ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 24, 319–327 (1992)

Simple Method to Prolong the Closed Bottle Test for the Determination of the Inherent Biodegradability

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Received November 25, 1991

A method to prolong the closed bottle test up to 200 days is described and validated. This prolonged closed bottle test has been used to determine the biodegradability of "recalcitrant" and toxic organic compounds. The results obtained in the prolonged closed bottle test are in accordance with those mentioned in the literature on biodegradation of organic compounds in waste water purification plants and on the isolation of microorganisms capable of utilizing these compounds as carbon and energy source. Furthermore, this test may prevent discrepancies and unexplainable results obtained in a 28-day test. The test has the potential to be used as an inherent biodegradability test when recognized by the authorities. © 1992 Academic Press, Inc.

INTRODUCTION

Biodegradability is an important property of chemicals which, besides adsorption, water solubility, volatility, etc., will govern their fate in the environment. Therefore, biodegradability tests on organic chemicals have to be conducted for the assessment of the impact on ecosystems. For the initial screening of ready biodegradability several methods have been defined by the OECD (1982) and EEC (1984). It is generally accepted that these tests are very stringent and that low percentages do not necessarily mean that a chemical is persistent in nature. For this reason, it is justified to perform other tests to demonstrate the biodegradability of a chemical (inherent biodegradability).

No single ready biodegradability test can be the most suitable for all chemicals. However, among screening tests the closed bottle test seems superior because of its simplicity, its low initial concentration of test compound (fewer problems with toxic chemicals), the techniques available to enable an accurate administration of insoluble test compounds, and the possibility of testing volatile compounds. Furthermore, it is very simple to prolong the closed bottle test to determine the "inherent" biodegradability. This prolongation may yield valuable additional information. Moreover, there are no scientific reasons to end biodegradability tests after 28 days. Strictly speaking, the biodegradation percentage at Day 28 is only important for the notification of chemicals to regulatory authorities.

In this article the method to prolong the closed bottle test is described and validated. This article is concluded with results of various chemicals tested in the prolonged closed bottle test and with a comparison of the results from microbial and waste water purification studies described in the open literature.

0147-6513/92 $5.00

MATERIALS AND METHODS

Chemicals

All reagent grade chemicals used were purchased from Janssen Chimica, Beerse, Belgium.

Inoculum

Secondary activated sludge was obtained from an activated sludge plant treating predominantly domestic waste water.

Closed Bottle Test

The closed bottle test was performed according to the test guidelines (EEC, 1984; OECD, 1982). Two minor deviations from these guidelines were introduced:

—Instead of an effluent/extract mixture, activated sludge was used as an inoculum as described by Blok *et al.* (1985). The inoculum was taken from an activated sludge plant. The sludge was preconditioned to reduce the endogenous respiration rates. To this end, the sludge (200 mg dry wt/liter) was aerated for a period of 7 days. The sludge was diluted to a concentration in the biochemical oxygen demand (BOD) bottles of 2 mg dry wt/liter.

—Ammonium chloride was omitted from the medium to prevent nitrification.

Determination of the Oxygen Concentration

The dissolved oxygen concentrations in the closed bottles were determined electrochemically using an oxygen electrode (WTW Trioxmatic EO 200) and meter (WTW OXI 530). The funnel used for the prolongation was obtained from Retch BV, Ochten, The Netherlands.

Calculation of the Biodegradation Percentages

The biodegradation was calculated as the ratio of the BOD to the theoretical oxygen demand (ThOD).

Selection of the Test Compounds

The test compounds were primarily selected from the papers by Gerike and Fischer (1979, 1981) and Painter and Bealing (1989) for four reasons. First, polyvinyl alcohol (PVA), carboxymethyl cellulose (CMC), an EOPO block polymer, and ethylenediaminetetraacetic acid (EDTA) were chosen for their recalcitrancy in all tests employed by Gerike and Fischer (1979, 1981) although these compounds are biodegradable. EDTA is biodegradable through an initial cometabolic transformation (Belly *et al.*, 1975; Tiedje, 1975) whereas PVA, CMC, and EOPO block polymers are susceptible to biodegradation by microorganisms using these organic compounds as carbon and energy source (Bryan and Harrison 1973; Kawai, 1987; Kawai *et al.*, 1977; Nishikawa and Fujita, 1975; Reese *et al.*, 1950; Sakazawa *et al.*, 1981; Suzuki *et al.*, 1973). Second, organic compounds such as diethylene glycol and triethanolamine with low results in ready biodegradability tests but shown to be biodegradable in inherent biodegradability tests were chosen (Gerike and Fischer, 1979, 1981). Third, chemicals with "discrepancies" among results obtained by participants of an EEC round robin test achieving the >60% biodegradation level in a ready biodegradability test were selected. Painter and Bealing (1989) listed thioglycolic acid, diphenic acid, tetrahydrofuran, t-butanol, pentaerythritol, hexamethylenetetramine, and 3-aminophenol as compounds with discrepancies. Last, hexadecyltrimethylammonium chloride was selected since this compound is recalcitrant in all ready biodegradability tests due to its bactericidal properties. On the other hand, true biodegradation of hexadecyltrimethylammonium chloride was observed with radiolabeled material (Larson, 1983).

RESULTS AND DISCUSSION

Determination of the Oxygen Concentration in the Prolonged Test

The closed bottle test described in the guidelines (EEC, 1984; OECD, 1982) is prolonged by measuring the course of the oxygen decrease in the bottles of Day 28 using a funnel. This funnel fits exactly in the BOD bottle to serve as an overflow reservoir. Subsequently, the oxygen electrode with built-in stirrer bar is inserted in the BOD bottle to measure the oxygen concentration (Fig. 1). After the withdrawal of the oxygen electrode, the displaced medium drains back into the BOD bottle whereupon the funnel is removed and the BOD bottle closed.

In order to validate the method with the funnel, the oxygen concentration in one bottle has been determined one after another at two oxygen concentrations (Fig. 2). These successive measurements show that only minor quantities of molecular oxygen are introduced into the bottles.

Endogenous Respiration of the Inoculum

Another important prerequisite to carry out the test successfully is the presence of sufficient molecular oxygen in the test bottles. Therefore, the microorganisms of the inoculum in the prolonged test should not consume too much molecular oxygen to permit additional substrate respiration. The inoculum used in the prolonged closed bottle test was proposed in an attempt to harmonize ready biodegradability tests (Blok *et al.*, 1985). The mean endogenous respiration of this inoculum has been calculated from seven test results. The endogenous respiration at Day 28 is 0.4 mg/liter with a negligible standard deviation. The endogenous respiration increases to 2.3 mg/liter with a standard deviation of 0.9 after approximately 200 days. This endogenous respiration is in general suitable to ensure that the concentration of oxygen does not fall below 0.5 mg/liter during the test period.

Biodegradation of "Recalcitrant" Compounds in the Prolonged Closed Bottle Test

The biodegradation of PVA, a water-soluble synthetic polymer, has been studied with pure and symbiotic cultures. The PVA utilizing isolated bacteria belong to the genera *Pseudomonas* and *Xanthomonas*. These studies clearly demonstrate the capacity of the microorganisms to degrade PVA (Nishikawa and Fujita, 1975; Sakazawa *et al.*, 1981; Suzuki *et al.*, 1973). Furthermore, over 90% of PVA is removed in activated sludge plants at COD loadings of 0.2–0.3 $kg/m^3 \cdot day$ of PVA containing waste water (Suzuki *et al.*, 1973). Despite this evidence of the capacity of microorganisms to utilize

FIG. 1. Schematic representation of the funnel and inserted electrode with built-in stirrer bar in a BOD bottle.

PVA as carbon and energy source, this compound is not degraded in OECD and EEC biodegradability tests (Gerike and Fischer, 1979, 1981). However, in the prolonged closed bottle test PVA (MW = 22,000) is degraded. Figure 3 shows a lag phase of approximately 25 days. After this period PVA is mineralized completely as illustrated by a biodegradation percentage of 62 at Day 108.

CMC is biodegradable as has been observed by the isolation of a gram-negative bacterium which is capable of liquifying CMC (Freeman *et al.*, 1948). Furthermore, Reese *et al.* (1950) demonstrated that CMC is metabolized by various microorganisms. These isolates indicate that the capacity to utilize CMC by aerobic microorganisms is widely distributed. In laboratory scale-activated sludge systems the utilization of CMC by microorganisms is substantial. Through the acclimatization of the microorganisms the CMC removal rate could be increased (Bryan and Harrison, 1973).

In the present study, the authors used CMC with a substitution grade of 0.7. On the basis of the above literature, biodegradation is expected. Indeed, the biodegradation

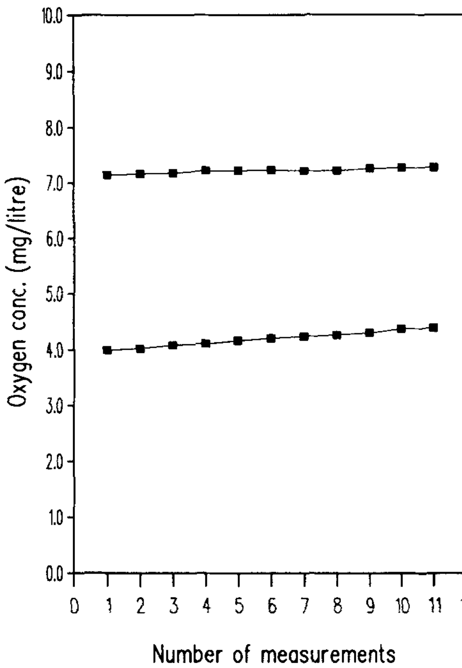

FIG. 2. Oxygen concentrations in the water phase of a BOD bottle at 10 successive determinations.

started immediately and this biodegradation was completed after approximately 110 days (Fig. 3).

Both polyethylene glycol (PEG) and polypropylene (PPG) with molecular weights up to 4000 are biodegraded by aerobic microorganisms using these polymers as carbon and energy source (Kawai, 1987). Kawai *et al.* (1977) also reported that a PPG utilizing strain grew on a few EOPO block polymers which contained a larger amount of PPG than PEG. The ready biodegradability of PEG is confirmed by many positive OECD/EEC screening tests, which is in accordance with the results of Kawai (1987). EOPO block polymers are not biodegraded in OECD/EEC tests (Gerike and Fischer, 1979, 1981) although Kawai *et al.* (1977) observed that a pure strain is capable of utilizing an EOPO block polymer as carbon and energy source. In the prolonged closed bottle test, an EOPO block polymer is degraded. After 98 days, the biodegradation percentage of this compound is 64 (Fig. 3). This biodegradation also indicates that valuable additional information may be obtained with the prolongation of the closed bottle test.

EDTA is degraded co-metabolically (Belly *et al.*, 1975; Tiedje, 1975), whereas microorganisms capable of growing on this substrate have not been isolated until now. Co-metabolism is only possible when bacteria are grown on another substrate which induces enzymes capable of transforming EDTA. Since only one substrate (EDTA) is present in the closed bottle, no biodegradation is expected.

Although some authors claimed degradation in OECD and EEC tests, most test results are negative (Gerike and Fischer, 1979, 1981; Zahn and Wellens, 1980). In the prolonged closed bottle test, no biodegradation has been observed (Fig. 3), which is in accordance with the present knowledge on EDTA biodegradation.

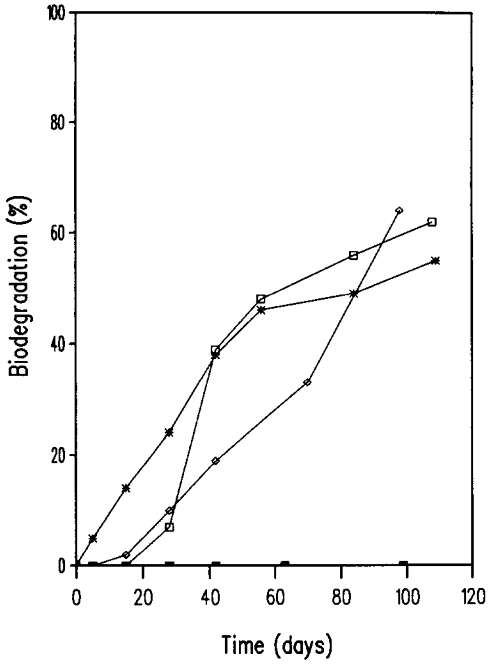

FIG. 3. Biodegradation curves of EDTA (■), PVA (□), CMC (*), and an EOPO block polymer (◇) in the prolonged closed bottle test.

Biodegradation of Compounds Having Discrepancies

Diethylene glycol and triethanolamine have been biodegraded in inherent biodegradability tests, whereas both compounds were recalcitrant in ready biodegradability tests employed by Gerike and Fischer (1979, 1981). Today triethanolamine is readily biodegradable (Table 1). Diethylene glycol does not reach 60% biodegradation within

TABLE 1

BIODEGRADATION PERCENTAGES OF VARIOUS ORGANIC COMPOUNDS

	Biodegradation percentage		
Test compound	Day 28	Day 42	Day 56
Diethylene glycol	46	66	—
Triethanolamine	73	78	—
Thioglycolic acid	67	—	—
3-Aminophenol	68	73	—
t-Butanol	10	63	67
Tetrahydrofuran	39	57	61
Diphenic acid	37	66	—
Pentaerythritol	64	71	—
Hexamethylenetetramine	70	>100	>100

28 days. However, this level is passed at Day 42, which demonstrates the complete mineralization of the compound (Table 1). This result indicates that the prolonged closed bottle test may be used to assess the "inherent biodegradability" at very low costs.

Comparing the results of the EEC round robin test (Painter and Bealing, 1989) only one compound has been assessed unanimously. Because of these disparate results at Day 28, eight chemicals from the ring test have been tested in the prolonged closed bottle test (Table 1). 3-Aminophenol, pentaerythritol, hexamethylenetramine, and thioglycolic acid are readily biodegradable in our closed bottle test. t-Butanol, tetrahydrofuran, and diphenic acid do not reach the pass level at Day 28. However, these compounds are completely mineralized at either Day 42 or Day 56 (Table 1). Using this additional information the possible formation of recalcitrant intermediates may be excluded and discrepancies among test results can be easily explained.

Organic compounds should be judged on their mineralization to H_2O, CO_2 and NH_3 since the oxidation of NH_3 is catalyzed by slow growing bacteria abundantly present in the environment. Hexamethylenetetramine contains a high percentage of nitrogen. This compound is readily biodegradable supposing the formation of NH_3 (Table 1). On the other hand, the biodegradation percentage is only 29% when calculating with the $ThOD_{NO_3}$. In the prolonged closed bottle test nitrification probably takes place between Day 28 and 56, resulting in increasing biodegradability percentages (60 at Day 56).

Biodegradation of Hexadecyltrimethylammonium Chloride

Due to the bactericidal properties of hexadecyltrimethylammonium chloride (HDTMAC) attempts to observe biodegradation in screening tests have been consistently unsuccessful (Gerike and Fischer, 1979, 1981). However, the addition of LAS reduced the toxicity of quaternary alkyl ammonium salts, thus enabling biodegradation in screening tests (Larson, 1983). Moreover, using radiolabeled octadecyltrimethylammonium chloride, Larson (1983) showed the ready biodegradability of monoalkyltrimethylammonium salts.

In the closed bottle test, a decrease of the endogenous respiration at the start of the test (Fig. 4) indicates the inhibitory effect of HDTMAC on microorganisms. After approximately 1 month, the microorganisms responsible for the degradation of HDTMAC are adapted to the test compound and subsequently HDTMAC is mineralized within 100 days. From this result, it can be concluded that the biodegradability of toxic compounds can be tested very easily in the prolonged closed bottle test.

CONCLUSIONS

The prolonged closed bottle test is very useful and it demonstrates the biodegradation of recalcitrant compounds. Also, resistance to biodegradation due to toxic effects of the test substance is often overcome, resulting in mineralization after Day 28 in the closed bottle test. Further discrepancies and misinterpretations can be excluded when using the prolonged closed bottle test. These conclusions prove that the prolonged closed bottle test is very robust and yields valuable additional information. This test has a very high cost–benefit ratio because the prolongation is not labor-intensive. The

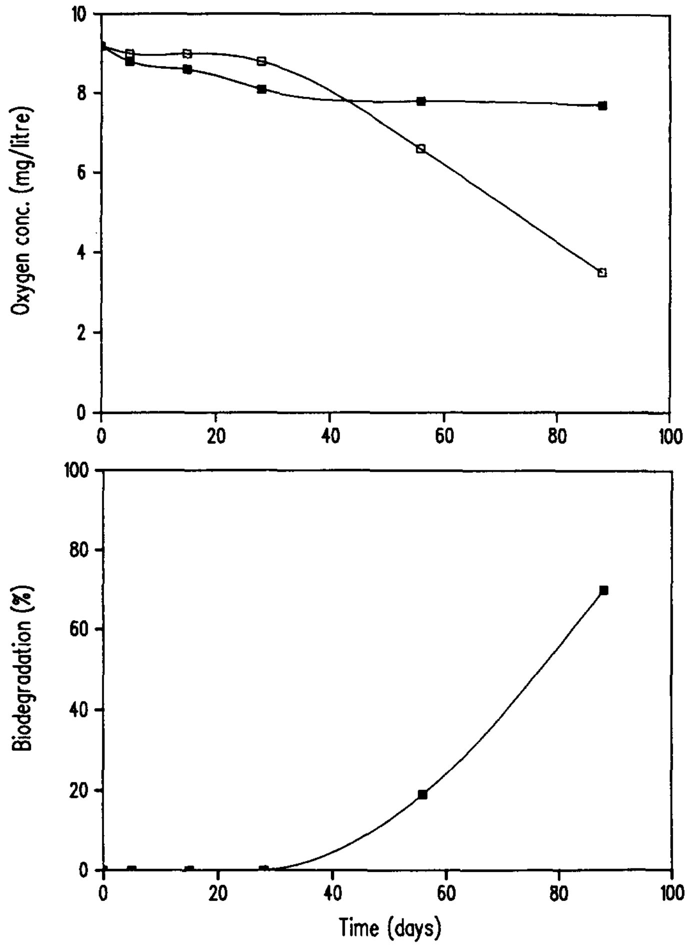

FIG. 4. Oxygen consumption in the closed bottle test with (□) and without (■) HDTMAC (top). Biodegradation curve of HDTMAC in the prolonged closed bottle test (bottom).

regulatory authorities should recognize this method, a simple available and inexpensive test that measures inherent biodegradability.

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