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Investigation of OECD 301F ready biodegradability test to evaluate chemical fate in a realistic environment

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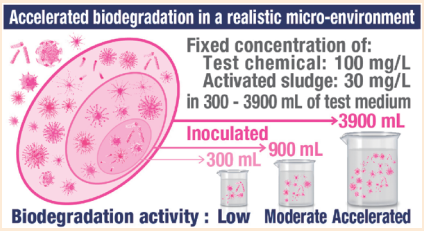
The OECD 301F ready biodegradability test has been approved for use under the Japanese Chemical Substances Control Law since 2018. This test uses activated sludge obtained from a sewage treatment plant instead of the standard activated sludge used for the 301C test. In addition, the test is allowed to add an inert support or emulsifying agent, and/or to change the volume of the test medium. In this study, we first confirmed that the standard sludge had lower biodegradation activities than the sludge taken from a sewage treatment plant. Second, we showed that biodegradation percentages were increased by adding suitable amounts of silica gel or Tween 80. Third, we found that the biodegradations were accelerated by only increasing the medium volume under the conditions that concentrations of chemical, silica gel, and sludge were held constant. These findings are expected to contribute to the appropriate evaluation of chemical fate in a realistic environment.



Keywords: OECD 301, biodegradability, activated sludge, silica gel, Tween 80, volume of test medium.

Introduction

Persistent, bioaccumulative, and toxic chemicals (PBTs) are difficult to degrade and remain in the environment for a long time, accumulate in organisms, and harm humans and/or wildlife. There is concern that when PBTs are discharged into the environment, they may pose serious risks to human health and ecosystems. Under regulatory frameworks such as the European regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)[1)] and the Japanese Chemical Substances Control Law (CSCL),[2)] chemicals must be evaluated before their manufacture or import from the viewpoints of biodegradation, bioaccumulation, and toxic properties. Biodegradation is generally recognized as the main pathway for removing chemicals from the environment and thus plays an important role in evaluating the environmental fate of chemicals. Ready biodegradability tests are laboratory-scale experiments for assessing the ready biodegradation of chemicals in an aqueous medium under aerobic conditions, and they highlight the rapid biodegradation of chemicals under most environmental conditions.[3)] Seven types of these tests have been listed in the Organisation for Economic Co-operation and Development (OECD) test guidelines,[4)] including 301C: modified MITI (I) (Ministry of International Trade and Industry, Japan), and 301F: manometric respirometry. The physicochemical properties of a test chemical, such as water solubility, volatility, and adsorptivity, are considered in choosing an applicable test. All of these tests require that a reference compound such as aniline be used to ensure the activity of activated sludge, and difference between the maximum and minimum degradation values of the test chemical should be within 20% at the end of the 28-day test period. These tests, except 301C, are required to be conducted in duplicate to evaluate the chemical fate qualitatively and to confirm that the activated sludge will degrade the reference compound more than 60% based on total theoretical oxygen demand or carbon dioxide evolution or more than 70% based on total dissolved organic carbon after 14 days.[4)] In contrast, the 301C test is required under the CSCL to be conducted in triplicate and to confirm that the activated sludge will degrade aniline as the reference compound by more than 40% based on total theoretical oxygen demand after 7 days as well as more than 65% after 14 days.[4)]

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These seven types of tests produce very conservative results that are not comparable to those under real environmental conditions.[3,5–7)]

In particular, the 301C test conventionally used for ready biodegradability tests under the CSCL is recognized as the most conservative with regard to the following aspects. First, the 301C test must be performed using the standard activated sludge,[8)] which is a mixed inoculum collected from 10 sites and cultivated with peptone and glucose as synthetic nutrients for more than a month at the Chemicals Evaluation and Research Institute (CERI, Japan). Inoculum collections at 10 sites consist of activated sludge from 4 municipal sewage treatment plants and surface water containing sediments from 3 rivers, 1 lake, and 2 inland seas in Japan. In contrast, for example, under the 301F test, activated sludge without any cultivation can be used on the day it is taken from a municipal sewage treatment plant. Since continuous cultivation of the activated sludge reduces microbial diversity[9)] and results in a decrease in degradation activity,[10)] the standard activated sludge used for the 301C test is regarded to have a lower degradation activity. Second, the 301C test must be performed at a concentration of 100 mg L^{-1} of a test chemical, even when it is insoluble in a test medium. This means that the bioavailability of the chemical to microorganisms is limited under this test condition, especially when the chemical is poorly water soluble. Chemicals in the real environment are generally adsorbed on organic and/or inorganic substances and usually exist dispersed at much lower concentrations. In fact, the ISO 10634 standard[11)] proposes four methods to improve the dispersibility of poorly water-soluble chemicals in a test medium: direct addition with continuous agitation, ultrasonic dispersion, adsorption on an inert support, and dispersion with an emulsifying agent. Third, the 301C test must be performed with 300 mL of test medium including 30 mg L^{-1} of the standard activated sludge. Microorganisms in the realistic environment are highly diversified and aggregate heterogeneously,[12)] and a small minority of microorganisms could take part in the degradation of a specific chemical.[3,13)] Therefore, the 300 mL of test medium including the specified amount of activated sludge may be too small to represent the degradation activity in all of the activated sludge. Taking into account the above limitations of the 301C test, the 301F test has been approved for use as a ready biodegradability test under the CSCL since 2018. The CSCL guideline for the 301F test[8)] permits the use of activated sludge obtained from a sewage treatment plant without any cultivation, to add inert support or an emulsifying agent, and to change the volume of the test medium. However, the newly introduced 301F test guideline does not describe the differences in chemical degradability between the standard activated sludge and the activated sludge obtained from a sewage treatment plant without any cultivation, does not specify what kind or amount of the inert support or emulsifying agent is appropriate in the test, and does not mention a suitable volume of the test medium. In this study, the effects on chemical degradability of using activated sludge with or without cultivation, of changing the amounts of silica gel as the inert support or Tween 80 as the emulsifying agent, and of increasing the volumes of the test medium were investigated to develop useful methods for evaluating the biodegradation potential in a realistic environment, especially for poorly water-soluble organic chemicals.

Materials and methods

1. Chemicals

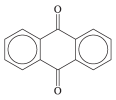
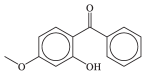
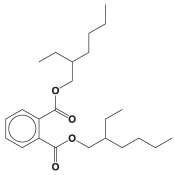
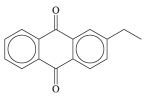
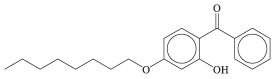
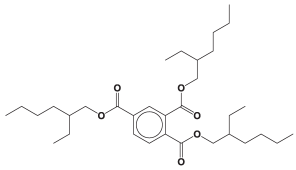
Anthraquinone (AQ), 2-hydroxy-4-methoxybenzophenone (OxB), bis(2-ethylhexyl)phthalate (Bis), 2-ethylanthraquinone (2-EA), 2-hydroxy-4-*n*-octyloxybenzophenone (OB), and tris(2-ethylhexyl)trimellitate (Tris), shown in Table 1, were purchased as test chemicals for ready biodegradability from Tokyo Chemical Industry (TCI, Japan). 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, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $FeCl_3 \cdot 6H_2O$, 0.5% phosphate solution, and aniline were purchased from FUJIFILM Wako Pure Chemical (Japan); 1 M sodium hydroxide solution was purchased from Kanto Chemical (Japan); and chloroform was purchased from Nacalai Tesque (Japan). Silica gel (5 to 25 μm particle size for thin-layer chromatography) was purchased from Sigma-Aldrich (USA), and Tween 80 was purchased from TCI. Water solubility for some of the test chemicals was estimated using EPI Suite™ ver. 4.11, a Windows-based suite of physical/chemical property and environmental fate estimation programs developed by the US EPA and Syracuse Research Corp.[14)]

2. Conventional ready biodegradability tests

Ready biodegradability tests were carried out according to OECD guideline 301C[4)] as follows: The standard activated sludge that was cultivated with peptone and glucose as synthetic nutrients for more than one month was purchased from CERI and was further cultivated with the synthetic nutrients at our laboratory until the initiation of each test. A mineral medium was prepared by mixing 30 mL of solution A, containing 21.75 g L^{-1} K_2HPO_4, 8.50 g L^{-1} KH_2PO_4, 44.6 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 1.70 g L^{-1} NH_4Cl; 30 mL of solution B, containing 22.5 g L^{-1} $MgSO_4 \cdot 7H_2O$; 30 mL of solution C, containing 27.5 g L^{-1} $CaCl_2$; and 30 mL of solution D, containing 0.25 g L^{-1} $FeCl_3 \cdot 6H_2O$, then adding water to a final volume of 10 L and adjusting the pH to 7.0 using 0.5% phosphate solution and 1 M sodium hydroxide solution. The biodegradability tests were performed in duplicate with 300 mL of test medium prepared by adding a test chemical and the standard activated sludge to the mineral medium adjusted to a concentration of 100 mg L^{-1} and 30 mg L^{-1}, respectively. The test media were stirred and incubated under diffuse light at 25±1°C for 28 days in a coulometer OM7000A (Ohkura Electric, Japan) that was able to measure the biochemical oxygen demand (BOD) of the test media.

Conventional ready biodegradability tests were carried out according to OECD guideline 301F[4)] as follows: Activated sludge was taken from a municipal sewage treatment plant receiving predominantly domestic sewage. A mineral medium was pre-

Table 1. Test chemicals and their reported water solubilities and biodegradation percentages

Test chemical	Water solubility (mg L^{-1})	Biodegradation percentage
Anthraquinone (AQ) 	1.35[15]	52.3% at 21 day in OECD 301C[16] 62% at 28 day in EU C.4-E[17]
2-Hydroxy-4-methoxybenzophenone (OxB) 	3.7[18]	4% at 28 day in OECD 301C[19] 62% at 28 day in EEC 79-831[20]
Bis(2-ethylhexyl)phthalate (Bis) 	0.27[21]	69% at 28 day in OECD 301C[22]
2-Ethylanthraquinone (2-EA) 	0.13269[a]	0% at 14 day in OECD 302C[23]
2-Hydroxy-4-*n*-octyloxybenzophenone (OB) 	0.03693[a]	0% at 14 day in OECD 301C[24] 5 to 6% at 28 day in OECD 301B[25]
Tris(2-ethylhexyl)trimellitate (Tris) 	0.00306[26]	4.2% at 28 day in OECD 302C[27]

[a] Estimated by EPI Suite™ ver. 4.11[14]

pared by mixing 100 mL of solution A, containing 8.50 g L^{-1} KH_2PO_4, 21.75 g L^{-1} K_2HPO_4, 67.21 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, and 0.50 g L^{-1} NH_4Cl, and adjusting the pH to 7.4; 10 mL of solution B, containing 27.5 g L^{-1} $CaCl_2$; 10 mL of solution C, containing 22.5 g L^{-1} $MgSO_4 \cdot 7H_2O$; and 10 mL of solution D, containing 0.25 g L^{-1} $FeCl_3 \cdot 6H_2O$, then adding water to a final volume of 10 L. The biodegradability tests were performed with 300 mL of a test medium prepared by adding a test chemical and the activated sludge to the mineral medium adjusted to a concentration of 100 mg L^{-1} and 30 mg L^{-1}, respectively. In accordance with the OECD and CSCL guidelines, the 301F tests were conducted in duplicate for each test chemical and in singlicate for aniline as the reference compound to ensure the activity of the activated sludge used. The test media were stirred and incubated under diffuse light at 22±1°C in the coulometer for 28 days.

3. Modified ready biodegradability test

In accordance with the OECD guidelines, the above conventional ready biodegradability tests were performed even when the test chemical was insoluble. In contrast, chemicals in the real environment usually are dispersed by adsorption on organic and/or inorganic substances. In order to improve the dispersibility of chemicals in the test medium and evaluate their biodegradability properly in a more realistic environment, the 301F test was modified by adding an inert support to the test medium. The ISO 10634 standard gives as an example 30 g of the support to 150 mL of 1 g L^{-1} of the test chemical solution in the chosen solvent, meaning 30 g of the support to 150 mg of the test chemical, and it states that the quantity of the support should be minimal to avoid or minimize surface area effects. In this study, silica gel was selected as the inert support. Since 300 mL of the test medium contained 30 mg of the test chemical for the 301F test, 6 g of silica gel was first added to the test medium in accordance

with the ISO 10634's example. Then the amount of silica gel was reduced from 6 g to 4.4, 2.8, 1.6, 0.8, or 0.4 g, corresponding to 11/15, 7/15, 4/15, 2/15, or 1/15 quantity of the ISO's value, respectively. One hundred and fifty milligrams of each test chemical was dissolved with 150 mL of chloroform in a beaker, and 2, 4, 8, 14, 22, or 30 g of silica gel was added and mixed well using a magnetic stirrer RCN-7 (EYELA, Japan) for 2 hr. The chloroform was thoroughly evaporated by using a rotary evaporator N-1000 (EYELA, Japan) at 45°C for about 10 min and then a vacuum oven AVO-250NB (ASONE, Japan) at 45°C for about 28 days. OxB, Bis, or 2-EA adsorbed on 2 mg of the silica gel was extracted with 1 mL of acetonitrile, while AQ, OB, or Tris was extracted with 1 mL of tetrahydrofuran. The extracted test chemical amounts were determined by HPLC analyses, and the results showed that almost all of the added test chemical was adsorbed on the silica gel. Based on the analytical results, the silica gel adsorbing 30 mg of the test chemical was added to 300 mL of the mineral medium for a ready biodegradability test.

Separately, the 301F test was modified by adding Tween 80 to the test medium, which was classified as a nonionic emulsifying agent, taking into account the ISO 10634 standard giving as an example polyethylene sorbitan trioleate.[11] One hundred and fifty milligrams of the test chemical was dissolved using a stirrer in about 20 mL of chloroform in a beaker for about 5 min, and 22.5, 60, 150, 450, or 2250 mg of Tween 80 was added to the beaker and stirred in a fume hood for about 3 hr to volatilize the chloroform. Residual chemicals in the beaker were transferred to a volumetric flask and diluted with distilled water. One milliliter of the solution was taken from the flask and diluted with acetonitrile for OxB, Bis, and 2-EA or tetrahydrofuran for AQ, OB, and Tris, and then the chemical amount was determined *via* HPLC analysis. Based on the analytical result, the solution containing the 30 mg of the test chemical was added to 300 mL of the mineral medium for a ready biodegradability test.

The 301F test was further modified by changing volume of the test medium from 300 mL to 900 or 3900 mL under the conditions that concentrations of the test chemical, silica gel, and activated sludge were kept at 100, 1333, and 30 mg L^{-1}, respectively. Specifically, after each test chemical was adsorbed onto silica gel at a ratio of 30 mg of the chemical to 0.4 g of the silica gel, 0.4, 1.2, and 5.2 g of the silica gel were added to 300, 900, and 3900 mL of the test medium including the activated sludge at a concentration of 30 mg L^{-1}, respectively, and the test media were incubated at 22±1°C for 28 days. In accordance with the OECD and CSCL guidelines, the modified 301F tests were conducted in duplicate for each test chemical and in singlicate for aniline as the reference compound to ensure the activity of the activated sludge used.

4. Chemical analyses

Spike and recovery tests were carried out as follows: The test chemical and microorganisms (as well as silica gel or Tween 80 in the case of the modified biodegradability test) were mixed in a test medium, an organic solvent was added to them at a ratio of 2 (organic solvent) to 3 (test medium), and ultrasonic dispersion for 1 min was repeated 3 times. The organic solvent was acetonitrile for OxB, Bis, and 2-EA and tetrahydrofuran for AQ, OB, and Tris. Next, centrifugation was performed, and the supernatant was subjected to HPLC analysis. The recovery percentage was calculated by dividing the recovered amount of test chemical determined *via* HPLC by the spiked amount. More than 80% of the test chemical was recovered from the test medium in all of the biodegradability tests.

Biodegradation percentages of aniline were determined by measuring the BOD after 7 and 14 days for the 301C tests and only after 14 days for the 301F tests. The percentages were calculated by dividing the BOD of aniline by the total theoretical oxygen demand of aniline. After the 28 days of exposure, the test chemical remaining in the test medium was extracted and subjected to HPLC analysis in the same manner as the spike and recovery test. The biodegradation percentage was calculated by dividing the remaining amount of test chemical determined *via* HPLC by the amount added at the beginning of the exposure. When performing quantitative evaluation, the biodegradation percentage was corrected by each recovery percentage obtained from the spike and recovery test. The biodegradation percentage was expressed as 0% when the test chemical remaining after the exposure was the same as the added amount.

All chromatographic experiments were carried out on a Shimadzu LC-10ADvp system with LabSolutions software (Shimadzu, Japan). The separation was achieved on an L-column2 ODS column (150 mm×2.1 mm, 5 μm; CERI, Japan). Mobile phase A, consisting of distilled water with 0.1% formic acid, and mobile phase B, consisting of acetonitrile with 0.1% formic acid, were pumped at flow rate of 0.2 mL/min. The gradient program of mobile phase B was set as follows: 0 to 16 min, linear gradient from 50% to 100%; 16 to 30 min, 100%; 30 to 45 min, 50%. The injection volume and detection wavelength were fixed at 10 μL and 254 nm, respectively. The column temperature was maintained at 40°C.

Results and discussion

The six test chemicals with water solubility of less than 100 mg L^{-1} (Table 1) were used for comparison of biodegradation percentages between the 301C, conventional 301F, and modified 301F tests. A combination of AQ and 2-EA, OxB and OB, or Bis and Tris had similar structures, but they differed in water solubility. According to previous studies, AQ, OxB, and Bis with a relatively high water solubility were judged to have "ready biodegradability" based on the results of OECD guideline 301C,[16,19,22] EU Method C.4-E,[17] and EEC Directive 79-831 tests.[20] 2-EA, OB, and Tris, having an additional side chain, were judged to have "not ready biodegradability" based on the results of OECD guideline 301C,[24] 301B,[25] and 302C tests.[23,27]

1. Influence on biodegradation percentages by changing the activated sludge

In this study, the 301C tests were performed at 25±1°C for 28

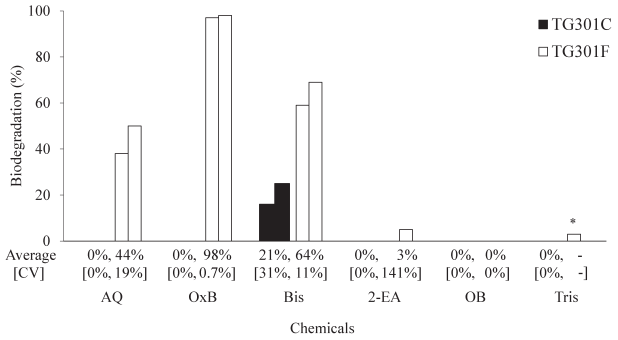


Fig. 1. Biodegradation percentages of test chemicals in 300 mL of test medium containing the standard activated sludge for the 301C tests (black bar) and containing activated sludge from a sewage treatment plant for the conventional 301F tests (white bar) after 28 days of incubation.

* Singlicate result due to autosampler problems with HPLC

days with 300 mL of the test medium containing each chemical and the standard activated sludge. They were conducted in duplicate at our laboratory in spite of the CSCL requirement (*i.e.*, in triplicate) because the degradability of the test chemicals had already been investigated, mainly by the same 301C tests (Table 1). As a result, the biodegradation percentages of Bis were 16% and 25%, and the difference between them was within 20%, which fulfilled the guideline requirement, but the other substances were not degraded at all (Fig. 1). For the tests of AQ, Bis, 2-EA, and Tris, it was confirmed at CERI two days before the tests began that the activated sludge degraded 77% and 95% of aniline based on the total theoretical oxygen demand after 7 and 13 days, respectively. For the tests of OxB and OB, it was confirmed at our laboratory in another test begun 2 days before that the activated sludge degraded 40% and 91% of aniline after 7.5 and 14 days, respectively, based on the total theoretical oxygen demand. Although the activated sludge degraded aniline almost to the degree required by the guideline, our results using the test chemicals were somewhat different from the previous 301C tests showing that the biodegradation percentages of AQ, OxB, Bis, and OB were 52.3%, 4%, 69%, and 0%, respectively (Table 1). It is well known that biodegradation percentages fluctuate, especially for the most conservative 301C test, because it is very difficult to maintain and control such low microbial activity of the standard activated sludge. In fact, the OECD guideline clearly states, "Realising that ready biodegradability tests may sometime fail because of the stringent test conditions, consistent positive test results (i.e., rapid and ultimate degradation) from test(s) should generally supersede negative test results."[28)]

In contrast, not only Bis but also AQ and OxB, which were judged to have "ready biodegradability" in the previous studies, were clearly degraded in the conventional 301F tests conducted in accordance with the OECD and CSCL guidelines: in duplicate at 22±1°C for 28 days with 300 mL of the test medium containing each chemical and activated sludge without any cultivation at our laboratory on the day it was taken from a sewage treatment plant. The result showed that the average biodegradation percentages of AQ, OxB, and Bis were 44%, 98%, and 64%, respectively (Fig. 1). It was confirmed in the 301F tests that the activated sludge degraded aniline by 60% for AQ and Bis and by 71% for OxB after 14 days, and the differences for each test chemical between the maximum and minimum degradation values were within 20%, which fulfilled the guideline requirements. Although a lower temperature generally reduced the biodegradation activity of microorganisms,[29)] the test chemicals were degraded more in the conventional 301F test than in the 301C test. It was reported that the microbial divergence index values and degradation activity of the standard activated sludge were lowered by culturing the sludge with peptone and glucose as synthetic nutrients.[9,10)] Our study confirmed that the activity of the standard activated sludge cultivated with the synthetic nutrients for more than one month was much lower than that of the activated sludge taken from a sewage treatment plant without any cultivation.

2-EA, OB, and Tris are more poorly water-soluble test chemicals and have been judged to have "not ready biodegradability" in previous studies. They were not degraded at all in our 301C tests conducted in duplicate with 300 mL of the test medium containing the standard activated sludge (Fig. 1). Even in the conventional 301F test conducted in duplicate with 300 mL of the test medium containing the activated sludge taken from a sewage treatment plant, their biodegradation percentages were 0% and 5% for 2-EA, 0% and 0% for OB, and 3% for Tris, which values were comparable to those in the 301C tests. It was confirmed in the 301F tests that the activated sludge degraded aniline by 60% for 2-EA and Tris and by 71% for OB after 14 days, and the differences between the maximum and minimum degradation values of each test chemical except Tris were within 20%, which fulfilled the guideline requirements. Only the singlicate result was obtained for Tris due to HPLC autoinjection problems. It was deemed that the change in the activated sludge had little effect on biodegradability, because the test chemicals with much lower water solubility had poor bioavailability to the microorganisms. It was thought that an increase in bioavailability to microorganisms was necessary to improve the degradability, especially for poorly water-soluble chemicals.

2. Influence on biodegradation by adding silica gel

In order to improve the bioavailability for poorly water-soluble chemicals, the ISO 10634 mentions "adsorption on an inert support" as one of the techniques for applying the test chemical to a test medium.[11)] In this study, silica gel was selected as the inert support, and 6 g silica gel was first added to the 300 mL of test medium in accordance with the ISO 10634's example. However, the amount of silica gel was found to be excessive in the context of dispersibility and fluidity. For that reason, an amount of silica gel that would be suitable was investigated by reducing the amount from 6 g to 4.4, 2.8, 1.6, 0.8, or 0.4 g. 2-EA and OB were chosen as test chemicals that had been judged to have "not ready biodegradability" and were confirmed to hardly degrade in our 301C or conventional 301F test (Fig. 1). This investigation

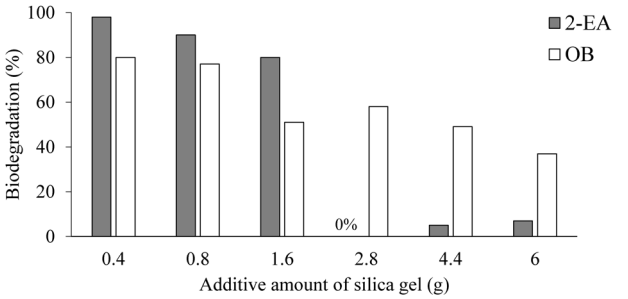


Fig. 2. Biodegradation percentages of 2-EA (gray bar) and OB (white bar) in 300 mL of test medium containing various amounts of silica gel additive and activated sludge from a sewage treatment plant for the modified 301F tests after 28 days of incubation.

was conducted in singlicate, and the degradation percentage of aniline was not determined because our purpose was to compare the degradation percentages of the test chemical among six amounts of silica gel under the same conditions, including the activity of the sludge. After the same amount of test chemical (30 mg) was adsorbed on each amount of silica gel and added to the test medium, twelve 301F tests were carried out for 28 days with the same activated sludge taken from the municipal sewage treatment plant. The results showed that 2-EA was hardly degraded with 2.8, 4.4, and 6 g of silica gel, but it was degraded by 98%, 90%, and 80% by reducing the amounts of silica gel to 0.4, 0.8, and 1.6 g of silica gel, respectively (Fig. 2). In contrast, OB was degraded to some extent even with 6 g of silica gel, and the biodegradation of OB was 80%, 77%, 51%, 58%, 49%, and 37% with 0.4, 0.8, 1.6, 2.8, 4.4, and 6 g of silica gel, respectively. OB has a long alkyl chain that could be biodegraded by many microorganisms,[30] and in fact it was slightly degraded in the 301B test,[25] while 2-EA was not degraded at all in the 302C test[23] (Table 1). It was considered that 2-EA needed more suitable amounts of silica gel to be degraded, in comparison with OB, which was degraded to some degree with silica gel ranging from 0.4 to 6 g. At any rate, biodegradation of the test chemicals was accelerated by reducing the amount of silica gel, probably owing to the improvement of dispersibility and fluidity. Timmer *et al.*[31] also observed that biodegradation rates decreased with increasing sorbent concentration, although their purpose was to mitigate the inhibitory effect of a cationic surfactant, cetylpyridinium chloride (CPC), on microorganisms by adding silica gel according to the OECD 310 ready biodegradability (headspace) test.[32] Biodegradation of CPC at 4 g/L of silica gel was slower than that at 0.8 g/L of silica gel, and the biodegradation slowed further at 20 g/L of silica gel. They considered that the inhibitory effects of CPC on microorganisms were mitigated by adding silica gel to the test medium, but a higher concentration of silica gel might reduce bioavailability, because CPC was highly adsorbed to the silica gel. In our study, the bioavailability of poorly water-soluble chemicals to microorganisms in the test medium was increased by adsorbing the chemicals onto silica gel as mentioned in the ISO standard,[11] but a higher concentration of silica gel might reduce the bioavailability because of the lower dispersibility and fluidity of silica gel in the test medium. Thus, we considered that an excess amount of silica gel should be avoided in the 301F test, even when investigating other chemicals. In short, 2-EA and OB, which had been judged to have "not ready biodegradability" based on previous studies and which were hardly degradable in our 301C and conventional 301F tests (Fig. 1), were considerably biodegraded by adsorption on an appropriate amount of silica gel in the modified 301F tests (Fig. 2).

Since 2-EA or OB were greatly degraded with 0.4 g of silica gel, the modified 301F tests for AQ, OxB, Bis, and Tris were designed to be conducted in duplicate under the same conditions as for OB and 2-EA, along with the conventional 301F tests without adding silica gel. While the biodegradation percentages in the 301F tests without adding silica gel were 38% and 50% for AQ, 97% and 98% for OxB, 59% and 69% for Bis, and 3% for Tris, those in the modified 301F tests with silica gel were 62% and 88% for AQ, 96% and 100% for OxB, 76% and 81% for Bis, and 10% and 11% for Tris (Fig. 3). It was confirmed in the conventional and modified tests that the activated sludge degraded aniline by 60% for AQ, Bis, and Tris and by 71% for OxB after 14 days, and the differences between the maximum and minimum degradation values of each test chemical, except in the case of AQ in the modified 301F test, were within 20%, which fulfilled the guideline requirements. Although the difference in AQ was 26% in the modified 301F test, it did not deteriorate the reliability of the results, because the coefficient of variation (25%) of the degradation percentages was not significantly different from that (19%) in the conventional 301F test; both of the biodegradation percentages, 62% and 88%, were higher than the 38% or 50% in the conventional test, and the average biodegradation percentage of 75% was 31% higher than that of 44% in the conventional test. These results showed that biodegradations of AQ, Bis, and Tris were accelerated by adding silica gel, and the acceleration could not be clearly observed for OxB because it was biodegraded by more than 97% even in the conventional 301F test. As chemicals discharged into the environment usually are dispersed by adsorption onto organic and/or inorganic substances, it is considered that the 301F test modified by adding silica gel is useful to properly evaluate the biodegradability

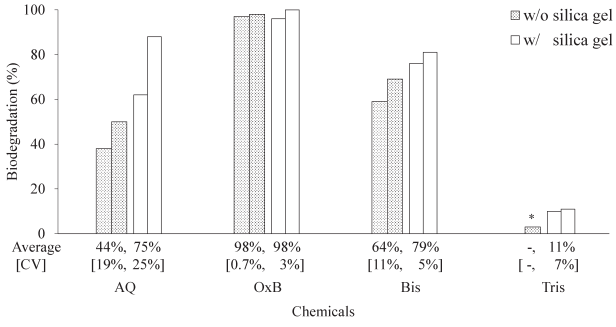


Fig. 3. Biodegradation percentages of AQ, OxB, Bis, and Tris in 300 mL of test medium without (dotted bar) and with (white bar) 0.4 g of silica gel for the conventional and modified 301F tests after 28 days of incubation.

* Singlicate result due to autosampler problems with HPLC

potential of chemicals.

3. Influence on biodegradation by adding an emulsifying agent

To improve the bioavailability of poorly water-soluble chemicals, "dispersion with an emulsifying agent" is also mentioned in ISO 10634 as one of the techniques for applying a test chemical.[11] In this study, a nonionic surfactant, Tween 80, was selected as an emulsifying agent, since it was known that nonionic surfactants had low antibacterial activity.[33] As is the case in the investigation of silica gel, 2-EA and OB were chosen as test chemicals because AQ, OxB, and Bis had already degraded in the conventional 301F test (Fig. 1), and Tris had hardly degraded even with the addition of silica gel (Fig. 3). Tween 80 was first added to the test medium at a concentration of 15 mg L^{-1}, which was close to its critical micelle concentration (CMC) of 13 mg L^{-1},[34] but it was observed that a homogeneous dispersion could not be achieved in the presence of 2-EA or OB at a concentration of 100 mg L^{-1}. Based on Nyholm's study,[35] in which Tween 80 was added at a concentration of 40 mg L^{-1}, we investigated 40, 100, 300, and 1500 mg L^{-1} Tween 80 in singlicate to find a suitable concentration from the viewpoint of biodegradability improvement for 28 days. The results showed that the biodegradation percentages of OB with Tween 80 concentrations of 40, 100, 300, and 1500 mg L^{-1} were 76%, 46%, 15%, and 33%, respectively, and 2-EA was degraded only with 40 mg L^{-1} of Tween 80 (Fig. 4), whereas the activated sludge degraded 67% of aniline after 14 days. In a similar fashion as with the silica gel, OB was degraded to some extent regardless of the concentrations of Tween 80, but 2-EA was degraded only at a certain concentration of Tween 80. These results indicated that both test chemicals were greatly degraded with around 40 mg L^{-1} of Tween 80. Zhang *et al.* (2012)[36] also found that biodegradation percentages decreased with increasing concentrations of Tween 80, although they used pyrene as a test chemical with a bacteria strain *Klebsiella oxytoca* PYR-1 isolated from the activated sludge of a cooking plant wastewater treatment facility that could effectively remove pyrene. They reported that the addition of Tween 80 at a CMC of 13.1 mg/L resulted in the most significant promotions for pyrene, but a higher concentration of Tween 80, 104.8 mg/L, inhibited the apparent degradation of pyrene. These findings indicated that emulsifiers promoted bioavailability due to the improvement in dispersibility, but an excess amount of emulsifier formed micelles that enclosed the test chemical, and hence the bioavailability of the test chemical was decreased. Thus, investigation of a suitable Tween 80 concentration in the 301F test should be conducted when evaluating other chemicals.

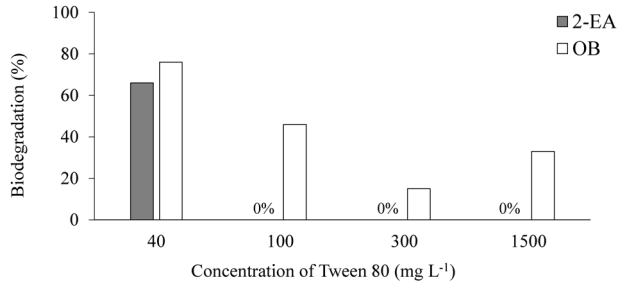


Fig. 4. Biodegradation percentages of 2-EA (gray bar) and OB (white bar) in 300 mL of test medium containing various concentrations of Tween 80 and activated sludge from a sewage treatment plant for the modified 301F tests after 28 days of incubation.

4. Influence on biodegradation by changing the test medium volume

It is known that a small minority of microorganisms play a key role in biodegrading a specific chemical, and some microorganisms heterogeneously crowd together into aggregates in activated sludge.[12] In fact, when the 301C and 301F tests with 300 mL of test medium had been carried out for many test chemicals at our laboratory in the past, high variabilities in the extent of biodegradation and/or lag phase had been observed between different tests and within test replicates for the same chemical. Since it was thought that 300 mL of test medium was too small to represent all kinds of microorganisms in the activated sludge taken from a sewage treatment plant, modified 301F tests were conducted in duplicate by increasing volume of the test medium under the conditions that concentrations of the test chemical, silica gel, and activated sludge were kept at 100, 1333, and 30 mg L^{-1}, respectively. 2-EA and Tris were chosen as test chemicals, because 2-EA needed more suitable amounts of silica gel or Tween 80 to be degraded in comparison with OB (Fig. 2 or 4), and Tris was hardly degraded among the six test chemicals with the same amount of silica gel (Fig. 3). In the results, the average biodegradation percentages of 2-EA in 300, 900, and 3900 mL of test medium were 90%, 85%, and 100%, respectively, and those of Tris were 10%, 20%, and 42%, respectively (Fig. 5). It was confirmed that the activated sludge degraded 88% and 67% of aniline after 14 days for the tests of 2-EA and Tris, respectively, and the difference between the maximum and minimum degradation values of each test chemical, except in the case of 900 mL for 2-EA, was within 20%, which fulfilled the guideline requirements. Although 70% and 99% of 2-EA were degraded in 900 mL of test medium, the difference did not deteriorate the reliability of the results, because the coefficient of variation decreased and the biodegradation percentages increased when increasing the volume of the test medium to 3900 mL. These results indicated that increases in the medium volume accelerated biodegradation of the test chemicals. Ingerslev *et al.*[37] also observed this tendency, from the viewpoint of lag phase, by changing test medium volume from 1.8 mL to 100 L with 4 mg/L of *p*-nitrophenol (PNP), reflecting the waiting time to begin preferential growth of specific degraders. By using water collected from a polluted river in Denmark, they found that increasing the total volume of the test medium resulted in decreased lag phases, and the phases were more constant above 180 mL of test medium. They considered that the initial population of degraders may be missing at small volumes or be too small to establish a surviving proliferating culture. While the lag phases for PNP as a readily degradable compound reached a plateau with more

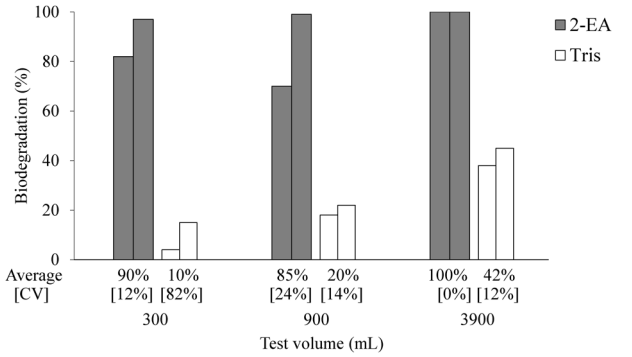


Fig. 5. Biodegradation percentages of 2-EA (gray bar) and Tris (white bar) in 300, 900, and 3900 mL of test medium with 0.4, 1.2, and 5.2 g of silica gel, respectively, for the modified 301F tests after 28 days of incubation.

than 180 mL of the river water, we tried to confirm the effect by increasing the medium volume to 3900 mL with other chemicals according to OECD 301F. Taking into account our results, it was suggested that a large volume of the test medium raised the probability of capturing a small minority of microorganisms playing a key role in biodegrading the relevant test chemical. Tris, including three branched alkyl chains and a benzene ring, was hardly degraded as observed in the conventional 301C and 301F tests (Fig. 1) because its bioavailability to microorganisms was limited due to its lower solubility,[38] and its degradation would require the existence of specific microorganisms biodegrading these parts.[37] Adding silica gel to the test medium enhanced the degradation of Tris to some extent by improving its dispersibility (Fig. 3), and increasing the test volume accelerated the biodegradation further by securing various microorganisms in the test medium (Fig. 5).

Conclusions

The OECD 301C tests produce very conservative results that are not comparable to those in real environmental conditions[39] from the viewpoints of (1) the biodegradation activity of microorganisms, (2) the concentration or dispersibility of test chemicals, and (3) the kinds and numbers of microorganisms. In order to develop suitable test methods for properly evaluating the biodegradability of chemicals in a realistic environment, we first compared the biodegradability of the standard activated sludge used in the 301C tests with that of the activated sludge without any cultivation. Second, we investigated the effect of dispersibility by adsorbing the chemical onto silica gel or by adding an emulsifying agent, Tween 80, to the test medium. Third, we introduced more kinds and greater numbers of microorganisms by increasing the volume of the test medium under the conditions that the concentrations of the test chemical, silica gel, and activated sludge were held constant. Our studies found that the use of activated sludge taken from the municipal sewage treatment plant resulted in improved degradation activity, adding silica gel or an emulsifying agent secured the bioavailability of chemicals to microorganisms, and only increasing the test medium volume accelerated the biodegradability of chemicals. These findings can be incorporated into an investigation of the 301F test that has recently been approved for use under the CSCL and is expected to contribute toward appropriate evaluation of chemical characteristics reflecting biodegradability potential in the actual environment.

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