MICROBIOLOGICAL CORROSION OF METALS—MARINE WOOD BORERS—RODENT ATTACKS ON STORED PRODUCTS—FOULING OF SHIPS BY BARNACLES—DETERIORATION OF STONE BY BACTERIA—ROTTING OF WOOD BY FUNGI—BACTERIAL BREAKDOWN OF ASPHALT—MILDEWING OF LEATHER—INSECT DAMAGE TO BOOKS—BIRD HAZARDS TO AIRCRAFT—FUNGI IN JET FUEL TANKS—TERMITES IN TIMBER—MICROBIOLOGICAL ATTACK ON RUBBERS PLASTICS AND PAINTS,— FUNGAL ETCHING OF GLASS.

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M. F. L. de Mele¹, R. C. Salvarezza¹ and H. A. Videla¹

Microbial Contaminants Influencing the Electrochemical Behaviour of Aluminium and Its Alloys in Fuel/Water Systems

Summary. Two of the most important microbial contaminants of jet fuel/water systems are Cladosporium resinae and Pseudomonas aeruginosa. It would be of great interest to know the role played by these microorganisms in the corrosion process of aluminium and its alloys. To elucidate the importance of each species on corrosion, we used the pitting potential as the electrochemical parameter simultaneously with evaluation of the biological variables: microorganism number and pH.

A marked decrease of pH and the pitting potential was observed in cultures of Cladosporium resinae but no appreciable change was found in these figures in the case of Pseudomonas aeruginosa. Experiments using mixtures of metabolites of both microorganisms confirm these results.

No direct action by cells on pitting potential values was demonstrated by our experiments.

Consequently the electrochemical behaviour of aluminium and its alloys in the presence of one or several species can properly be followed by the use of the pitting potential as the main experimental parameter.


Es ist von grossen Interesse zu wissen, welche Rolle diese Mikroorganismen in der Korrosionsproces des Aluminums und seiner Legierungen spielen.

Um die Wirkung jeder einzelnen Spezies auf die Korrosion zu klaren, haben wir das Lochfrasspotential als elektrochemischen Parameter benutzt, in Abhängigkeit der biologischen Werte wie die Mikroorganismenzahl und des pH werts.

Im Fall von Cladosporium resinae ergab sich eine bemerkenswerte Erniedrigung des pH Wertes und Potentials, die aber bei Pseudomonas aeruginosa nicht gefunden wurden. Versuche mit Mischungen aus den metabolischen Produkten beider Spezies bestätigen die oben genannten Ergebnisse. In keinem Versuch wurde eine direkte Wirkung der Zellen auf das Lochfrasspotential beobachtet.

Die Ergebnisse zeigen, dass das elektrochemische Verhalten des Aluminiums und seiner Legierungen in Anwesenheit einer oder mehrerer mikrobiologischer Spezies mittels des Lochfrasspotentials als bezeichnenden Parameter untersucht werden kann.

Introduction

Several attempts have been made to find a mechanism to explain the microbiological corrosion of aluminium and its alloys by microbial contaminants of jet fuel in fuel/water systems (Hedrick, Crum, Reynolds and

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the relative importance of each one of the biological species involved in the process (cf. Parbery, 1968) and the complex interrelation between biological and electrochemical parameters.

In recent years relevant progress has been made in knowledge of the microbiological aspects of the problem as well as on the development of different systems of protection (Parbery, 1971a, b; Sheridan, 1972; Tanaka, Shimizu and Fukui, 1968; Park, 1973; Cooney and Felix, 1972). Notwithstanding this, the electrochemical aspects of the process have not yet been properly studied.

Several studies on the corrosion of aluminium and its alloys in different inorganic media have used potentiostatic polarization techniques to study the corrosion process. The pitting potential (Ep) obtained from these curves is used as one of the principal electrochemical parameters, especially in the case of aluminium and its alloys which commonly present localised corrosion (Muller and Galvele, 1977; De Micheli, 1978). Pitting and intergranular corrosion is frequently found with these metals in the presence of microorganisms in fuel/water systems (Hedrick, Miller, Halkias and Hildebrand, 1964; Hedrick, 1970).

The pitting potential of a metal can be defined in a potentiostatic polarization curve as the potential below which the metal surface remains passive and above which pitting nucleates on the metal surface (Galvele, 1977). It is well known that the increase of the concentration of an aggressive substance in a medium produces a decrease in the Ep value (Galvele and De Micheli, 1970).

There is a scarcity of information on the metabolites produced during the growth of mixed cultures of bacteria and fungi in fuel/water systems as well as on the interaction between these microorganisms. For this reason we have concentrated our efforts on the study of the individual influence of each isolated species, especially those more frequently found in the microbial sludge of fuel storage tanks, such as Cladosporium resinae and Pseudomonas aeruginosa on the electrochemical parameters.

In our own earlier research work (Salvarezza and Videla, 1978) we have studied the role of Cladosporium resinae in the corrosion of aluminium 99.99% and two alloys. In that work the direct effect of metabolic products on the Ep decrease was reported.

The purpose of the present work was to study the influence of the microorganism number, the pH, and metabolites, on Ep values of aluminium 99.99% and its alloys for pure cultures of Ps. aeruginosa. To obtain a better understanding of the role of different microorganisms involved in the corrosion process, C. resinae was also included. Attempts were made to understand the behaviour of both species and their mixtures.

Experimental

Pure cultures of Ps. aeruginosa and C. resinae isolated from fuel storage tanks were used.

The composition of the solution employed as electrolyte and culture medium was:

- CaCl₂ - 1.8 x 10⁻⁴ M
- MgSO₄ - 1.7 x 10⁻³ M
- (NH₄)₂SO₄ - 7.6 x 10⁻³ M

is deionized water. Initial pH adjusted to 7.00 by addition of either H₂SO₄ 0.1 N, or NaOH 0.1 N.

Fungal and bacterial culture were performed in erlenmeyer flasks of 500 ml. The fuel to medium ratio was 1/1 (100 ml of sterile medium and 100 ml of JPI as carbon source) for the fungus and 4/1 (200 ml of sterile medium and 50 ml of JPI) for the bacteria.

Static conditions were chosen for C. resinae but in the case of Ps. aeruginosa cultures were agitated during the first 24 hours to provide an adequate oxygen supply. After growth was initiated, static conditions were used for this organism also. Temperature was maintained at 28°C. To estimate fungus growth, the mycelium weight method was adopted, while for bacteria the viable count method (nutirient agar) was employed.

Electrochemical measurements were made in a pyrex glass cell using aluminium 99.99%, SAE 332 and 2024 chemically polished probes of 1 cm² exposed area.

In Table 1 the composition of the two alloys is shown.

<table>
<thead>
<tr>
<th>Alloy designation</th>
<th>Cu</th>
<th>Mg</th>
<th>Zn</th>
<th>Si</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAE 332</td>
<td>3.50</td>
<td>0.53</td>
<td>0.08</td>
<td>10.00</td>
<td>0.40</td>
</tr>
<tr>
<td>2024</td>
<td>3.80</td>
<td>1.53</td>
<td>0.09</td>
<td>0.15</td>
<td>0.32</td>
</tr>
</tbody>
</table>

The counter electrode was a platinum wire. The potential of the working electrode was referred to a saturated calomel electrode through a Luggin capillary.

The experimental temperature was 28°C. Potentiostatic polarization curves were performed changing potential values by steps of 10 mV each minute. A previous de-aeration of the electrolyte with pure nitrogen was carried out before the anodic curve was run.

When polarization curves were performed to obtain Ep in the presence or in the absence of cells, two aliquots coming from the same culture were used. One of these was filtered through Gelman membranes (0.2 µm pore diameter) in order to remove the cells.

Results

The relation between the growth of *C. resinae* and the pH variation of the medium can be seen in Figure 1. The marked pH decrease is a consequence of the fungus growth; pH values in the range 4.0 to 5.0 are reached. A different effect is found for the strain of *Ps. aeruginosa*. Figure 2 shows the pH variation vs. the microorganism number for this bacteria. In this case, the pH values are between 6.0 and 7.0 for a wide range of microorganism concentration.

The Ep vs. pH relation for Al 99.99%, SAE 332 and 2024 alloys in the presence of *C. resinae* can be seen in Figure 3. In this figure, the values obtained at pH 7.0 correspond to the sterile medium. In all cases, the Ep values found in contaminated media (pH 4.0 and 5.0) are more cathodic than those corresponding to sterile controls. If aluminium and its alloys are compared, the highest values of Ep correspond to the aluminium.

On the other hand, Figure 4 shows the Ep variation as a function of the microorganism number in pure cultures of *Ps. aeruginosa* for Al 99.99% and the two alloys. Ep values obtained are similar to those corresponding to sterile controls except for the 2024 alloy which shows a small decrease of Ep.

In Figure 5, anodic polarization curves for Al 99.99% in active cultures of *Ps. aeruginosa* are compared to similar curves obtained using the cell free solutions. Figure 6 shows similar experiments for *C. resinae*. It could be deduced from these results that similar EP values are obtained either for active cultures or cell-free solutions.

Occasionally, potentiostatic polarization curves without electrolyte de-aeration were performed. The Ep values were similar to those found with previous pure nitrogen treatment.

To clarify the role played by different microorganisms in the process, several mixtures obtained from pure cultures were made. In these mixtures Ep values were determined. Figure 7 shows anodic polarization curves for Al 99.99% performed in a sterile medium, in a *Ps. aeruginosa* culture and in a mixture of metabolites. Figures 8 and 9 show similar results for SAE 332 and 2024 alloys.

Discussion

The electrochemical nature of the corrosion process of metals remains valid whether biological agents are present or not (Booth, 1971). Consequently, the direct or indirect participation of microorganisms in the process could be followed by the evolution of the electrochemical parameters such as corrosion potential and Ep. In the case of aluminium and its alloys the Ep value assumes a particular importance (Wexler and Galvele, 1974).

Several authors have observed a marked decrease of

**Figure 4.** Relation between $E_p$ and number of organisms in media containing *Pseudomonas aeruginosa.*

- Aluminium 99.99% - ○
- SAE 332 - ■
- 2024 - ×

**Figure 5.** Anodic polarization curves for aluminium 99.99% in *Cladosporium resinae* with and without cells.

- With cells - ■
- Without cells - ×

**Figure 6.** Anodic polarization curves for aluminium 99.99% in *Pseudomonas aeruginosa* media with and without cells.

- With cells - ■
- Without cells - ×

**Figure 7.** Anodic polarization curves for aluminium 99.99%.

- In sterile control - ○
- In *Ps. aeruginosa* culture - ■
- In mixture of metabolites (1/1 equivolume mixture) - ×

pH due to metabolites produced during the growth of *C. resinae* (Hendey, 1964; Parbery, 1968). Different research works are related to the metabolic breakdown of hydrocarbon by various species of bacteria. The principal pathway of n-alkanes degradation involves the terminal oxidation through the alcohol and the aldehyde to the monooic acid. The fatty acids produced enter in the tricarboxylic acid cycle via acetyl CoA. Only recently some information has been given on the metabolic utilization of hydrocarbon by several species of fungi. Citric, cisconitic, isoconitic, α-keto-glutaric and oxal-acetic acids are mentioned among other metabolic products in the case of *C. resinae* (McKenzie, Akbar and Miller, 1977; Walker and Cooney, 1973). Different authors attribute the main responsibility for the corrosion process to these acids. It has already been reported that the formation of citric acid by *C. resinae* on undecane, reached 91% of the total carboxylic acids produced in 17 days of culture (Lin, Iida and Itzuka, 1971).

Our results, using Ep as the electrochemical parameter to evaluate the degree of medium aggressivity, show that the metabolites produced by the fungus during growth are the principal agents of the Ep decrease (Salvarezza and Videla, 1978).

Nevertheless, the electrochemical behaviour of aluminium and its alloys in the presence of citric acid is different from that corresponding to active cultures of *C. resinae*. This suggests that unidentified metabolic products could probably play a relevant role in the corrosion process. In this respect, the selective attack on different mineral components of alloys (e.g. magnesium) is very significant.

When other species of microorganisms such as bacteria of genus *Pseudomonas* are present, the scope of the influence of the metabolites on the process becomes more complex.

Unlike *C. resinae*, the strain of *P. aeruginosa* used in this work does not produce significant changes of the medium pH. While the cells are viable, the pH values remain near neutrality. Coincidently, Bushnell and Haas (1941) using different strains of the genus *Pseudomonas* metabolizing hydrocarbons, reported a slight acidification of the medium.

From our experimental results, it can be seen that bacterial growth has a behaviour for aluminium and its alloys dissimilar from that of *C. resinae*. On this subject, it is interesting to remark the correspondence of these results with those of Guillaume, Brisou et al., (1977) on the action of different bacteria on copper, zinc, nickel and aluminium in a sea water environment. An inhibiting action by living bacteria, is indicated by these authors. Only in the case of death of the bacteria are metabolic products which are free in the medium able to produce a corrosion effect.

On this subject our results performed in the absence of cells show no difference with reference to Ep values, from those obtained in the presence of microorganisms. This suggests an Ep dependence on metabolites production.

The use of electrochemical parameters (such as Ep or corrosion potential) enables us to follow the modification of the medium aggressivity simultaneously with the evolution of biological variables.

In our case the Ep seems to be a good experimental parameter to decide the influence of each microbiological species on the corrosion process. Likewise, this parameter is useful to distinguish between the direct action of biological species and metabolic products on corrosion.

References


**EVALUATION OF A NEW LABORATORY DECAY TECHNIQUE USING SERPULA LACRYMANS**

J.D. Thornton

**Summary.** The work describes mass loss and moisture content data obtained every three weeks during decay of *Pinus radiata* D. Don sapwood by *Serpula lacrymans* Gray in covered metal trays. Each tray contained 36 blocks placed above the agar substrate by means of glass tubes. These conditions were conducive to steady, continual mass loss increases throughout the 12-week test. Statistical analysis of data from four replicate trays inoculated with a single isolate indicates that for the technique to detect differing decay by several cultures it would be necessary to have tray replication for each culture. Although no significant effect of either tree origin or within-tray block location was detected, it is thought wise in future to adopt complete block randomization within each tray.

**Introduction**

Standard laboratory testing of the natural durability of timber comprises chiefly agar-flask (Cartwright and Findlay 1958) and soil-jar (ASTM D2017-71, 1971) methods, or variations on these two techniques. Essentially these standards employ one test block per vessel and are, therefore, labour-intensive techniques. This problem can no doubt be lessened somewhat by the adoption of multiple-block test vessels and it was thought desirable to develop a suitable test method for use with basidiomycetous fungi. Preliminary studies (Thornton 1979a) formulated a method using glass boiling tubes to support blocks above the agar. This paper reports testing and evaluation of this multiple-block decay technique using sapwood of *P. radiata* (radiata pine) and employing several trays, each with the same fungal isolate. Although this technique is intended as a normal 12-week decay test, for evaluation purposes sequential harvesting of blocks was performed.

**Materials and Methods**

**Timber substrate**

Sapwood of three trees of *Pinus radiata* was machined to produce blocks 19 x 19 x 25 mm, with the last measurement along the grain. Blocks from each of the trees were then selected so that they all had similar, narrowly spaced growth rings and visually appeared low in resin content of latewood bands.

**Test fungi**

The culture used was an isolate of the true dry rot fungus *Serpula lacrymans* (DEP 16508) which was originally obtained from decayed floorboards of a Melbourne residence in 1977, with the aid of the specific isolation medium of Coggins and Jennings (1975).

Between its isolation and use in the present test the culture was maintained on 1.25% malt extract (1.0 – 1.25% agar) at 18–20°C.

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Evaluation of a new laboratory decay technique using *Serpula lacrymans*, J.D. Thornton.

Decay technique

The method used is illustrated in Figure 1. Briefly this involves the pacing of large boiling tubes (which for the present test were of internal diameter 27 mm, length 180 mm, and external diameter across the mouth of 36 mm) in the base of a large metal tray so that there is a gap (approximately 3 mm) between adjacent tubes along their length. A solution of 1.0% malt extract (15% agar) is poured into the tray to reach a height approximately equal to the radial measurement of the tubes. After autoclaving, the inoculum is allowed to grow so that it reaches the uppermost point of the tubes' outer surfaces. Sterilized test blocks are then placed above the spaces between adjacent tubes so that each row of blocks is supported by two adjacent tubes. An aluminium foil cover is placed over the top of each tray prior to incubation at 20 ± 1°C.

For a normal test of 12-weeks' duration, test blocks would usually be randomly placed within each tray. However, for the present tests, where some blocks were to be removed after three, six, nine and 12 weeks'"
Evaluation of a new laboratory decay technique using *Serpula lacrymans*, J.D. Thornton.

incubation, blocks were segregated into three groups, each of which corresponded to the same tree of origin. This restriction of randomization was performed solely for ease and speed of block removal to exclude contaminating microorganisms. The layout of blocks and their times of removal from test are shown in Figure 2.

Results relate to four replicate trays, each inoculated with *S. lacrymans* culture 16508, and three sterile trays from which the fungus was omitted.

Block conditioning and reconditioning was to 12 per cent equilibrium moisture content in every case and, following each removal time, appropriate measurements were made in order to calculate the mass losses and moisture contents of all blocks at removal.

Results and Discussion

Table 1 presents data illustrating between-tray moisture content variation of timber in the absence of decay. By the end of three weeks' incubation blocks have increased from their initial 12 per cent moisture content to the value expected for fibre saturation (around 32 per cent) and thereafter increase only a few per cent throughout the remaining test periods. This uniformity of moisture content of uninoculated timber contrasts with those variable moisture contents obtained in this laboratory using standard soil-jar testing (Tam and Thornton 1978; Thornton 1979b).

Data concerning block mass loss following inoculation with *S. lacrymans* are given in Table 2. A standard three-way analysis of variance was performed on the mass loss values of all replicate blocks. This showed a significant effect (at the 0.1% level) of time on mass loss. It would of course be expected that mean mass loss values would increase fairly uniformly with time. However, this contrasts with determinations made in a soil-jar test with radiata pine sapwood blocks and the white rot fungus *Fomes lividus* (Kalch.) Sacc. where the author found a marked reduction in the rate of mass loss at later measurement times with no significant differences between the 10 and 12 week determinations, even though mass losses had by then reached only around 30 per cent (Thornton 1979b). Whether or not *F. lividus* would exhibit continual mass loss increases using the present test method is not known at the moment. Statistical analysis showed the only other significant variable to be that of the tray itself (at the 0.1% level), this variation being entirely due to the significantly lower (at the 0.1% level) mass losses achieved in tray number 2 (Table 2). Whether or not *F. lividus* would exhibit continual mass loss increases using the present test method is not known at the moment. Statistical analysis showed the only other significant variable to be that of the tray itself (at the 0.1% level), this variation being entirely due to the significantly lower (at the 0.1% level) mass losses achieved in tray number 2 (Table 2). This finding of greater variation between trays than within trays was also obtained using the soil-tray technique (each tray containing nine blocks) of Greaves (1977). These results show, therefore, that in order to detect significant differences between a number of fungi, or mixed populations of fungi, using multiple-block techniques, replication of containers is essential.

Data concerning block moisture content at removal from incubation with *S. lacrymans* isolate DEP 16508

| Tray no. | Time in weeks | Table 1
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>32(2)</td>
<td>35(3)</td>
</tr>
<tr>
<td>2</td>
<td>31(1)</td>
<td>34(4)</td>
</tr>
<tr>
<td>3</td>
<td>33(3)</td>
<td>34(5)</td>
</tr>
</tbody>
</table>

Table 2

Decay of radiata pine blocks after inoculation of trays with the same isolate of *S. lacrymans*.

| Tray no. | Time in weeks | Table 2
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5.2(6.6)</td>
<td>21.4(6.1)</td>
</tr>
<tr>
<td>2</td>
<td>1.7(2.1)</td>
<td>14.8(6.6)</td>
</tr>
<tr>
<td>3</td>
<td>4.8(5.6)</td>
<td>21.3(6.5)</td>
</tr>
<tr>
<td>4</td>
<td>4.2(5.3)</td>
<td>21.0(4.0)</td>
</tr>
</tbody>
</table>

* Mass loss values presented are those following correction for sterile controls.

| Tray no. | Time in weeks | Table 3
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>43(4)</td>
<td>54(4)</td>
</tr>
<tr>
<td>2</td>
<td>42(4)</td>
<td>46(5)</td>
</tr>
<tr>
<td>3</td>
<td>42(2)</td>
<td>54(7)</td>
</tr>
<tr>
<td>4</td>
<td>42(3)</td>
<td>50(3)</td>
</tr>
</tbody>
</table>

are presented in Table 3. After only three weeks' incubation, mean block moisture contents were higher than any recorded for the sterile controls (Table 1),
Evaluation of a new laboratory decay technique using *Serpula lacrymans*, J.D. Thornton.

which would be expected since block decay (Table 2) had begun during this three-week period. Again a standard analysis of variance was performed on moisture content readings. Significant effects (at the 0.1% level) on moisture content were found to occur with tray and time. As with mass loss data (Table 2) the significant tray variation was due entirely to significantly lower (at the 0.1% level) moisture contents of blocks in tray number 2 (Table 3).

The data presented here concern a single fungus species which may be said to exhibit exceptional powers of colonization and translocation. However, a range of Basidiomycetes have now been used with this technique and reproducibly high mass losses have been achieved (Thornton, unpublished data).

Although this technique was designed initially for natural durability testing, it is currently in use in this laboratory for the screening of potential preservative formulations against Basidiomycetes (Thornton and Greaves 1978). The use of preservative treated wood probably necessitates a higher inoculum potential than for untreated timber, and volatile preservatives would be expected to cause problems in a multiple-block situation. For these reasons this technique has so far been used only with leached blocks and with the addition of “feeder strips” above the glass supports (Thornton and Greaves 1978).

Conclusions

The multiple-block decay technique assessed here with *S. lacrymans* provided a continuous and steady decay of the sapwood of the softwood timber *P. radiata* to reach a reasonable value for the decay at the end of 12 weeks’ test period. In the absence of fungus the timber increased from an initial moisture content of 12 per cent to a fibre saturation value (30 to 34 per cent) within three weeks. At this moisture content, initial fungal attack could normally be expected to occur. Using this technique blocks do not become too wet for decay to be expected to continue throughout the duration of a standard laboratory test period.

Although no significant mass loss variation due to either tree origin or spatial distribution of blocks within tray was detected, when using the technique as a 12-week decay test it would be wise to randomly distribute all blocks within each tray.

If, using this technique, direct comparisons of different fungi are required then tray replication is essential for each and every isolate employed.

Acknowledgements

The author acknowledges the technical assistance of Ms. O. Collett. Thanks are also due to Dr. D. Kildea, Division of Mathematics and Statistics, CSIRO, for statistical analyses.

References


THE ANTI-MOLLUSCAN BORER EFFICACY OF SOME BENZYPHENOLS

John D. Bultman and Leonard Jurd

Summary. A series of benzyl monoalkyl- and dialkylphenols were evaluated for their ability to protect pine from teredinids and pholads. The most effective compound at 0.5% concentration in the wood was 2-benzyl-4,6-di-t-butylphenol (I); removal of the t-butyl group from the 6-position of the phenolic ring completely destroyed this activity toward teredinids, and decreased it toward pholads. The other phenols were less effective than I, however wood impregnated at a concentration as low as 0.8% with those compounds containing a 1-phenylethyl substituent was only lightly attacked by teredinids; wood impregnated with phenols containing a 4-methoxybenzyl or benzyl substituent was only lightly attacked by pholads. The mode of action of the active benzylphenols is not known.

Introduction

As part of a study on the natural resistance of tropical American woods to wood-destroying organisms (Bultman and Southwell, 1976; Southwell and Bultman, 1971; Bultman, 1974), a series of benzyl monoalkyl- and dialkylphenols, many of which have shown repellent or other biological activity against terrestrial organisms, (Jurd, 1976; Fridantseva and Voldkin, 1974), have received a preliminary evaluation as potential anti-marine-borer wood preservatives.

Experimental

Pine sapwood discs (6.5 mm x 50 mm diameter), previously dried by a solvent extraction procedure (Stamm, 1964) to increase their porosity, were impregnated with acetone solutions containing sufficient of each of the benzylphenols to provide a concentration of the solute in the wood varying from less than one to nearly four per cent. A modified Bethel full-cell vacuum/pressure technique was employed (Hunt and Garriott, 1953). The treated wood was exposed in the marine environment at Naos Island, Canal Zone (Pacific) for three months. Representative of the marine borer population at this site are Teredo panamensis, T. bartschi, and Martesia straita; no limnophiles have been observed at this site for several years. The treated wood was sufficiently immersed to be one meter below the water surface at low tide. Wood damage was estimated using a subjective rating system which combined the results of a visual inspection of a number of x-ray prints with the average number of organisms in the specimens, as determined by microscopic examination.

Results and Conclusion

Several of the benzylphenols provided some protection against teredinids, pholads or both, as shown in Table 1. However, the most effective compound was...

2-benzyl-4,6-di-t-butylphenol (I), a compound also used as a stabilizer and anti-oxidant for rubber and polymeric hydrocarbons (Green, 1965; Spacht, 1960). Wood impregnated with I at concentrations of 1.8 to 3.8 per cent was undamaged (Figure 1). At 0.9 per cent, the lowest concentration employed, the wood discs were invaded only by one small teredinid and four small pholads, although there were many abandoned entry pits in the wood surface. While the other phenols were less effective than I, most of the wood impregnated with compounds containing a 1-phenylethyl substituent, e.g., 2-(1-phenylethyl)-4-ethyl phenol (II), at a concentration as low as 0.8 per cent was only lightly attacked (rating = 1) by teredinids; wood treated with most of the phenols containing a 4-methoxybenzyl substituent, e.g., 4-(4-methoxybenzyl)-2,6-di-t-butylbenzophenol (III), or a benzyl substituent, was only lightly attacked by pholads. Interestingly, the removal of the 6-t-butyl substituent from I destroyed its activity against teredinids.

The mode of action of the active benzylphenols on teredinid and pholad larvae is not known. They may be acting as larvicides, or they may function on the wood surface as repellents which discourage the settlement of the larval forms. Highly hindered phenols such as I and III, are insoluble in water, are relatively inert chemically, and can be synthesized cheaply and easily from phenol. These compounds, therefore, may prove to be of practical use as preservatives for wood in the marine environment. Further studies on the relationship between the molecular structure of benzylphenols and antiborer efficiency are in progress.

References


Green, H.A. (1965). Synthetic polymeric hydrocarbon compositions stabilized with an arylmethylene ether

Figure 1. X-ray photographs of pine sapwood disks. Those on the left contain 1.8% of 2-benzyl-4,6-di-t-butylphenol, those on the right are untreated controls. The damage to the controls was caused primarily by teredinids (shipworms).
Table 1

Summary of the exposure data for the benzylphenols showing the average damage rating for each set of treated wood. Rating scale: 0 = no apparent damage, 1 = light damage, 2 = moderate damage, and 3 = heavy damage.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Solute Concentration</th>
<th>Average Rating of teredinids pholads</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-benzyl-4,6-di-t-butylphenol</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>1</td>
</tr>
<tr>
<td>2-(1-phenylethyl)-4,6-di-t-butylphenol</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>2</td>
</tr>
<tr>
<td>2-(4-methoxybenzyl)-4,6-di-t-butylphenol</td>
<td>0.7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>2</td>
</tr>
<tr>
<td>4-(1-phenylethyl)-2,6-di-t-butylphenol</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>2-(1-phenylethyl)-4-ethylphenol</td>
<td>3.1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>1</td>
</tr>
<tr>
<td>4-(4-methoxybenzyl)-4-ethylphenol</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>2-[1-(4-methoxyphenyl)-propyl]-4,6-di-t-butylphenol</td>
<td>3.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>1</td>
</tr>
<tr>
<td>2-benzyl-4-methylphenol</td>
<td>3.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>4-benzyl-2-butylphenol</td>
<td>3.6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>Acetone controls</td>
<td>-</td>
<td>3</td>
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<tr>
<td>Untreated controls</td>
<td>-</td>
<td>3</td>
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CG ....................................................... 6
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FJ Timber .......................................... 1
FL Bacterial attack ............................... 1
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CO-OXIDATION OF 2,4-DICHLOROPHENOXYPENTACETATE BY PSEUDOMONAS SP.

S.R. Bauer1, E.M. Wood2 and R.W. Traxler2

Summary. A Pseudomonas species was isolated by enrichment culture on sodium benzoate. It did not utilize 2,4-dichlorophenoxycacetate (2,4-D) as a sole source of carbon and energy but did co-oxidize 2,4-D when growing on sodium benzoate. The p-chloro group was removed from the ring structure. Studies with 14C-labeled 2,4-D showed removal of the acetate moiety of the co-substrate. Radiochromatography demonstrated the formation of a co-oxidation product.

Co-oxidation du 2,4-dichlorophenoxycacetate par Pseudomonas sp. Un Pseudomonas a été isolé en culture enrichie sur benzoate de sodium. On n’a pas utilisé le 2,4-dichlorophenoxycacetate (2,4-D) comme seule source de carbone et d’énergie mais la co-oxydation du 2,4-D par croissance sur benzoate de sodium. Le groupe chloro en position Para a été enlevé de la structure du noyau. Des études avec du 2,4-D marqué en 14C ont montré la coupure de la partie acétate du co-subsatrate. Une radiocromatographie a montré la formation d’un produit de co-oxidation.

Introduction

When organic molecules are introduced into soil or water environments, many different microbiological phenomena determine the fate of the molecules. The material may be completely oxidized to carbon dioxide and water, it may be transformed without modification of the carbon skeleton, it may be partially oxidized or it may not be modified by the resident microbial population.

The phenomenon of co-oxidation was introduced by Leadbetter and Foster (1959) to describe oxidation of ethane to acetic acid by Pseudomonas methylacida deriving growth energy and carbon from methane. Some confusion exists today regarding this process since it has been modified and expanded in concept (Jensen, 1963; Horvath and Alexander, 1970; Raymond Jamison and Hudson, 1967).

The phenomenon of co-oxidation requires the involvement of two substrates, a growth substrate and a second substrate which is not modified in the absence of the growth substrate. In addition, there is some accumulation of a partially oxidized product of the non-growth substrate.

To the practicing microbiologist concerned with the fate of organic molecules, co-oxidation is a real phenomenon. It may be a practical event, however, rather than a specific physiological event explained by a universal mechanism. The explanation of the phenomenon may in different systems be related to different physiological mechanisms such as repression, enzyme specificity, adaptation or membrane permeability.

This report describes the ability of a soil pseudomonad to co-oxidize 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of sodium benzoate, and will be used as a basis for determining the mechanisms of co-oxidation of this system.

Materials and Methods

Isolation and culture conditions

The Pseudomonas sp. used in this study was isolated by enrichment culture from garden soil in M9 medium (Roberts et al., 1955) containing 0.1% (w/v) of sodium benzoate at pH 7.0. The isolate was identified as a member of the genus Pseudomonas by comparison of its characteristics with those of the genus description in Bergey’s Manual (1974). Bacteria were grown in 250 ml of medium in a 1 liter flask on a gyrotory shaker at 120 rpm for 24 hours at 27°C. The cells were harvested by centrifugation at 2840 RCF for 15 minutes, washed three times and suspended in 0.02 M phosphate buffer, pH 7.0. Reactions were performed in Warburg flasks containing 5-7 mg cells (dry weight) suspended in phosphate buffer and the compound(s) tested. The total flask content was 3.2 ml. Growth experiments were performed in 250 ml flasks containing 50 ml of medium.

14C-labeled experiments.

Experiments using labeled 2,4-D (ring-UL-14C, (Received November 1978; in revised form, February, 1979).
carboxyl-\textsuperscript{14}C, acetic-\textsuperscript{14}C; Mallinckrodt) were performed in 250 ml flasks containing 50 ml of medium at 27°C in a gyratory water bath shaker. Each flask contained 1.08 mg of unlabeled 2,4-D (Eastman Organic Chemicals), 14.18 mg of sodium benzoate (Mathewson, Coleman & Bell), 0.32 mg of \textsuperscript{14}C-labeled 2,4-D, and 20 mg of cell suspension. Air was passed through ascariate (Arthur H. Thomas Co.) to remove CO\textsubscript{2}, then through the reaction flask to carry metabolic CO\textsubscript{2} into two, 5 ml methanol: monoethanolamine (4:1) traps. The traps were changed at 2 hr. intervals to prevent over saturation and all traps from a reaction vessel combined before counting. The total volume from the traps was divided into 2.5 ml aliquots and added to 12 ml of scintillation cocktail. The cells were collected at the end of the experiment by centrifugation, washed on anurn before counting. The total volume from the traps was divided into 2.5 ml aliquots and added to 12 ml of scintillation cocktail. The cells were collected at the end of the experiment by centrifugation, washed on an

\textit{Pseudomonas sp.}, isolated on sodium benzoate, was shown previously (Bauer and Traxler, 1977) to grow on either sodium benzoate or a mixture of sodium benzoate and 2,4-D but not 2,4-D as a sole source of carbon and energy. Oxygen uptake by cell suspensions on the mixture of sodium benzoate and 2,4-D was shown to be greater than on sodium benzoate alone and oxygen uptake on 2,4-D alone was equal to the endogenous oxygen uptake of the isolate (\textit{loc. cit.}). This information suggested that the \textit{Pseudomonas} utilized 2,4-D as a co-oxidizable substrate when growing on sodium benzoate.

\textbf{Results and Discussion}

Growth of this \textit{Pseudomonas} sp. on the indicator medium of Loos (1975) indicated that chloride was removed from 2,4-D. A quantitative test in growth medium containing both sodium benzoate and 2,4-D indicated that 24% of the available chloro groups were removed from the 2,4-D molecule (Table 1). The organism was able to partially dechlorinate 2,4-D in the absence of the growth substrate indicating that this reaction is not dependent upon co-oxidation. However, there was a significant increase in the amount of chloride released from the ring structure if sodium benzoate was present. Evans, Smith, Fernly and Davies, (1971) showed that in the dissimilation of 2,4-D the first metabolic step was the removal of the p-chloro group.

\begin{table}[h]
\centering
\caption{Chloride ion release by a \textit{Pseudomonas} sp. from 2,4-D in 7 day flask experiments}
\begin{tabular}{lcc}
\hline
Substrate(s) & \text{\textmu} \text{moles} & \% available \\
\hline
2,4-D & 1.006 & 8 \\
2,4-D + Sodium benzoate & 3.08 & 24 \\
Sodium benzoate & 0.0 & 0 \\
2,4-D is 2,4-dichlorophenoxyacetate. & & \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{2,4-D lost from Warburg vessel after 3 hours incubation}
\begin{tabular}{lccc}
\hline
Substrate(s) & \text{\mu}2,4-D & \text{\mu}2,4-D & \% lost \\
& added & recovered & \\
\hline
2,4-D & 84 & 81.1 & 3.5 \\
2,4-D + Sodium benzoate & 84 & 63.9 & 23.9 \\
2,4-D is 2,4-dichlorophenoxyacetate. & & & \\
\hline
\end{tabular}
\end{table}

\textbf{Décchlorination}

Chloride ion release was demonstrated in the indicator medium of Loos (1975) and by a modification of the Mohr method (Skoog and West, 1969) in which the silver nitrate was reduced 10-fold to increase the sensitivity of the reaction.

\textbf{Analytical techniques}

2,4-D was quantitated as the ethyl ester in a Tracor Mikrotek model 220 gas chromatograph equipped with a 1.5% OV-17/1.95% QF-1 on 100/120-mesh Supelcoport 4' x 6' glass column at 200°C with the nitrogen carrier at 70 ml/min. The electron capture detector was set at 325°C. The 2,4-D was extracted from the aqueous medium by diethyl ether at pH 3.0, washed by transferring back to the water phase at pH 10.0 and re-extracted into ether at pH 3.0. The final ether phase was protected with 1 ml of 0.01% mineral oil in hexane and then evaporated to dryness. The acids were esterified by the method of Woodham, Mitchell, Loftis and Collier, (1971).

Paper chromatography was used to distinguish 2,4-D and possible metabolites. Whatman No. 1 paper was prewashed in distilled water and dried before spotting. The solvent system was n-butanol saturated with 1.5N NH\textsubscript{4}OH. The indicator spray was 0.04% (w/v) bromocresol purple in formaldehyde ethanol (1:5) followed by exposure to ammonia vapor. When \textsuperscript{14}C-labeled substrates were used, the solvent was run for 16 cm and each solvent path was cut into 1 cm segments for analysis in the liquid scintillation counter (Nuclear Chicago, Mark 1).

The co-oxidation system showed 24% loss of available 2,4-D (1 mg/flask) by gas chromatographic analysis (Table 2) during the short term experiments. This data does not represent total potential degradation of 2,4-D in the system. The appearance of a partial oxidation product at 14 cm in a system designed to separate the aromatic chloro compounds (Fig. 2) suggests that the product contains at least one chloro group.

**Carbon dioxide release**

In order to establish the moiety of the 2,4-D modified in this co-oxidation system, 2,4-D $^{14}$C-labeled in the carboxyl group, methylene carbon or uniformly ring-labeled was amended with unlabeled sodium benzoate and metabolic CO$_2$ measured for radioactivity (Table 3).

Measurement of $^{14}$C radioactivity associated with the cells and culture filtrate demonstrated that there was 2,4-D uptake by the cells but that the major portion of the 2,4-D or its metabolic product was located in the culture filtrate. There was $^{14}$CO$_2$ produced from both the carboxyl- and methylene-labeled 2,4-D indicating that the acetate group was oxidized in this system. There was no $^{14}$CO$_2$ detected from 2,4-D labeled in the ring indicating that co-oxidation does not result in dissimilatory ring cleavage products.

**Radiochromatography**

The filtrates obtained from sodium benzoate+2,4-D (labeled)- grown cultures were extracted to recover 2,4-D and its co-oxidation products. The extracts were chromatographed on paper which was then cut into 1 cm segments for radioactivity measurement. The results from the 2,4-D labeled in the carboxyl or methylene positions are shown in Figure 1.

There were no differences in the peak geometries of the radioactive compounds recovered from co-oxidation filtrates generated from acetate-labeled 2,4-D. The uniform peak geometries mean the compounds detected from each flask represented unreacted 2,4-D. The only compounds which were detected by this method contain either all or part of the acetate group.

---

**Figure 1.** Radio-chromatograph of culture filtrates from sodium benzoate-2,4-D labeled in the COOH or -CH$_2$- positions.

- $\bigcirc$ Reference 2,4-D
- $\bullet$ COOH labeled 2,4-D
- $\triangle$ -CH$_2$- labeled 2,4-D

**Figure 2.** Radio-chromatograph of culture filtrates from sodium benzoate-2,4-D labeled in the ring or COOH positions.

- $\blacktriangle$ Reference 2,4-D
- $\blackcircle$ COOH labeled 2,4-D
- $\blacklozenge$ Ring labeled 2,4-D

Table 3
Distribution of $^{14}$C activity in co-oxidation from 2,4-D labeled in various positions

<table>
<thead>
<tr>
<th>Label Position</th>
<th>Fraction</th>
<th>DPM $^{14}$C Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ring UL</td>
<td>CO$_2$</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Cells</td>
<td>$5.5 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>Filtrate</td>
<td>$1.7 \times 10^6$</td>
</tr>
<tr>
<td>-COOH</td>
<td>CO$_2$</td>
<td>$7.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Cells</td>
<td>$3.2 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>Filtrate</td>
<td>$6.0 \times 10^6$</td>
</tr>
<tr>
<td>-CH$_2$-</td>
<td>CO$_2$</td>
<td>$2.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Cells</td>
<td>$3.1 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>Filtrate</td>
<td>$5.1 \times 10^3$</td>
</tr>
</tbody>
</table>

attached to a chlorinated benzene nucleus. This data indicated that any co-oxidation product formed from the sodium benzoate-2,4-D system did not contain the acetate moiety of 2,4-D.

Figure 2 is a similar plot of the co-oxidation mixture containing ring-UL-2,4-D. In this case the radiochromatogram shows a modified peak geometry and the formation of a new peak at 14 cm from the origin. The new peak represents a chlorinated benzene compound with at least one polar group and lower molecular weight than 2,4-D. This observation, coupled with evidence for decarboxylation of the acetate moiety, no evidence for disimilatory ring cleavage and dechlorination from the ring moiety, suggests that this Pseudomonas sp. carried out a co-oxidation reaction in which the acetate moiety is disimilated to carbon dioxide and one chloro group is removed from the benzene nucleus.

Acknowledgements

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References


A REPETITIVE DIE-AWAY TEST COMBINING SEVERAL BIODEGRADABILITY TEST PROCEDURES

J. Blok

Summary. A method is described to screen chemical compounds for their biodegradability, and for comparative studies. The reproducibility of the method is given and the advantages are illustrated. The method combines measurement of dissolved organic carbon and oxygen uptake. This makes the test more useful than any other comparable method. Repetitive incubation answers questions concerning adaptation time and degradation rate.

Essai répétitif de dégradation lente combinant plusieurs modes opératoires de biodegradabilité. On décrit une méthode de dépistage de composés chimiques pour étudier leur biodegradabilité et les comparer. On indique la reproductibilité de la méthode et on met en évidence ses avantages. La méthode combine la mesure du carbone organique dissous et l'assimilation d'oxygène. Ceci rend l'essai plus utile que toute autre méthode comparable. Des incubations répétées répondent aux questions concernant le degré d'adaptation et la vitesse de dégradation.

Introduction

Biodegradation studies are conducted for different reasons. Frequently the potential biodegradability is measured as a property of a compound for the environmental assessment of new products. Especially for the screening of new chemicals and for comparative studies, some standardized and reproducible laboratory methods are required.

In addition, biodegradation may be studied as a process in some environmental situation. For this purpose simulation methods must be used. In some cases this becomes a research task, but for general use five different simulation methods could be standardized for such conditions as: activated sludge, surface water, sea water, anaerobic sludge, or soil. (Blok, 1975; Gilbert and Watson, 1977).

A final answer to difficult questions about biodegradation can be provided by field studies, but simulation and field studies take much time and are expensive. One of the merits of a screening method is that it reduces the amount of research to be done. This is based on the experience that most substances which are degraded in a screening method are also degraded in simulation methods and in the environment. Therefore, the screening method should not have an extremely high biodegradation potential. Further studies need to be made only with compounds that are repeatedly not degraded in a range of screening tests.

The choice of a screening method for biodegradability depends on the purpose of the study, the analytical possibilities, toxicity, water solubility, and vapor pressure of the compound. The main criterion for a screening method is a reproducible analytical procedure. Specific analyses for special compounds, or functional group analyses for certain chemical-related compounds, are possible. In this way a primary degradation or a loss of functionality is determined. For the environmental assessment this primary degradation may be of great interest, especially when the original structure gives rise to certain environmental problems such as foaming or toxicity. Finding an analytical procedure for all new chemicals to be tested, however, is difficult. Moreover, the environmental assessment is not complete with a primary degradation; the ultimate biodegradation to carbon dioxide, minerals, water and biomass, is the only safe criterion. Incomplete mineralisation gives rise to questions about residual toxicity and accumulation.

The analytical methods for ultimate biodegradation are: Chemical Oxygen Demand (COD), Dissolved Organic Carbon (DOC), carbon dioxide evolution, and oxygen uptake. Recent experience gained with DOC analysis and with dissolved oxygen measurement is such that they can be considered to be the most appropriate methods for biodegradation studies.

Although commercial instruments are sensitive to a level below 5 ppm, the smallest significant concentration difference that can be measured is 5 ppm DOC because of variations in blank values. Dissolved oxygen concentrations, however, may be differentiated at a level of 0.1 ppm. When 5 mg DOC/litre is the residue of a

1 Akzo Research, Corporate Research Department Arnhem, Netherlands.

(Received, November 1978; in final form, January 1979).
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Figure 1. Percent oxidation of kerosine and diethylhexylphosphoric acid (Depha) in an RDA test, impregnated on millipore filters.

Fresh material is added each week up to and including the fifth week and the height of each point represents the percentage of this material, together with that remaining unoxidised from the previous week, which is oxidised during the week.

degraded substance, the level of the test compound before degradation should be 50 mg DOC per litre. For some compounds this concentration may already be toxic to fresh water microorganisms. In this case the DOC method is not applicable.

The main disadvantage of DOC analysis is the limitation to water-soluble compounds. Many chemicals to be tested adsorb to the sludge or glass walls, precipitate during the test, or are insoluble, or nearly insoluble, in water. When these circumstances exist the so-called bioelimination, instead of biodegradation, is measured. This disadvantage can be overcome by the simultaneous measurement of the oxygen uptake, which is real proof of the oxidation of the compound.

The theoretical oxygen uptake by a compound at a level of 50 mg DOC/litre will normally vary between 100 and 150 mg O₂/litre. This amount, however, is not soluble in water. This paper describes a method which makes it possible to measure simultaneously the oxygen uptake and the residual amount of dissolved organic carbon at a level of 50 mg DOC/litre.

A typical complication with biodegradation studies is the phenomenon of adaptation. During the degradation of a compound the product could be more or less recalcitrant to further degradation. A rapid degradation at the end of the incubation time may mean an adaptation to the original molecule, but it may also point to an increased biodegradability of the breakdown products. For the interpretation of the results for practical situations this is of great importance. It should be known whether the incubation time is necessary because of the slow degradation rate or because of the time required for the induction of adaptation. This problem can be overcome by repetitive incubation. The adapted flora is fed with a new sample of the original compound and if a real adaptation has occurred the degradation rate increases with repetitive incubations. Using this experimental technique, the incubation period for each increment of the newly-added substance is only one week. The biodegradation potential of this test is more in accordance with relevant environmental situations.

A repetitive incubation has some further advantages. Because of the repetitions the statistical value of the final result is higher. Also, the adsorption capacity of the system is gradually saturated and the toxicity of non-degradable compounds, or residual compounds, may be measured at different levels. Furthermore, when some information about metabolites is necessary, the repetitive incubation gives an accumulation of a measurable amount of the recalcitrant metabolite.

The test method described in this paper is an attempt
A repetitive die-away test combining several biodegradability test procedures. J. Blok.

to combine several existing methods. The Sapromat method (Steinecke, 1968), the Japanese MITI method, and the Closed Bottle test of Fischer (Fischer, 1973), are all based on oxygen uptake. The disadvantages of the Sapromat type of apparatus are its expense and the carbon dioxide depletion which could result in a limitation for certain bacterial species. The Closed Bottle test is not very accurate because of the low concentration but can be recommended for toxic compounds.

The revised OECD screening test using DOC analysis (I.S.O. 1978) and the French AFNOR test (AFNOR, 1977) are very similar to each other. These methods do not measure the oxygen uptake. Checking for physico-chemical eliminations is done by a sterile control, but this control does not contain any sludge. The sterilisation procedures and the bacterial counting advised do not seem to be strictly necessary. The OECD screening test should be carried out at the level of 10 mg DOC/litre. This level decreases during biodegradation and becomes too low for accurate measurement. The recommended filtration procedure could give contamination with organic substances from the filter. Therefore the filters must be washed extensively with sample, which requires big samples. The same holds also for the possible adsorption of organics on the paper surface. Centrifugation for 5 minutes at 5000g is much easier, only small sample volumes are needed and a clear supernatant is produced.

The OECD screening test and the AFNOR test are strictly limited to watersoluble non-adsorbing compounds with low volatility.

The new elements in the method described in this paper are:

1. The simultaneous measurement of oxygen uptake and residual amount of organic carbon.
2. The repetitive incubation.

In this way most drawbacks of other existing methods are removed.

Experimental

Test Principle

The test compound is brought together with polyvalent aerobic microorganisms in a mineral medium. This medium contains some vitamins and an inhibitor of nitrification. The concentration of the test compound is about 50 mg/litre expressed as organic carbon. Each week the oxygen uptake and the amount of dissolved organic carbon (DOC) are measured. After these measurements have been made a further quantity of the test compound is added. The process is repeated at weekly intervals, an equal quantity of the test compound being added on each occasion after the measurements have been made. This is continued for four weeks; on the next two weekly occasions measurements are made but test compound is not added.

Incubation takes place in the dark at neutral pH and 20°C on a rotary shaker (200 rpm). The flasks are two thirds filled with liquid leaving one third their volume of air. Oxygen is measured with a dissolved oxygen electrode and the total oxygen uptake is calculated from the per cent decrease in dissolved oxygen multiplied by the amount originally present in the water and air together.

Mineral Medium

The mineral medium is obtained by diluting 1 : 1000 of equal quantities of the stock solutions A - F of composition given below with distilled water. The constituents suffice for the formation of about 150 - 200 mg/litre biomass or for the ultimate biodegradation of about 1 g/litre of test compound.

Solution A: 2.83 g per litre
KH2PO4
K2HPO4
Na2HPO4.2aq

Solution B: 7.5 g per litre
MgSO4.7aq
CaCl2
FeCl3.6aq

Solution C: 127 g per litre
NH4Cl

Solution D: 2 g per litre
Allylthiourea

Solution E: 5 g per litre
Yeast extract

Solution F: 1.2 g per litre
H3BO4
CuSO4.5aq
KI
MnSO4
NaMoO4
ZnSO4.7aq

Preparation of fresh inoculum

to obtain a polyvalent inoculum, various sources of aerobic organisms are used together. To 70 ml tap water the following are added:

10 ml stock solution A
10 ml fresh activated sludge
10 ml fresh river sludge
500 mg fresh compost or garden soil.

This mixture is homogenised with an ultrasonic mixer for 10 - 20 seconds and paper-filtered. The filtrate is used as soon as possible, in any event within one hour. 3 ml of this inoculum is added to each 600 ml of mineral medium.

Incubation procedure

The test is carried out in brown incubation bottles of 280 ± 1 ml with stirrers provided with ground glass stoppers (n 19/26) each containing the appropriate quantity of mineral medium plus test substance and inoculum. Each determination is carried out in triplicate.

Each test series should include a blank (without test
compound but with inoculum) and a reference and a toxicity control (made by combining test compound and reference). The test compound is added to the mineral medium from a stock solution, or directly by weighing in the case of a water-insoluble compound. The concentration should not exceed a theoretical oxygen demand of 100–150 mg/litre. The reference measurement is made with sodium acetate. 5 ml of a stock solution containing 30.94 g/litre NaAc3aq is added to 600 ml mineral medium. Each flask is numbered and contains a teflonised magnetic stirrer. Each flask is charged with 187 ml of the aqueous mixture, carefully closed, and placed on a rotary shaker for one week.

Oxygen measurement

Each flask is placed on a magnetic stirrer in the 'off' position. The stopper is removed and the calibrated oxygen probe is immediately put in its place. The probe passes through a rubber stopper which closes the flask while the measurement is made. The stirrer is switched on and the oxygen concentration is read when the value is stable. Because the volume of the electrode is greater than that of the stopper some air is displaced when the electrode is inserted. No problems have been met in practice due to possible entry of fresh air during this process.

Dissolved organic carbon measurement

A mixed sample of 7.5 ml is prepared by taking 2.5 ml from each of three flasks. The samples are centrifuged for 5 minutes at about 5000g. The supernatant is decanted and acidified with one drop of 2N H2SO4. Carbon dioxide is stripped with a nitrogen flow through a glass capillary for three minutes. The amount of organic carbon is then determined with a commercial DOC analyser.

Reincubations

After sampling and dissolved oxygen measurements the pH is measured and corrected if necessary. The water is aerated by bubbling air through for 15 minutes until saturation: 1.6 ml of the stock solutions of test compound and/or reference material is added. The volume is adjusted by addition of mineral medium to 187 ml and the flasks are closed for the next one week incubation period on the shaker. This procedure is followed after 7, 14, 21, 28 days but after 35 days and 42 days the measurements are made but no new test compound is added. This completes the test.

Calculation of the results

The blank value (DOCth) is subtracted from the measured amount of dissolved organic carbon (DOCt). The added amount of organic carbon (TOCadded) is calculated; for water-insoluble compounds this is a theoretical amount based on the dry weight addition. The results are expressed as per cent elimination of the dissolved organic carbon, calculated as shown:

$$\text{per cent eliminated} = \frac{\text{TOC}_{\text{added}} - (\text{DOC}_{\text{t}} - \text{DOC}_{\text{th}})}{\text{TOC}_{\text{added}}} \times 100$$

The oxygen uptake is corrected for that in the blank and related to a measured chemical oxygen demand or to a theoretical oxygen demand (TOD) of the amount added. The maximum oxygen uptake per litre of flask volume is 2/3 of this TOD.

The total oxygen per litre of flask volume at 20°C and 76 cm Hg before the incubation is:

- In air: $1/3 \times 1/22.4 \times 273 \times 32 \times 0.21 = 93$ mg O2
- In water: $2/3 \times 8.8 = 6$ mg O2

Total $99$ mg O2

This figure has a standard variation of +2 mg because of variations in the atmospheric pressure.

The per cent degradation follows from:

$$\text{per cent degradation} = \frac{\text{DO}_{\text{th}} - \text{DO}_{\text{t}}}{\text{DO}_{\text{st}} / 2/3 \times \text{TOD}} \times 100$$

where:

- DOth = dissolved oxygen in blank after incubation
- DOt = dissolved oxygen in test after incubation
- DOst = dissolved oxygen at saturation (known from the temperature) after incubation
- TOD = Total (or Theoretical) oxygen demand of the substance added at the beginning of incubation (mg/litre water).

Remarks on the method

1. If the oxygen uptake in the toxicity control is lower than in the reference the test must be repeated with a lower concentration of the test compound.

2. The dissolved oxygen should not fall below 1 mg/litre during incubation because otherwise the per cent degradation cannot be calculated. If this occurs the next incubation should take place with addition of less test compound.

3. For volatile compounds the liquid must not be aerated, only the air in the flask should be replaced. This can be done by pouring the contents into a clean flask and back into the test flask. The dissolved oxygen concentration is measured before the incubation without waiting to reach equilibrium with the gas phase. The dissolved oxygen deficit (saturation value minus the measured value) times 2/3 is subtracted from 99 in the formula.

4. Water-insoluble fluids can be added by impregnating them on a cellulose-acetate millipore filter. This may be done directly on a microbalance. Dilutions of water-insoluble compounds can also be made in diethylether or ethanol. They are adsorbed volume-
A repetitive die-away test combining several biodegradability test procedures. J. Blok.

trically on a millipore filter and the solvent is evaporated before placing the filter in the flask.

5. An increased oxygen uptake in the blanks together with a decrease of the pH points to nitrification. In that case the next incubation should be done with an extra addition of 2 ppm allylthiourea.

6. The method can be modified to permit lower concentrations which will give less accurate weekly DOC measurements but the same reproducibility after 5 repetitive incubations. This is achieved by increasing the water fraction in the flask. With 9/10 volume parts of water and 1/10 volume parts of air, five repeated additions of 10 ppm DOC are made. The formula then is:

\[
\text{per cent oxidation} = \frac{(\text{DOC}_{tb} - \text{DOC}_{t})}{0.9 \times \text{TOD}}\times 100
\]

Results

The method has been applied to a readily degradable reference, namely sodium acetate (NaAc), and a poorly degradable reference, namely triethylenetetramine (ttta). For this test, blank, NaAc, and tta were tested six times in triplicate to measure the reproducibility and standard deviation. The results are given in tables 1 – 4.

From these data it can be concluded that the oxygen uptake with NaAc is about 80% of the theoretical oxygen demand. As 98% DOC was removed this means that about 20% of the compound is not oxidised but is found back in the synthesised biomass. So an oxidation of 80% can be regarded as a biodegradation of 100%.

The reproducibility of the results is good. The least controllable factor is the inoculum composition. For the less degradable compounds, especially, this will vary the time required for adaptation. To check this factor sodium acetate is not an appropriate substance.

The standard deviation of the DOC measurements show that indeed about 5 ppm DOC is the lowest level to obtain an accurate measurement.

The results with tta show that no oxidation takes place. Some reduction of the DOC related to the amount of TOC added takes place in the first weeks. The repetitive incubations, however, show some degree of saturation. In separate studies it has been shown that a primary degradation takes place without oxidation and that two of the four main groups are split off and can be found back as ammonia. The metabolite has the same organic carbon content but is less adsorbed to soil, glass, or sludges and is also less toxic to Daphnia magna.

The test has also been performed with two poorly water-soluble compounds, namely diethylhexylphosphoric acid (Dehpa) and kerostine. These compounds were impregnated on a millipore filter. Figure 1 gives the oxygen uptake expressed as a percentage of the theoretical oxygen demand. DOC measurements were not made in these cases. The tests were continued for 56 days. It can be seen from the figure that the repetitive incubation gives an increased oxidation capacity up to 80% in one week because of the adaptation during the previous six weeks. The time necessary for the induction of an adapted flora, however, is evidently different for these two compounds.

Discussion

Biodegradability is an important factor for the environmental assessment of a new product. An incomplete or slow biodegradation has to be put in the context of overall world production, distribution pattern, water-solubility of product and metabolites, volatility, adsorption to soil as well as sludges, and toxicity.

The new test described is a good tool for obtaining part of this knowledge. The results can be used to make a selection for further research or simply to avoid expensive studies. The accumulated metabolites can be employed for analytical studies or for toxicity tests with fish and Daphnia etc., and the accumulated sludge can be used for further respirometric studies. The applicability to water-insoluble and volatile compounds is attractive because so many organic compounds are poorly water-soluble.

This test method can easily be modified to permit lower concentrations in the case of toxic compounds and to permit higher concentrations of sludge and test compound in order to be more akin to sludge tests such as those described by Zahn and Wellens (1974) and Pitter (1976).

Without difficulty a modification for sea water is possible. When inoculation is done with special sludges from soil columns, river bottoms, or purification plants, the results may be used to show some level of adaptation to special compounds which these sludges may contain.

The repetitive incubation is a step towards semi-continuous test methods (Fischer, Gerike and Holtman 1975). This makes the test more easy to compare with these systems and with nature situations.

The test can be conducted in multiple for a series of compounds without necessitating enormous investments. When the DOC measurement is carried out with an automatic injection system the test can be performed on a routine basis which requires two man/days per test compound.

1 In our experience millipore membranes give no oxygen uptake or release of dissolved organic carbon. For security, however, membranes could be added to the blank.
A repetitive die-away test combining several biodegradability test procedures. J. Blok.

### Table 1
Oxygen uptake in the RDA test, related to the theoretical oxygen demand of sodium acetate added in five weekly quantities each of 47 mg/litre TOC

<table>
<thead>
<tr>
<th>Incubation week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{O_t}$ x mg/litre</td>
<td>8.50</td>
<td>8.55</td>
<td>8.75</td>
<td>8.63</td>
<td>8.51</td>
<td>8.60</td>
</tr>
<tr>
<td>n = 6 s</td>
<td>0.09</td>
<td>0.08</td>
<td>0.07</td>
<td>0.12</td>
<td>0.10</td>
<td>0.11</td>
</tr>
</tbody>
</table>

| $D_{O_t}$ x mg/litre | 2.90 | 2.41 | 2.78 | 2.86 | 3.07 | 7.06 |
| n = 6 s | 0.19 | 0.30 | 0.39 | 0.33 | 0.69 | 0.45 |

$$\frac{D_{O_t} - D_{O_{t}}}{{D}_{O_{st}}}$$

**Integral oxygen uptake mg/1 flask**

|     | 63 | 132.5 | 200 | 265 | 326 | 344 |

**TOD$_{added}$ mg/1 flask**

|     | 84 | 167 | 251 | 334 | 418 | 418 |

**% oxydation**

|     | 75 | 79 | 80 | 79 | 78 | 82 |

Mean value 79 ± 5%.

### Table 2
Dissolved organic carbon in the RDA test related to the amount of sodium acetate added in five weekly quantities each of 47 mg/litre TOC

<table>
<thead>
<tr>
<th>Incubation week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{O_{C_t}}$ x mg/litre</td>
<td>2.0</td>
<td>4.5</td>
<td>2.0</td>
<td>4.0</td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td>n = 6 s</td>
<td>0.55</td>
<td>9.75</td>
<td>1.25</td>
<td>1.50</td>
<td>2.0</td>
<td>1.25</td>
</tr>
</tbody>
</table>

| $D_{O_{C_t}}$ x mg/litre | 3.5 | 9.0 | 3.5 | 7.0 | 7.0 | 4.0 |
| n = 6 s | 2.7 | 1.5 | 2.2 | 2.6 | 3.8 | 0.8 |

**% elimination**

|     | 97 | 95 | 99 | 98 | 99 | 100 |

$$\left[ 1 - \frac{D_{O_{C_t}} - D_{O_{C_{tb}}}}{TOD_{added}} \right]$$

Mean value 98 ± 2%.

### Table 3
Oxygen uptake in the RDA test related to the theoretical oxygen demand of triethylenetetramine added in five weekly quantities of 38 mg TOC/litre. Blank values as in Table 1.

<table>
<thead>
<tr>
<th>Incubation week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{O_t}$ x mg/litre</td>
<td>8.63</td>
<td>8.24</td>
<td>8.61</td>
<td>8.65</td>
<td>8.67</td>
<td>8.28</td>
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<tr>
<td>n = 6 s</td>
<td>0.11</td>
<td>0.14</td>
<td>0.09</td>
<td>0.08</td>
<td>0.09</td>
<td>0.12</td>
</tr>
</tbody>
</table>

| $D_{O_{C_t}}$ | 0.00 | 0.04 | 0.02 | 0.00 | 0.00 | 0.04 |
| $D_{O_{st}}$ | 0.64 | 0.70 | 0.68 | 0.66 | 0.62 | 0.18 |

**Integral oxygen uptake mg/1 flask**

|     | 0.0 | 4.0 | 6.0 | 6.0 | 10.0 |

**TOD$_{added}$ mg/1 flask**

|     | 100 | 200 | 300 | 400 | 500 | 500 |

**% oxydation**

|     | 0.0 | 2.2 | 2.6 | 3.8 | 0.0 | 4.0 |

Mean value 2 ± 2%.

### Table 4
Dissolved organic carbon in RDA test related to the amount of triethylenetetramine added in five weekly quantities. Blank values are the same as in Table 2

<table>
<thead>
<tr>
<th>Incubation week</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<tr>
<td>$D_{O_{C_t}}$ x mg/litre</td>
<td>28</td>
<td>62</td>
<td>101</td>
<td>154</td>
<td>196</td>
<td>185</td>
</tr>
<tr>
<td>n = 6 s</td>
<td>0.8</td>
<td>1.1</td>
<td>1.7</td>
<td>4.7</td>
<td>6.0</td>
<td>5.9</td>
</tr>
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</table>

**% elimination**

|     | 32 | 24 | 13 | 0  | 0  | 4  |

### References


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<td>Environmental Biogeochemistry 4th International Symposium on Environmental Biogeochemistry.</td>
<td>Canberra, Australia</td>
<td>Conference Secretary, Australian Academy of Science, P.O. Box 783, Canberra City, ACT 2601, Australia.</td>
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<td>3–7 September 1979</td>
<td>Actinomycete Biology International Symposium on Actinomycete Biology.</td>
<td>Cologne, Germany</td>
<td>Professor Dr. D.P. Schaal, Hygiene-Institute der Universität, Goldenfesstrasse 21 5000 Koln 41 Germany.</td>
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<td>18–21 September 1979</td>
<td>Extracellular Products of Microorganisms Joint NWEC/FEMS/SGM meeting. Symposia on: Extracellular Enzymes; Bacterial Toxins.</td>
<td>Dublin, Ireland.</td>
<td>Dr. W.M. Fogarty, University College, Dept. of Industrial Microbiology, Ardmore, Stillorgan Road, Dublin 4, Ireland.</td>
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All enquiries which involve a literature search will be charged at £10 each inclusive of photocopying up to 40 pages (anything over 40 pages will be charged at the rate of 8p per page). If the search time involved exceeds 2 hours subsequent time will be charged at £6 per hour. British enquirers will be advised by telephone if it is found that more than 2 hours or extra photocopying is needed. Overseas enquirers will be sent the results of up to 2 hours' work and up to 40 pages of photocopying, if appropriate, and advised if further work is likely to yield further results.

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The Centre will supply single copies of papers from the literature collection at a cost of 8p per page, minimum charge £1 (U.K.), £2 (Overseas), providing that a photocopy declaration is signed in compliance with the 1956 British Copyright Act.

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The Centre undertakes a wide variety of contract research projects including testing of products by standard or specially developed methods. Organisms available for use in research and testing include a comprehensive collection of micro-organisms, insects including termites, and a colony of wild strain mice. Contract research rates are very competitive.

SPECIALISED BIBLIOGRAPHIES
Specialised bibliographies on specific aspects of biodeterioration and biodegradation are produced from the document collection from 1965. A list of titles and prices currently available is published in most issues of the International Biodeterioration Bulletin, or may be had on request.

JOURNALS PUBLISHED BY THE CENTRE
Three quarterly journals are published:

International Biodeterioration Bulletin (IBB) A scientific journal for publication of original works, including reviews and book reviews on all aspects of biodeterioration and biodegradation. Each issue also contains the Biodeterioration Society Newsletter which includes short abstracts of papers presented at meetings of the Society in Great Britain and Ireland, and also details of forthcoming meetings, conferences and symposia.

Biodeterioration Research Titles (BRT) A bibliographic journal which presents, in classified form, references to published literature on all aspects of biodeterioration and biodegradation. About 2000 references per annum.

Waste Materials Biodegradation Research Titles (WMB) A bibliographic journal similar to BRT dealing with all aspects of the biological treatment of solid and liquid wastes and the biodegradation of waste materials in nature. About 1800 references per annum.

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