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Brief Report

Establishing a ready biodegradability test system using OxiTop® to evaluate chemical fate in a realistic environment

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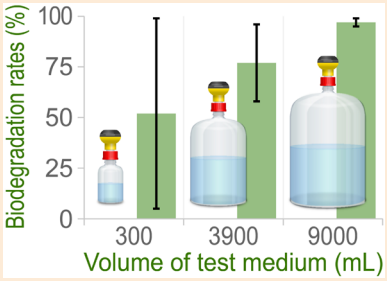
The purpose of this study is to propose the use of OxiTop® for measuring biochemical oxygen demand (BOD) under the Japanese Chemical Substances Control Law in order to properly evaluate chemical fate in a real environment. In our previous study, the biodegradation of test chemicals was accelerated by both adsorbing the chemical to silica gel with chloroform and increasing the medium volume from 300 to 3900 mL in the OECD 301F test using a coulometer. However, the biodegradability of these chemicals could not be evaluated based on BOD due to chloroform residue in the silica gel, or the medium volume could not be increased further due to the oven size of the coulometer. In this study, we established an evaluation system using OxiTop® based on BOD by increasing the medium volume to 9000 mL. Based on triplicate testing, increasing the medium volume accelerated biodegradation and decreased variation in BOD.



Keywords: OECD 301, biodegradability, coulometer, OxiTop®, test medium volume.

Introduction

Persistent, bioaccumulative, and highly toxic chemicals (PBTs) have adverse effects on human health and ecosystems; hence, they are strictly regulated in many countries. Under the Japanese Chemical Substances Control Law (CSCL),[1)] persistence should be evaluated by conducting a ready biodegradability test in accordance with Organisation for Economic Co-operation and Development (OECD) guideline 301C or 301F.[2)] The 301C test must be performed in a test bottle with 300 mL of test medium including 100 mg L^{-1} of the test chemical and 30 mg L^{-1} of the standard activated sludge that is a mixed inoculum collected from 10 sites in Japan and cultivated with peptone and glucose as synthetic nutrients for more than a month at the Chemicals Evaluation and Research Institute (Japan). In contrast, the 301F test can be performed with any test medium volume including 100 mg L^{-1} of the test chemical and 30 mg L^{-1} of activated sludge obtained from a municipal sewage treatment plant without any cultivation. In addition, according to the 301F guideline, it is permissible for the test chemical to be adsorbed on an inert support such as silica gel before being added to the test medium. In both the 301C and 301F tests, the persistence of the test chemical is evaluated by the BOD as well as by the amount of residual chemical remaining and its degradation products after the exposure period. The BOD represents the amount of oxygen consumed by microorganisms in activated sludge to degrade the chemical in the test medium during an exposure period. Theoretical oxygen demand (ThOD) is the calculated amount of oxygen required to oxidize a test chemical to its final oxidation products. When the BOD is more than 60% of the ThOD, and the amounts of the chemical and its degradation products are less than 1% of the added chemical, the test chemical is judged as "readily biodegradable" under the CSCL.[3)] While the amounts of the test chemical and its degradation products are mainly determined using high-performance liquid chromatography (HPLC), the BOD has been exclusively measured using a coulometer manufactured by Ohkura Electric in Japan. A coulometer consists of a thermostatic oven that accommodates six 300 mL test bottles, a manometer that detects the drop of inner pressure in the bottle caused by the consumption of oxygen, an electrolyzer that adds oxygen to the bottle to replenish the amount of

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oxygen consumed, and a BOD recorder.

In our previous report,[4] tris(2-ethylhexyl) trimellitate (Tris), which had been determined to be "not readily biodegradable" under the CSCL,[5] was subjected to ready biodegradability tests under various conditions, and the amounts of Tris were determined using HPLC after 28 days of exposure. While Tris was not degraded at all in the 301C test with the standard activated sludge, 3% of Tris was degraded in the 301F test using the activated sludge obtained from a municipal sewage treatment plant without any cultivation. We confirmed that the activated sludge used in the 301F test had higher biodegradation activity than that of the standard activated sludge used in the 301C test. In addition, in the 301F test, Tris was degraded by 11% by absorbing it on silica gel, and further degraded by 42% by both absorbing it on silica gel and increasing the test medium volume to 3900 mL. We confirmed that the modified 301F test, which absorbed a test chemical on silica gel, was useful for improving the bioavailability of chemicals with poor water solubility, such as Tris, and increasing the test volume further accelerated the biodegradation through the introduction of a greater diversity of microorganisms into the test medium. However, one problem and one question arose concerning these tests. The problem was that the degradability of Tris could not be evaluated by the BOD because the test medium included not just Tris but also chloroform. Tris was dissolved with chloroform to mix with silica gel, the chloroform was thoroughly evaporated using an evaporator and a vacuum oven, and Tris adsorbed on the silica gel was added to the test medium in a test bottle. However, since a small amount of chloroform inevitably remained, we could not distinguish the BOD caused solely by the degradation of Tris from the recorded BOD. As a result, the persistence of the test chemical was evaluated by the amounts of the chemical remaining as determined by HPLC, but evaluation by BOD was not possible. The question arising from our previous study is, how can the degradation of Tris be accelerated when the test medium volume is increased to more than 3900 mL, because many more species of microorganisms exist in a real environment than exist in test conditions. The volume of 3900 mL was determined based on the size of the coulometer's thermostatic oven, and it was impossible to accommodate a test bottle with a volume greater than 3900 mL in the coulometer.

The purpose of this study was to build a test system in which the biodegradation properties of a test chemical could be evaluated by not only the amount determined after exposure but also the BOD measured during the exposure in the 301F test with a test medium volume of more than 3900 mL. Since a test medium volume exceeding 3900 mL could not be examined using the coulometer, we chose OxiTop® as the BOD measuring device. OxiTop® is a cap-shaped pressure meter attached to a test bottle containing the test medium and measures the BOD by detecting the degree to which air pressure in the bottle decreases.[6] Therefore, if OxiTop® is used, we are able to increase the bottle size without limitation, provided that the thermostatic oven is able to accommodate the bottle. OxiTop® is known to be a highly reliable device for determining the BOD of chemicals.[7,8] However, published information on studies using OxiTop® to evaluate the fate of chemicals in the environment is still scarce,[6] and there are no studies comparing results using OxiTop® with those using a coulometer. In addition, as OxiTop® does not supply any oxygen consumed by the degradation of chemicals to the bottle, it is necessary to ensure sufficient headspace in the test vessel.[6] First, we compared the BODs for seven test chemicals obtained using the coulometer and OxiTop® in both the 301C and 301F tests to confirm the applicability of OxiTop® under the CSCL. Second, we had to determine what was an appropriate headspace volume so that the oxygen shortage in the bottle did not inhibit biodegradation for OxiTop®. Third, we evaluated the biodegradation properties of a test chemical using OxiTop® not only by the amount determined after exposure but also by the BOD measured during the exposure in the 301F test with a test medium volume exceeding 3900 mL.

Materials and methods

1. Chemicals

Sodium benzoate and sodium hydroxide solution were purchased from Kanto Chemical (Japan). Aniline, distilled water (HPLC grade), formic acid, acetonitrile (HPLC grade), tetrahydrofuran (stabilizer free, special grade), K_2HPO_4, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, NH_4Cl, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $FeCl_3 \cdot 6H_2O$, and 0.5% phosphate solution were purchased from FUJIFILM Wako Pure Chemical (Japan). Sodium acetate, benzyl alcohol, phenol, 2,4-dichloroaniline, 2,3-dichlorobenzoic acid, and tris(2-ethylhexyl) trimellitate (Tris) were purchased from Tokyo Chemical Industry (TCI, Japan).

2. BOD measuring system

Two different systems for measuring BOD were used: Coulometer OM7000A (Ohkura Electric, Japan) and OxiTop® (WTW, Germany). In both systems, microorganisms in a test bottle convert oxygen to carbon dioxide (CO_2) during the biodegradation of a test chemical. Since a CO_2 trap in the bottle absorbs CO_2, both systems detect a decrease in air pressure in the bottle. The coulometer is a large instrument with a thermostatic oven that accommodates six test bottles, six manometers that detect the decrease of air pressure in each bottle, six electrolyzers that add oxygen to each bottle to replenish the consumed oxygen, and a BOD recorder. OxiTop® is a cap-shaped pressure meter attached to a test bottle and measures BOD without generating any oxygen simply by detecting the degree to which air pressure in the bottle decreases.[6] After a test bottle is sealed with an OxiTop® cap, the bottle is incubated in a thermostatic chamber (IN804, Yamato Scientific, Japan).

3. 301C and 301F tests using a coulometer and OxiTop® with 300 mL of test medium

The 301C and 301F tests were conducted using a coulometer and OxiTop® for sodium benzoate, aniline, sodium acetate, benzyl alcohol, phenol, 2,4-dichloroaniline, and 2,3-dichlorobenzoic

Table 1. Method for calculating the TODs of test chemicals

Test chemical	Complete oxidation reaction	[A]*a)*	[B]*b)*	TOD*c)*
Sodium benzoate	$C_7H_5O_2Na+7.5O_2 \rightarrow 7CO_2+2.5H_2O+0.5Na_2O$	7.5	144.1	1.67
Aniline	$C_6H_7N+7O_2 \rightarrow 6CO_2+2H_2O+NH_3$	7	93.13	2.41
Sodium acetate	$C_2H_3O_2Na+2O_2 \rightarrow 2CO_2+1.5H_2O+0.5Na_2O$	2	82.03	0.78
Benzyl alcohol	$C_7H_8O+8.5O_2 \rightarrow 7CO_2+4H_2O$	8.5	108.14	2.52
Phenol	$C_6H_6O+7O_2 \rightarrow 6CO_2+3\ H_2O$	7	94.11	2.38
2,4-Dichloroaniline	$C_6H_5NCl_2+8.25O_2 \rightarrow 6CO_2+2.5H_2O+NO_2+2Cl$	8.25	162.02	1.63
2,3-Dichlorobenzoic acid	$C_7H_4O_2Cl_2+7O_2 \rightarrow 7CO_2+2H_2O+2Cl$	7	191.01	1.17
Tris(2-ethylhexyl)trimellitate	$C_{33}H_{54}O_6+43.5O_2 \rightarrow 33CO_2+27H_2O$	43.5	546.78	2.55

a) Number of oxygen *b)* Molecular weight (g/mol) *c)* TOD (mg-O_2/mg-chemical) = [A] × (32 g-O_2/mol)/[B]

acid. Specifically, the volume of the test medium was 300 mL in accordance with the 301C test guideline, and it was poured into a 500 mL test bottle for the coulometer and a 1140 mL test bottle for OxiTop®. Each test bottle was a standard bottle supplied by Ohkura Electric or WTW. Concentrations of the test chemical and the activated sludge were 100 and 30 mg L^{-1}, respectively, in the test medium. The test medium was incubated at 25±1°C in the 301C test and 22±2°C in the 301F test. Each test was conducted in duplicate until the degradation of the test chemical progressed and the BOD curve approached a plateau.

4. 301C test or 301F test using OxiTop® with different headspace volumes

Ready biodegradability tests were carried out according to the OECD 301C guideline for sodium benzoate and the OECD 301F guideline for aniline and benzyl alcohol with different headspace volumes by pouring the test medium at volumes of 300, 400, 500, 600 700, 800, 900, or 1000 mL into a 1140 mL test bottle capped with an OxiTop®. Each test medium included 100 mg L^{-1} of the test chemical and 30 mg L^{-1} of the activated sludge. The test bottles were stored in a thermostatic chamber at 25±1°C in the 301C test and 22±2°C in the 301F test. Each test was conducted in singlicate until the degradation of the test chemical progressed and the BOD curve approached a plateau.

5. 301F test using OxiTop® with a larger volume of test medium

The 301F test was conducted for Tris using OxiTop® by pouring the test medium at volumes of 300, 3900, or 9000 mL into a 560, 7700, or 18000 mL test bottle, respectively. These test bottles, having similar diameters and heights, were specifically designed and produced for this experiment. The test medium included 100 mg L^{-1} of Tris and 30 mg L^{-1} of the activated sludge, and each test was conducted in triplicate at 22±2°C for 28 days.

6. Chemical analysis

The amount of Tris in the test medium after 28 days was determined by the method described in our previous report[4]; tetrahydrofuran was added to the medium at a ratio of 2 (tetrahydrofuran) to 3 (test medium), and the supernatant after centrifugation was subjected to HPLC analysis. Spike and recovery tests were carried out in triplicate, and the average recovery percentage was 103±3%. The biodegradation percentage was calculated by subtracting the remaining amount of Tris, as determined by HPLC, from the amount added at the beginning of the exposure.

7. Calculation of oxygen amount

Since oxygen exists in the headspace and the test medium, the amount of oxygen in the bottle (O_{2_bottle}) is given by Eq. 1. In contrast, the ThOD in the bottle is given by Eq. 2.

$$O_{2\_bottle} = (V_{bottle} - V_{medium}) \times M_{O2} \times R_{O2} \div (R \times T) + DO \times V_{medium} \quad (1)$$

$$ThOD = C_{chemical} \times V_{medium} \times TOD \quad (2)$$

where V_{bottle} is the test bottle volume (L), V_{medium} is the test medium volume (L), M_{O2} is the molecular weight of oxygen (32000 mg mol^{-1}), R_{O2} is the percentage of oxygen in the air (21%), R is the gas constant (0.082 atm L K^{-1} mol^{-1}), T is the temperature (298 or 295 K for the 301C or 301F test, respectively), DO is the dissolved oxygen in water (8.53 mg L^{-1} at 298 K and 8.11 mg L^{-1} at 295 K), $C_{chemical}$ is the concentration of the test chemical in the test medium (100 mg L^{-1}), and TOD is the total oxygen demand (mg of oxygen per mg of test chemical) of the test chemical. The TOD values for the test chemicals are listed in Table 1.

Results and discussion

1. 301C and 301F tests using a coulometer and OxiTop® with 300 mL of test medium

Since the 301C and 301F tests have been exclusively conducted using a coulometer under the CSCL, it was not known if OxiTop® could reproduce the same results as the coulometer. In this study, seven chemicals were used to confirm the homology of the BOD curves between the coulometer and OxiTop® in both the 301C and 301F tests. While the test medium volume was fixed at 300 mL, the capacity of the test bottle for the coulometer was 500 mL (a standard bottle supplied by Ohkura Electric), and that for OxiTop® was 1140 mL (a standard bottle supplied by WTW). Based on previous assessments or studies,[9–15] sodium benzoate, aniline, sodium acetate, benzyl alco-

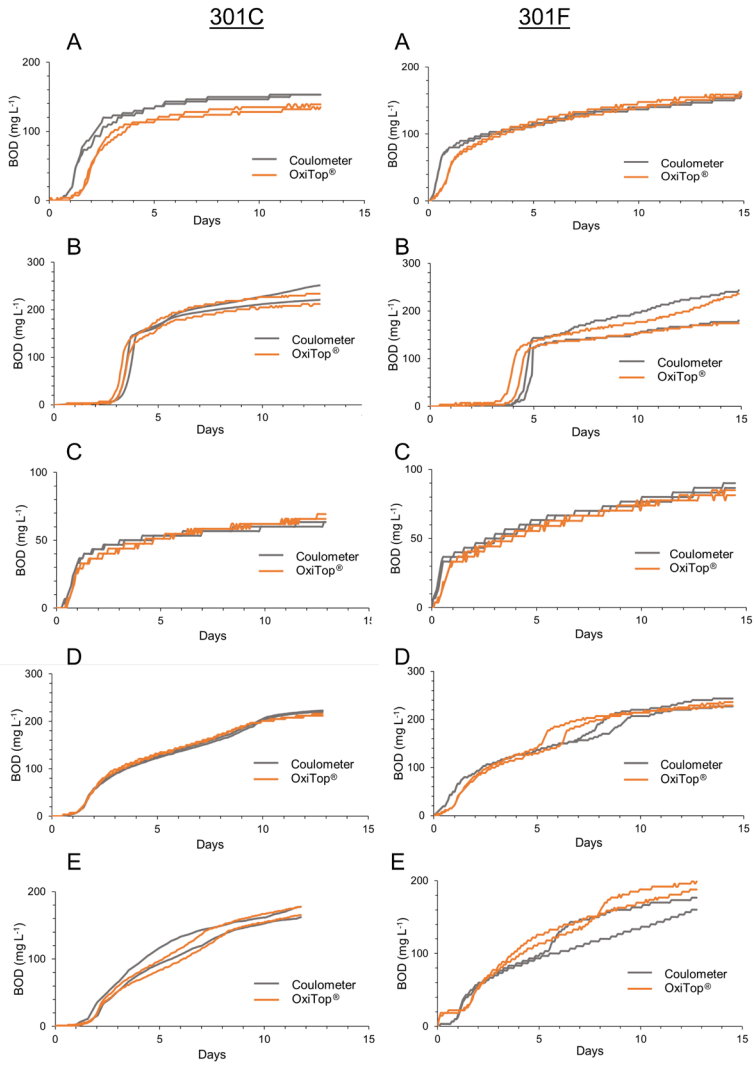

Fig. 1. BOD curves of sodium benzoate (A), aniline (B), sodium acetate (C), benzyl alcohol (D), and phenol (E) in the 301C and 301F tests by pouring 300 mL of test medium into a 560 mL test bottle for the coulometer or a 1140 mL test bottle for OxiTop®

hol, and phenol were judged to be readily biodegradable, while 2,4-dichloroaniline and 2,3-dichlorobenzoic acid were judged to be not readily biodegradable.

Figure 1 shows the BOD curves in the 301C and 301F tests using the coulometer and OxiTop® for sodium benzoate (A), aniline (B), sodium acetate (C), benzyl alcohol (D), and phenol (E). The time that elapses before the BOD curve starts to rise is called the lag phase; the slower the degradation, the longer the lag phase. For the respective five test chemicals, it was confirmed that no large variation in the BOD curves or the lag phases between duplicated data were observed in the 301C test or 301F test using the coulometer or OxiTop®. In addition, average BOD values (%) against ThOD for the five chemicals at the end of exposure were almost the same between the coulometer and OxiTop® for both tests (Table 2). Although the standard activated sludge used in the 301C test was different from the activated sludge used in the 301F test, similar BOD curves and lag phases were obtained from these tests. It was considered that the standard activated sludge and the activated sludge taken from the plant both contained specific degraders for these chemicals. From these results, we confirmed that the BOD curves obtained using the coulometer in both the 301C and 301F tests could be reproduced by using OxiTop®; based on the BOD curves, these chemicals were judged to be readily biodegradable, as determined previously.

Experiments identical to those conducted for the five chemicals above were conducted for 2,4-dichloroaniline and 2,3-dichlorobenzoic acid, which were judged to be not readily biodegradable in previous studies.[14,15] No differences between the 301C and 301F tests were observed in the BOD curves, and the BOD curves for neither the coulometer and OxiTop® were observed to rise during the 28-day exposure. In addition, at the end of the exposure, the average BOD values (%) against the ThOD were similar for both the coulometer and OxiTop® in the

Table 2. Average percentage ratio of BOD to ThOD of each test chemical at the end of exposure in the 301C and 301F tests using the coulometer and OxiTop®

Test chemical	BOD (%) against ThOD			
	301C		301F	
	Coulometer	OxiTop®	Coulometer	OxiTop®
Sodium benzoate	92	82	95	96
Aniline	98	93	88	85
Sodium acetate	81	87	113	106
Benzyl alcohol	88	85	93	92
Phenol	71	72	71	81
2,4-Dichloroaniline	4	6	7	9
2,3-Dichlorobenzoic acid	20	18	21	19

301C and 301F tests (Table 2). Thus, we verified that the BOD curves obtained using the coulometer in both the 301C and 301F tests were comparable to those obtained using OxiTop® for these chemicals; based on the BOD curves, these chemicals were judged to be not readily biodegradable, as determined previously.

The coulometer exclusively used under CSCL is a large and relatively expensive instrument, and the sizes of test bottles available are limited due to the size of the coulometer's thermostatic oven. On the other hand, OxiTop®, which is a cap-shaped pressure meter attached to a test bottle, is a compact and relatively inexpensive device. Because OxiTop® has reproduced BOD values similar to those of the coulometer, it is expected that OxiTop® would be utilized for the 301C and 301F studies under the CSCL in the future.

When microorganisms consume oxygen in a test bottle to degrade a test chemical, the coulometer adds the same amount of oxygen consumed into the bottle to keep the pressure in the bottle constant. On the other hand, because OxiTop® does not add any oxygen into the bottle, it was anticipated that the degradation of a chemical using OxiTop® might be suppressed due to a shortage of oxygen in the test bottle. In the above studies, since the size of a standard bottle for OxiTop® was 1.14 L (V_{bottle}) and the test medium volume was 0.3 L (V_{medium}), the amount of oxygen in the bottle (O_{2_bottle}) was calculated to be 234 mg for the 301C test and 236 mg for the 301F test, in accordance with Eq. 1. The ThOD for benzyl alcohol, which has the highest TOD among the seven chemicals, was calculated to be 75.6 mg, in accordance with Eq. 2. Therefore, when the volume of the test medium was 300 mL, the standard bottle size for OxiTop® was considered to have sufficient oxygen in the headspace to degrade the seven test chemicals. As a result, BOD values similar to those obtained with the coulometer could be obtained with OxiTop®, provided there was sufficient oxygen in the test bottle.

2. 301C test or 301F test using OxiTop® with different headspace volumes

When the acceleration of chemical degradation is confirmed by an increase in the test medium volume, the coulometer is limited due to the size of the test bottle; however, OxiTop® does not have this size limitation. When using OxiTop®, however, it is important to be aware of the amount of oxygen in the test bottle. According to Eq. 1, the amount of oxygen in the bottle depends primarily on the headspace volume: for example, in the case of a 1.14 L bottle (V_{bottle}) and a test medium volume of 0.3 L (V_{medium}), as used in the 301C test, 231 mg and 3 mg of oxygen exist in the headspace and the test medium, respectively. For that reason, we endeavored to ascertain whether degradation was seriously suppressed in the 301C and 301F tests by varying the headspace volume when the test medium volume was increased using OxiTop®. A test medium volume of 300, 400, 500, 600, 700, 800, 900, or 1000 mL was poured into the 1140 mL standard test bottle for sodium benzoate in the 301C test and for aniline and benzyl alcohol in the 301F test. The ThODs required to completely oxidize these three chemicals are different, with benzyl alcohol requiring the highest TOD among the seven chemicals (Table 1). Aniline or sodium benzoate is used as a reference substance, as it is readily biodegradable in 301C and/or 301F tests.

Figure 2 shows the results when varying the headspace volume. The plateaus of the BOD values significantly decreased when the test medium was more than a certain volume for the respective test chemicals. According to the OECD[2] and the CSCL[3] guidelines, when the BOD value during a test period reaches 60% of the ThOD for a test chemical in the 301C test or the 301F test, the test chemical is considered to be degraded completely (and the remaining 40% is assumed to have been assimilated by microorganisms). The 60% values of ThODs for sodium benzoate, aniline, and benzyl alcohol are indicated as blue lines in Fig. 2. If the amount of oxygen in the test bottle at the beginning of the test is less than 60% of the ThOD for a chemical (*i.e.*, $O_{2\_bottle} < \text{ThOD} \times 60\%$), it is anticipated that the test chemical might not be degraded completely. To satisfy the $O_{2\_bottle} < \text{ThOD} \times 60\%$ based on Eqs. 1 and 2, the V_{medium} is calculated to be more than 854, 766, or 753 mL for sodium benzoate, aniline, or benzyl alcohol, respectively. In contrast, if the amount of oxygen in the bottle at the beginning of the test is equal to or more than the ThOD for a test chemical ($O_{2\_bottle} \geq \text{ThOD}$), it is assumed that the biodegradation of the test chemical will not be suppressed due to an oxygen

shortage in the bottle. To satisfy the $O_{2\_bottle} \geq$ ThOD based on Eqs. 1 and 2, the V_{medium} is calculated to be equal to or less than 723, 621, or 608 mL for sodium benzoate, aniline, or benzyl alcohol, respectively.

For sodium benzoate in the 301C test, the BOD curves were almost the same for the test volumes from 300 to 700 mL (*i.e.*, $V_{medium} \leq 723$ mL); however, the plateaus of the BOD curves significantly decreased as the volume increased for test volumes of 900 and 1000 mL (*i.e.*, $V_{medium} > 854$ mL). For aniline in the 301F test, the highest BOD was for a test volume of 300 mL, the second was for 600 mL, and the BOD curves were similar for test volumes of 400, 500, and 700 mL, meaning that variations in the BOD curves for $V_{medium} \leq 621$ mL were not caused by varying the head space. However, the BOD curves for the test volumes from 800 to 1000 mL (*i.e.*, $V_{medium} > 766$ mL) were suppressed as the test volumes increased. For benzyl alcohol in the 301F test, the BOD curves were similar for test volumes from 300 to 500 mL, the plateaus of the BOD curves for the test volumes from 600 to 1000 mL decreased as the test volume increased, but significant suppression was observed for test volumes from 800 to 1000 mL (*i.e.*, $V_{medium} > 753$ mL). These findings clearly showed that the biodegradation of the test chemicals was strongly suppressed when the amount of oxygen in the bottle at the beginning of the test was less than 60% of the ThOD, but that negligible suppression was observed when the amount of oxygen was equal to or greater than the ThOD of the chemical being tested. These results made it clear that a headspace volume that ensured an oxygen amount of at least 60% of the ThOD, or preferably more than the ThOD, was necessary when performing a ready biodegradability test using OxiTop®.

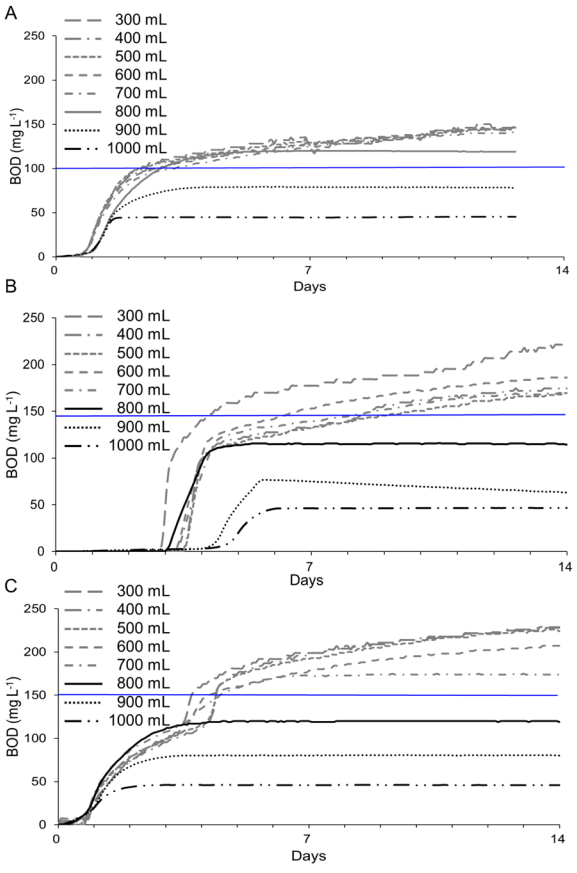

Fig. 2. BOD curves of sodium benzoate (A) in the 301C test, and aniline (B) and benzyl alcohol (C) in the 301F tests with varying headspace volume by pouring 300 to 1000 mL of the test medium into a 1140 mL test bottle. The blue line indicates the 60% value of ThOD for each chemical.

3. 301F test using OxiTop® with a larger volume of test medium

Since the test medium volume is not specified in the 301F test,[2,3] in the previous study using a coulometer, we found that the biodegradation of test chemicals (including Tris) adsorbed onto silica gel was accelerated by increasing the volume of the test medium. However, the residual chloroform in the silica gel made it difficult to determine the BOD caused solely by the degradation of the test chemical, and the thermostatic oven of the coulometer cannot accommodate any available test bottle where the volume of the test medium is more than 3900 mL. Therefore, we endeavored to investigate biodegradability of Tris without adsorbing onto silica gel by increasing the test medium volume using OxiTop®, and to evaluate the biodegradability based on the BOD curves and the remaining amount of Tris. The test medium volume was set to 300, 3900, or 9000 mL, and the corresponding test bottle size able to accommodate these volumes was 560, 7700, or 18000 mL, respectively. The O_{2_bottle} for the 560, 7700, or 18000 mL test bottle was calculated from Eq. 1 to be 74.7, 1087, or 2573 mg respectively, while the ThOD calculated from Eq. 2 was 76.5, 995, or 2295 mg, respectively. This meant that the headspace volume could supply 98%, 109%, or 112% of the ThOD for test mediums of 300, 3900, or 9000 mL, respectively. With these headspace volumes, the degradation of Tris would not be seriously suppressed due to oxygen shortage.

Figure 3 shows the BOD curves of Tris determined in triplicate for the respective three test mediums. For the test volume of 300 mL, large variations among the triplicate determinations were observed as follows: the lag phases were 7, 19, and ≥28 days; the percentage ratio of BOD to ThOD at day 28 was 34±23% based on measurements of 54%, 39%, and 9%; and a biodegradation rate of Tris determined by HPLC was 52±47% based on measurements of 96%, 56%, and 3%. When test medium volumes were increased to 3900 mL, these variations were decreased, and biodegradation was accelerated; the lag phases were shorter overall (*i.e.*, 8, 13, and 18 days), the percentage ratio of BOD increased to 53±8% based on measurements of 59%, 57%, and 44%; and the biodegradation rate of Tris was 77±19%, based on measurements of 98%, 71%, and 62%. When the test medium volume was further increased to 9000 mL, these variations were minimized, and biodegradation was further accelerated: the lag phases were similar (*i.e.*, 7 days); the percentage ratio of BOD reached 56±2% based on measurements of 58%, 56%, and 53%; and the biodegradation rate of Tris increased to 97±2%, based on measurements of 99%, 97%, and 96%. The degradation of Tris including three branched–alkyl

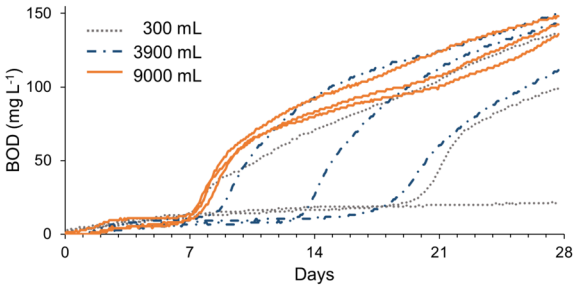

Fig. 3. BOD curves of Tris in the 301F test using OxiTop® by pouring 300, 3900, or 9000 mL of the test medium into a 560, 7700, or 18000 mL test bottle, respectively

chains and a benzene ring would require specific microorganisms that could degrade these parts. By increasing the test medium volume to 9000 mL, a sufficient number of these microorganisms could be introduced into the test bottle, the degradation of Tris was accelerated, and the reproducibility of test results was improved in triplicate determinations. In addition, it became clear that the biodegradation activity was increased just by increasing the test medium volume without adding silica gel. Ingerslev *et al.* (2000)[16] also found that the test volume played an important role when biodegradation took place in a closed system in which there was only a small total population of degraders, such that important microorganisms were lacking or were unable to proliferate. In addition, Martin *et al.* (2017)[17,18] showed that the use of more environmentally relevant cell numbers improved the accuracy of characterizing potentially environmentally persistent chemicals in freshwater screening tests. In a real environment, a greater diversity of bacterial flora coexist with chemical substances; hence, the biodegradation activity is expected to be higher than under the conditions of this study. Since OxiTop® can allow test medium volumes to be increased, provided the test bottles can be accommodated in a thermostatic chamber, the biodegradation potential should closely coincide with what would be seen in a real environment, as long as the headspace ensures that there is enough oxygen for microorganisms to degrade the test chemical.

Conclusions

OxiTop® is a cap-shaped pressure meter that can be attached to a test bottle of any size and that allows the test medium volume to be increased virtually without limitation. Although OxiTop® is considered to meet applicable regulatory requirements under the CSCL, the coulometer has been exclusively utilized for 301C and 301F tests in Japan. In this study, by conducting 301C and 301F tests with a standard bottle having a capacity of 1140 mL, including 300 mL of test medium, we were able to demonstrate that the chemical profiles of seven chemicals obtained using the coulometer could be reproduced using OxiTop®. Next, for no serious suppression of biodegradation to occur, we ascertained that when using OxiTop® with the standard 1140 mL bottle including the test medium at volumes of 300 to 1000 mL, the test bottle at the beginning of the 301C or 301F test must contain an amount of oxygen equivalent to at least 60% or more of (or preferably equal to) the respective ThOD of the three chemicals being tested. Finally, we designed and produced test bottles containing an amount of oxygen in their headspace similar to the ThOD of Tris for test medium volumes of 300, 3900, and 9000 mL, and we showed that the biodegradability of Tris was accelerated by increasing the test medium volume using OxiTop®. While the coulometer is a large and relatively expensive instrument, OxiTop® is compact and relatively inexpensive. Therefore, we recommend the use of OxiTop®, even for the 301C test in which the medium volume is fixed. When increasing the medium volume for the 301F test, OxiTop® is a suitable device, as the size of the test bottle can be freely increased. This technique does not artificially accelerate the biodegradation rate of test chemicals; rather, it more closely reflects what occurs in a real environment in which a much greater number and diversity of microorganisms exist. We recommend this technique, as it allows chemical fates to be evaluated more accurately under realistic conditions. Future research will be needed to clarify a greater diversity of microorganisms when the test medium volume is increased.

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