rotary shaker at a shaking speed of 140 rpm. The incubation period was usually 16 to 20 hours.

Main culture and test cultures

A main culture was prepared by inoculating 200 to 300 ml of growth medium with 5 to 6 ml of a well grown preculture. The main culture was grown in a 1000 ml Erlenmeyer flask also with a baffle at the bottom. The conditions for cultivation were the same as for the preculture. After 1 to 2 hours of incubation the main culture usually reached the early exponential growth phase (OD$_{530}$ = 0.1 to 0.15) and was divided into test cultures of 20 ml volume in 100 ml Erlenmeyer flasks (Fig. 1). These flasks contained different amounts of the test compound and were incubated as described above. When possible test compounds were added as neutralized stock solutions. In a preliminary test 1, 10, 100 and 1000 mg/l of a test compound were used to estimate the range of concentrations needed for a definitive test. In a definitive test at least five concentrations in a logarithmic series were used.

Control cultures containing growth medium but no test compound were used to calculate the extent of inhibition. In general all test cultures were duplicated.

Measurement of growth and stabilization of samples

At regular intervals 1 ml samples were withdrawn from the cultures, transferred into Eppendorf vials containing 20 μl of a sodium azide solution (100 g/l) and thoroughly mixed. The samples were stored in this way at room temperature until measurement of growth. Other treatment methods included the addition of 30 μl NaOH (0.5 M), 20 μl HgCl$_2$ (10 g/l) or 25 μl HCl (5 M). Growth was estimated by measuring the optical density at 530 nm (OD$_{530}$) with a spectrophotometer against non-inoculated growth medium. The number of cells per ml of culture correspon-
Preculture prepared from activated sludge; incubation overnight

Main culture inoculated with preculture; preincubation for 1 to 2 hours to an OD530 of 0.1 to 0.15

Main culture is divided into test cultures; test compounds are added at different concentrations

Samples from test cultures are withdrawn at 30 to 60 minute intervals and treated with sodium azide

At the end of the test period (4 to 5 hours) the OD530 values are determined

Fig. 1. Flow diagram of the growth inhibition test

ding to a certain OD530 was estimated by microscopic enumeration with the aid of a Thoma counting chamber.

Calculation of inhibition of growth
Inhibition of growth was generally calculated by measuring the OD530 after a certain incubation period in the late exponential growth phase. The extent of inhibition is obtained by

\[ I = \frac{\left( \text{OD530 control} - \text{OD530 test} \right) \times 100}{\text{OD530 control}} \]

where I is the extent of inhibition (%), OD530 control is the OD530 of the control culture without test substance and OD530 test is the OD530 of the test culture. It is important to note that growth inhibition is calculated in this way in terms of inhibition of biomass production.
3. Results

Optimization of growth medium

The growth medium described by Painter (1987, personal communication) was optimized by adding 200 mg/l MgSO₄·7H₂O and 5 ml/l of a trace element solution according to Janssen et al. (1984). Without these supplements the growth of the inoculated bacteria was rather irregular, but the supplementation clearly stabilized growth properties. Therefore, the modified medium was used for all further experiments. At an incubation temperature of 25°C the generation time of the bacteria ranged between 1.0 and 1.25 hours, resulting in a growth rate of 0.69 to 0.55 h⁻¹. For cultures originating both from municipal and industrial sludge, growth was optimal at pH 7.0 to 7.5. At pH 6.0 growth was reversibly inhibited, resulting in a growth rate of 0.36 h⁻¹ whereas at pH 8.5 the growth rate was about 0.6 h⁻¹.

The relationship between the number of cells per ml and the optical density at 530 nm is shown in Fig. 2. It is obvious that there is no discrepancy between the different inocula indicating that the measurement of optical density is a reliable parameter for estimating growth even of different bacterial cultures.

![Graph showing the relationship between biomass production and OD530.](image_url)

**Fig. 2.** Relationship between biomass production (expressed as number of cells per ml) and OD530. The results were obtained with different inocula in a Thoma counting chamber.

Optimization of test performance

*Treatment of test cultures.* For a toxicity test one main culture (volume 200 to 300 ml) was prepared by inoculating the growth medium with a preculture grown overnight. It proved to be necessary to grow this culture for 1 to 2 hours to the early exponential growth phase (OD₅₃₀ = 0.10 to 0.15) before this main culture was divided into the different test cultures (volume: 10 to 20 ml). Otherwise growth of the different test cultures proved to be irregular, causing difficulties
in the calculation of growth inhibition. Therefore, the preincubation period was important for obtaining reliable and reproducible results.

*Treatment of samples for growth measurement.* The test compounds were added in the early exponential growth phase after a preincubation period of 1 to 2 hours. As the stationary phase was usually reached after 6 hours, about 4 to 5 hours remained for estimating growth inhibition. Therefore it was necessary to obtain a sufficient number of growth data to prepare reliable growth curves. To perform several assays at the same time it was useful for samples to be taken at different times and stored for measurement of the OD₅₃₀ until the test had been finished. To prevent further growth of the stored samples it was advantageous to add sodium azide up to a final concentration of 2 g/l. This treatment immediately stopped growth of the bacteria and the samples could be stored up to 24 hours. A treatment with HCl, NaOH, HgCl₂ caused rather irregular OD₅₃₀ measurements (Figs. 3 and 4B). Storage of the samples on ice is also possible but is not as safe as a sodium azide treatment. Therefore the sodium azide treatment method was used for all further studies. As shown in Figs. 4A and 5 reliable growth curves were obtained in this way.

**Evaluation of the test method with different reference chemicals**

The growth inhibition test was used to estimate the toxicity of different phenolic compounds. As an example a definitive test with 2,3-dichlorophenol is shown in Fig. 5. The data obtained in these tests are summarized in Table 1.

**Table 1.** Toxicity data with different phenolic compounds determined in the growth inhibition test. The different effective concentrations causing 20, 50 and 80 % growth inhibition (EC values) are indicated.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inoculum prepared from municipal activated sludge</th>
<th>Inoculum prepared from industrial activated sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₂₀ (mg/l)</td>
<td>EC₅₀ (mg/l)</td>
</tr>
<tr>
<td>Phenol</td>
<td>600 &gt; 1000 &gt; 1000</td>
<td></td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>50 92 &gt; 100</td>
<td></td>
</tr>
<tr>
<td>2.3-Dichlorophenol</td>
<td>30 60 &gt; 100</td>
<td></td>
</tr>
<tr>
<td>2.4-Dichlorophenol</td>
<td>22 53 100</td>
<td></td>
</tr>
<tr>
<td>2.5-Dichlorophenol</td>
<td>5 30 60</td>
<td></td>
</tr>
<tr>
<td>3.4-Dichlorophenol</td>
<td>20 26 42</td>
<td></td>
</tr>
<tr>
<td>3.5-Dichlorophenol</td>
<td>5 10 100</td>
<td></td>
</tr>
<tr>
<td>2.4-Dinitrophenol</td>
<td>430 700 &gt; 1000</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3. Treatment of samples for storage until estimation of the OD$_{530}$. Samples were treated with sodium azide and ice (A), NaOH, HCl and Hg(II)chloride (B) and the OD$_{530}$ was followed. Details of the treatment methods are described in the Materials and Methods section.

Fig. 4. Preliminary growth inhibition test with 2,3-dichlorophenol. The inoculum was prepared from municipal activated sludge. Samples were treated with sodium azide (A) and NaOH (B).
Fig. 5. Definitive growth inhibition test with 2,3-dichlorophenol. The inoculum was prepared from municipal activated sludge. The preincubation period before addition of 2,3-dichlorophenol was 1.5 hours. Growth pattern at different concentrations (A) and inhibition of growth based on biomass production (B) are shown.

Comparing the EC50 values obtained it is obvious that there is no clear discrepancy between the two different inocula. 3,5-Dichlorophenol proved to be most toxic with an EC50 of 10 mg/l and 5 mg/l for municipal and industrial inoculum. The lowest toxicity was observed for phenol with an EC50 of >1000 mg/l and 800 mg/l respectively. Due to its high toxicity 3,5-dichlorophenol therefore seems to be an appropriate reference substance for a sensitivity control of the test system.

The same reference substances were also tested in short-term respiration tests according to the method proposed by the OECD (1984). The data obtained are summarized in Table 2.

In the short-term respiration test an EC50 of 10 mg/l was obtained with 3,5-dichlorophenol the most toxic chemical compound tested. Phenol exhibited an EC50 of 300 mg/l and exhibited the lowest toxicity of the compounds studied.

4. Discussion

The data presented show that the methodology used is appropriate for determining the toxicity of chemical compounds to growing sewage bacteria. The results of the test give an accurate indication of the likely effect of a test compound to activated sludge bacteria. By performing the test at an incubation temperature of 20 to 25°C the usual temperature range for other bacterial toxicity and biodegradation tests was met (OECD, 1984; OECD, 1993). Nevertheless, the growth rate proved to be sufficient for completing the test in 6 to 8 hours. When using different types of sludge as a source of inoculum it was advisable to check the sensitivity of the microorganisms...
Table 2. Toxicity of different phenolic compounds in the short-term respiration test. The different effective concentrations (EC values) causing 20, 50 and 80 % inhibition are indicated. The tests were performed with activated sludge from the BASF wastewater treatment plant.

<table>
<thead>
<tr>
<th>Substance</th>
<th>EC20 (mg/l)</th>
<th>EC50 (mg/l)</th>
<th>EC80 (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>100</td>
<td>300</td>
<td>800</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>3</td>
<td>25</td>
<td>160</td>
</tr>
<tr>
<td>2,3-Dichlorophenol</td>
<td>32</td>
<td>120</td>
<td>320</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>30</td>
<td>55</td>
<td>80</td>
</tr>
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<td>54</td>
<td>85</td>
<td>300</td>
</tr>
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<td>15</td>
<td>38</td>
</tr>
<tr>
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<td>6</td>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>190</td>
<td>250</td>
<td>800</td>
</tr>
</tbody>
</table>

using a well-known reference substance (for example, 3.5-dichlorophenol). Furthermore, 3.5-dichlorophenol has been recommended as a reference substance for bacterial toxicity tests by the ISO and OECD (OECD, 1984; ISO, 1986).

After addition of the test compound the growth pattern of the inoculum varied in two ways: (I) growth inhibition after addition of the test compound could be followed by an increase in the growth rate, indicating a reversible type of inhibition and acclimatisation processes and (II) growth inhibition without a subsequent increase in the growth rate in the test period, indicating an irreversible type of inhibition. As it was difficult to calculate growth inhibition in the case of a reversible inhibition process by means of the growth rate it seemed to be appropriate to estimate toxicity by measuring the biomass production in a test period of 4 to 6 hours. Furthermore, it was possible to determine the type of inhibition by plotting the single growth curves of the test cultures.

The data in Table 1 show that the inhibitory potential of the tested phenols in the growth inhibition tests increased in the following sequence: phenol < 2.4-dinitrophenol < 3-chlorophenol < 2.3-dichlorophenol < 2.4-dichlorophenol, 2.5-dichlorophenol < 3.4-dichlorophenol < 3.5-dichlorophenol. By contrast, in the respiration test a higher toxicity of phenol, 3-chlorophenol and 2.4-dinitrophenol and a lower toxicity of 2.3-dichlorophenol and 2.5-dichlorophenol was found, indicating a different substrate sensitivity of the test system.

Previous studies on the toxicity of phenol and 3.5-dichlorophenol are in good agreement with the data obtained from the growth inhibition studies (Dutka et al., 1983; Klecka et al., 1985, Pagga, 1985). The toxicity of 2.4-dichlorophenol was similar both in the growth inhibition test and the respiration test. Comparable data have been described by Klecka et al. (1985), who stated an
EC₅₀ of 49.5 mg/l. Concerning 2.5-dichlorophenol Ewald (1987) described an inhibition of dehydrogenase activity by 50 to 60 % at a concentration of 20 mg/l, confirming the data of the growth inhibition tests (EC₅₀ = 30 to 35 mg/l). For 2.4-dinitrophenol a pH dependent inhibition of dehydrogenase activity has recently been described (EC₅₀ = 10 mg/l at pH 6.0) (Strotmann et al., 1993a). By contrast, in the growth inhibition tests a significant lower toxicity was found (EC₅₀ = 700 to 740 mg/l), also indicating a different substrate sensitivity of the growth inhibition test.

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5. References


Broecker, B. and Zahn, R. (1977). The performance of activated sludge plants compared with the results of various bacterial toxicity tests - a study with 3.5-dichlorophenol. Water Res. 11: 165-172


