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Comparative evaluation of trimethylated α-, β-, and γ-cyclodextrins as optimal dispersants for ready biodegradability testing of poorly water-soluble substances

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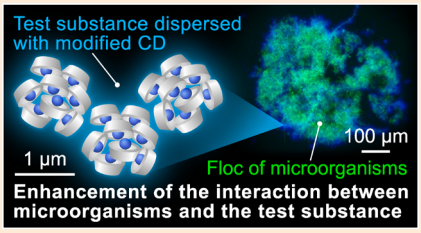
Supplementary material

We investigated whether various modified cyclodextrins (CDs) and emulsifiers could be applied as dispersing agents in ready biodegradability tests of poorly water-soluble substances. Trimethylated *α*-, *β*-, and *γ*-CDs and partially methylated *β*-CD were not biodegraded in the test period but accelerated the biodegradation of octabenzone and anthraquinone. The process by which trimethylated *α*-, *β*-, and *γ*-CDs enhance the biodegradation of test substances has been partially uncovered. These CDs create inclusion complexes with the substances, which then coalesce into larger aggregates. These aggregates disperse throughout the testing medium and attach to clusters of activated sludge, known as flocs. This close contact with the sludge speeds up the breakdown of the hydrophobic substances being tested.



Keywords: cyclodextrin, emulsifier, poorly water-soluble substance, ready biodegradability test, metagenomic analysis.

Introduction

Human activities release various chemical substances into the environment. These substances are biodegraded by microorganisms. However, the biodegradation speed differs for each substance. The substances that are biodegraded slowly can remain in the environment for a long time. If these persistent substances can bioaccumulate in living organisms and are toxic, they can be very harmful to human health and ecosystems.[1,2] That's why many countries perform tests to check how quickly these substances can be biodegraded.[3–6] In Japan, under the Chemical Substances Control Law (CSCL), the biodegradability of substances is evaluated using a ready biodegradability test according to Organisation for Economic Co-operation and Development (OECD) test guideline 301C.[3,7] The 301C test is conducted at a concentration of 100 mg L^{-1} for any substance.[3] Thus, the biodegradability of poorly water-soluble substances must be evaluated in an inadequately dissolved state. Since revision of the CSCL in 2019, the OECD test guideline 301F can also be used for the evaluation of biodegradability in Japan.[3,7] In this test, auxiliary substances, as referred to additives, such as emulsifiers, solvents, and carriers may be added to improve the contact between microorganisms and the test substance in the test medium.[3,7] The CSCL and the OECD 301 require that additives should not be toxic to microorganisms and must not be biodegraded (here, this is referred to as the "ideal standard"), although they do not provide any examples of these additives.[3,7] The OECD 301 refers to the International Organization for Standardization (ISO) 10634 standard. Like the CSCL and the OECD 301 standards, the ISO 10634 standard states that the additive should be non-biodegradable. However, ISO 10634 adds a realistic standard that the biodegradation of additive does not exceed 10% compared to a test compound (here, this is referred to as the "realistic standard").[8,9]

The ISO 10634 uses polyethylene sorbitan trioleate (Tween®)

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as an example of an additive emulsifier.[8,9] In a previous study, we demonstrated that Tween® 80 promotes the biodegradation of poorly water-soluble substances such as 2-ethylanthraquinone and octabenzone (OB).[10] However, Tween® 80 itself was found to be biodegradable, and its biodegradation rate exceeded the realistic rate according to the ISO 10634 standard.[11] The ISO 10634 also lists other emulsifiers, such as block copolymers of ethylene oxide (EO) and propylene oxide (PO), with hydrophilic-lipophilic balance (HLB) values of 9 (Pluronic P10300® also known as Pluronic® P-103) and 13.5 (Pluronic P9400® also known as Pluronic® P-94).[8,9] These emulsifiers have polyether groups as their main structures, which tend to be biodegraded.[12] Therefore, additives that meet the ideal standard of the CSCL or the OECD 301, or the realistic standard of the ISO 10634, are seemingly difficult to find. Furthermore, even if an additive meets the realistic standard, one cannot argue that the biodegradability of a test substance is appropriately evaluated when the microorganisms that biodegrade the additive accelerate the biodegradation of the test substance. In such cases, it will be necessary to determine whether the microorganisms biodegrading the additive are the same as those biodegrading the test substance.

In this study, we searched for additives that disperse poorly water-soluble substances and appropriately evaluated their biodegradability. To be appropriate, an additive must meet the following conditions: 1) it must be non-biodegradable, or if it is biodegradable, it must meet the realistic standard of the ISO 10634; 2) if it is biodegradable, the microorganisms degrading the additive must not be the same as those degrading the test substance; and 3) it must not be toxic to microorganisms. In our study, OB and anthraquinone (AQ) were chosen as poorly water-soluble test substances because the biodegradation of both OB and AQ is accelerated by addition of the additive.[10] As to the additive, the following materials were investigated: a) ISO reference materials: Tween® 85 and 15 types of Pluronic® covering a wide range of HLB values. b) Nonylphenols: IGEPAL® CO-520, CO-630, and 4-nonylphenyl-polyethylene glycol (4-NP) as a polyoxyethylene nonylphenyl ether with repeating EO structures. c) Per- and polyfluoroalkyl substances (PFAS): perfluoropentanoic acid (PFPA), perfluorohexanoic acid, and perfluoroheptanoic acid, which are believed to have emulsifying properties and to be difficult to biodegrade. d) Modified cyclodextrins (CDs): cyclic oligosaccharides of α(1,4) linked D-glucose.[13] They are unique in having a hydrophilic outer surface and a hydrophobic inner cavity[13] and being emulsifiers *i.e.*, able to form an inclusion complex with a hydrophobic guest substance.[13] CDs are utilized as stabilizers of pharmaceutical or cosmetic substances[14,15] and as bioremediators because they are thought to increase the bioavailability of organic pollutants to microorganisms in the environment.[16] However, it has been reported that CDs themselves are readily degradable regardless of the number of glucose molecules[17–19] and have not been reported to be usable as additives in ready biodegradability tests. In this study, we investigated the influence of several types of CDs with modified hydroxyl groups on the ready biodegradability test.

We first examined the intrinsic biodegradability of the above additives and, using next-generation sequencing (NGS), we observed changes in microbiota prompted by the addition of the additives. Next, to determine which additive is appropriate, we examined: 1) whether the additive accelerated the biodegradation of the test substance and showed toxicity to microorganisms, 2) whether microorganisms degrading the additive were the same as those degrading the test substance, using microbial community analysis. Furthermore, to know how the modified CD improves the contact between microorganisms and the test substance, we first investigated the state of equilibrium between the test substance and the modified CD. Then we observed the states of the test substance, the modified CD, and activated sludge in the test medium during the degradation of the test substance.

Materials and methods

1. *Materials*

OB, AQ, and Tween® 85 were purchased from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile, and distilled water (HPLC grade, respectively), tetrahydrofuran (stabilizer free, special grade), formic acid, 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, 1 M sodium hydroxide, and perfluorohexanoic acid were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Methanol, distilled water (LC-MS grade, respectively), and aniline were purchased from Kanto Chemical (Tokyo, Japan). Anthracene, Pluronic® L-31, L-35, L-61, L-64, L-81, L-121, F-68, F-108, F-127, P-105, P-123, P-124, 10R5, 17R4, and 31R1, IGEPAL® CO-520 and CO-630, 4-nonylphenyl-polyethylene glycol (4-NP), perfluoropentanoic acid (PFPA), perfluoroheptanoic acid, trimethylated β-CD (composed of seven glucose units linked *via* alpha-1,4 connections, permethylated), methylated β-CD (1.6–2.0 mol of substitution per anhydroglucose unit, hereafter referred to as "partially methylated β-CD"), (2-hydroxypropyl)-β-CD (0.6 M substitution), and β-CD sulfated sodium salt (12–15 mol of sulfate substituted per mole of β-CD) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Trimethylated α- and γ-CDs were prepared by Sanyu Chemical (Hyogo, Japan), in which α- and γ-CDs (composed of six and eight glucose units, respectively) were exhaustively methylated with excess methylating agent. The purities of trimethylated α- and γ-CDs were determined at 99.7% and 99.8%, respectively, by a Shimadzu LC-MS 2020 (Shimadzu Corp., Kyoto, Japan). SYTO™ 9 Green Fluorescent Nucleic Acid Stain was purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Spectra/Por® Micro Float-A-Lyzer® (Molecular weight cut-off (MWCO): 8–10 kDa) was purchased from Repligen (Waltham, MA, U.S.A.).

2. *Ready biodegradability test*

The ready biodegradability test was carried out in accordance with OECD 301F guideline[3] as follows: activated sludge was collected from a sludge return line at a municipal sewage treatment

plant (Osaka, Japan) receiving predominantly domestic sewage. The collection days were Dec 25 in 2020 (for the intrinsic biodegradability test of the ISO reference materials, nonylphenols, and PFAS); Apr 17 (for the biodegradability test of OB with Tween® 85, Pluronic® P-123 and PFPA) and Dec 20 in 2021 (for the intrinsic biodegradability test of the modified CDs); Mar 30 (for the biodegradability test of OB or AQ with trimethylated or partially methylated β-CD) and Aug 2 in 2022 (for the biodegradability test of OB or AQ with trimethylated α-, β-, or γ-CD). Activated sludge was aerated just after its collection until the initiation of any tests. The test medium was prepared as described in our previous report,[20] where the final concentrations of K_2HPO_4, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, NH_4Cl, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $FeCl_3 \cdot 6H_2O$ were 85, 217.5, 672.1, 5, 22.5, 27.5, and 0.25 mg L^{-1}, respectively. The final concentration of activated sludge in the test medium was 30 mg L^{-1}, and the test substance was added up to a concentration of 100 mg L^{-1} either directly or using the dispersion method described in the following sections. After the test medium was added up to 900 mL in a 1200 mL glass vessel (diameter 90 mm, height 250 mm), the mixture of activated sludge and test substance was stirred and cultivated in darkness at 22±1°C. Biological oxygen demand (BOD) was measured continuously with a coulometer OM7000A (Ohkura Electric, Saitama, Japan). The biodegradation rate of OB or AQ, without any additive, was calculated with Eq. (1).

$$\text{Biodegradation rate}_{\text{test}}\ (\%) = \frac{\text{BOD}_{\text{test}} - \text{BOD}_{\text{control}}}{\text{ThOD}_{\text{test}}} \times 100 \quad (1)$$

where BOD_{test} is the BOD (measured value, mg) of the test substance and activated sludge in the test medium. $BOD_{control}$ is the BOD (measured value, mg) of activated sludge in the test medium. $ThOD_{test}$ is the theoretical oxygen demand (calculated value, mg), which is total amount of oxygen required to oxidize the test substance. Table 1 shows $ThOD_{test}$ values of OB and AQ. The biodegradation rate of OB or AQ, with an additive, was calculated with Eq. (2).

$$\text{Biodegradation rate}_{\text{test+additive}}\ (\%) = \frac{\text{BOD}_{\text{test+additive}} - \text{BOD}_{\text{control}}}{\text{ThOD}_{\text{test}}} \times 100 \quad (2)$$

where $BOD_{test+additive}$ is the BOD (measured value, mg) of test substance, the additive, and activated sludge in the test medium in a vessel (hereafter referred to as the "test+additive vessel"). The biodegradation rate of an additive relative to the $ThOD_{test}$ value was calculated with Eq. (3).

$$\text{Biodegradation rate}_{\text{additive}}\ (\%) = \frac{\text{BOD}_{\text{additive}} - \text{BOD}_{\text{control}}}{\text{ThOD}_{\text{test}}} \times 100 \quad (3)$$

where $BOD_{additive}$ is the BOD (measured value, mg) of the additive and activated sludge in the test medium. The ISO 10634 guidance for the realistic standard is that the additive biodegradation does not exceed 10% compared to the test compound biodegradation. It is interpreted that Biodegradation $rate_{additive}$ given by Eq. (3) should not exceed 10%. The biodegradation rates in Figs. 1, 2, 6, S1–S4, and S10 are calculated with Eqs. (1), (2), or (3). The intrinsic biodegradation rate of an additive at the end of cultivation was calculated with Eq. (4).

$$\text{Biodegradation rate}_{\text{additive itself}}\ (\%) = \frac{\text{BOD}_{\text{additive}} - \text{BOD}_{\text{control}}}{\text{ThOD}_{\text{additive}}} \times 100 \quad (4)$$

where $ThOD_{additive}$ is total amount of oxygen required to oxidize the additive (calculated value, mg). Table S1 shows $ThOD_{additive}$ for the ISO reference materials, nonylphenols, PFAS, and modified CDs. The biodegradation rates in Table 2 were calculated with Eq. (4).

Table 1. Method for calculating the theoretical oxygen demands (ThODs) of test chemicals

Test substance	Complete oxidation reaction	[A][a]	[B][b]	[C][c]	ThOD[d]
Octabenzone	$C_{21}H_{26}O_3+26O_2 \rightarrow 21CO_2+13H_2O$	26	326	100	230
Anthraquinone	$C_{14}H_8O_2+15O_2 \rightarrow 14CO_2+4H_2O$	15	208	100	208

[a] Number of oxygen molecules, [b] molar mass (g mol^{-1}), [c] concentration (mg L^{-1}), [d] ThOD (mg) = $MM_{O2} \times A \times C \times V_{\text{test volume}}/B$ = 32.0 (g mol^{-1}) × A × C (mg L^{-1}) × 0.9 (L)/B (g mol^{-1})

3. *Dispersion with additives*

All additives listed in Table 2 were investigated for intrinsic biodegradability. The ISO reference materials, nonylphenols, and PFAS were added at the minimum concentration at which OB was dispersed almost completely. The modified CDs were added at molar equivalent concentrations to those of OB. Table 2 shows these concentrations in the test medium. The biodegradation rate of each additive was investigated, without OB, for 28 days in singlicate.

To investigate the effectiveness of the modified CDs as the appropriate additive, trimethylated α-, β-, or γ-CD or partially methylated β-CD was added to a glass vial with OB or AQ at a molar equivalent concentration, respectively. The mixture was homogenized at 12,000 rpm for 30 min with an Omni Tissue Homogenizer (OMNI International, GA, U.S.A.) after distilled water was added up to a half volume of each CD. Using test medium, the homogenate was washed into the test vessel and ultrasonicated at 38 kHz for 5 min in an ultrasonic bath (SND, Nagano, Japan). The ready biodegradability tests were conducted over a 42-day period in three test vessels, three test+additive vessels, two additive vessels, and two control vessels.

Tween® 85, Pluronic® P-123, or PFPA was chosen as the additive for the ready biodegradability test of OB. The final concentration of Tween® 85, Pluronic® P-123, or PFPA was 40, 100, or 500 mg L^{-1}, respectively. Both OB and each additive were added to a test vessel and stirred with a hot stirrer HS-5BH (AS ONE Corporation, Osaka, Japan) at about 40°C. The test medium for OECD 301F was gradually added and the mixture was emulsified by continuous stirring and then ultrasonicated at 38 kHz for 5 min in the ultrasonic bath.

Table 2. Properties, initial concentration in the test vessel, and biodegradation rate of emulsifiers or cyclodextrins, and the taxonomic families that showed increased abundance

Additives			Molecular weight[a)]	%EO[b)]	HLB	Concentration ($mg\,L^{-1}$)	Biodegradation $rate_{additive\ itself}$ (%)[c)]	Families with increased abundance[d)]			
ISO materials	Tween® 85		1,895	—	11	40	73	—			
	Pluronic®	L-121	4,400	10	1	188	31	*Sphingomonadaceae*	*Verrucomicrobiaceae*	*Xanthomonadaceae*	
		L-81	2,800	10	2	953	9.5	*Sphingomonadaceae*	*Verrucomicrobiaceae*		
		L-61	2,000	10	3	458	24	*Sphingomonadaceae*	*Verrucomicrobiaceae*	*Cytophagaceae*	
		L-31	1,100	10	4	1164	11	*Sphingomonadaceae*			
		P-123	5,800	30	8	100	12	*Sphingomonadaceae*			
		P-124	2,000	40	12–18	164	19	*Sphingomonadaceae*	*Chitinophagaceae*		
		L-64	2,900	40	15	1693	5.1	*Sphingomonadaceae*	*Verrucomicrobiaceae*	*Criblamydiaceae*	
		P-105	10,000	50	15	111	91	*Sphingomonadaceae*	*Verrucomicrobiaceae*	*Criblamydiaceae*	
		L-35	1,900	50	18.5	1166	10	*Sphingomonadaceae*			
		F-127	12,000	70	18–23	167	14	*Sphingomonadaceae*	*Parachlamydiaceae*		
		F-108	14,600	80	27	360	11	*Sphingomonadaceae*			
		F-68	8,400	80	29	769	4.2	*Sphingomonadaceae*			
		31R1	3,300	10	2.0–7.0	196	70	*Sphingomonadaceae*	*Verrucomicrobiaceae*		
		17R4	2,700	40	7.0–12.0	833	14	*Sphingomonadaceae*	*Verrucomicrobiaceae*	*Cytophagaceae*	
		10R5	2,000	50	15	1169	12	—			
Nonylphenols	IGEPAL®	CO-520	441	—	10	100	54	*Sphingomonadaceae*	*Chitinophagaceae*	*Fimbriimonadaceae*	*GU230460_f*
		CO-630	617	—	13	83	63	*Sphingomonadaceae*	*Xanthomonadaceae*	*Parachlamydiaceae*	*Flavobacteriaceae*
	4-nonylphenyl-polyethylene glycol		1,101	—	20	102	56	*Sphingomonadaceae*	*Xanthomonadaceae*	*Parachlamydiaceae*	
PFAS	perfluoropentanoic acid (PFPA)		264	—	—	500	−3.8	—			
	perfluorohexanoic acid		314	—	—	500	−0.42	—			
	perfluoroheptanoic acid		364	—	—	500	0.88	—			
Modified CD	*α*-cyclodextrin	trimethylated	1,225	—	—	375	−0.83	—			
	β-cyclodextrin	trimethylated	1,430	—	—	438	0.079	—			
		methylated (partially)	1,310	—	—	401	0	—			
		2-hydroxypropylated	1,380	—	—	380	19	*Caldilineaceae*	*Devosia_f*		
		sulfated sodium salt	2,519	—	—	693	9.1	*HQ014634_f*			
	γ-cyclodextrin	trimethylated	1,634	—	—	501	0.28	—			

% EO, % ethylene oxide; HLB, hydrophilic-lipophilic balance values; ISO, International Organization for Standardization; PFAS, Per- and polyfluoroalkyl substances; CD, cyclodextrin. [a)] Average, [b)] ratio of ethylene oxide (wt%) in a molecule, [c)] calculated with Eq. (4), [d)] family abundance has increased more than 5% compared to that in the control of each test

4. Metagenomic analysis

Activated sludge containing about 4.5 mg of suspended solids was collected and centrifuged at 15,000 rpm for 20 min in a centrifuge CF16RXII (Eppendorf Himac Technologies, Ibaraki, Japan). After removing the supernatant, the sludge pellet was stored at −20°C. Bioengineering Lab (Kanagawa, Japan) conducted DNA extraction, PCR, and next-generation sequencing of the sludge pellet, as described in our previous report.[20] The library for the V3–V4 hypervariable region of the 16S rRNA was prepared using the two-step tailed PCR method, and sequencing was performed with read lengths of 2×300 bp using the MiSeq System and the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, U.S.A.). After removing of the primer, sequences with a quality value of less than 20 were removed. Sequences with a length of less than 40 bases and their paired sequences were discarded. After removing the chimeras and denoising the sequences obtained using the DADA2 plugin in Qiime2 (ver.2022.8),[21] an amplicon sequence variant (ASV) table and the representative sequences were output. The EzBioCloud 16S database was used for taxonomic classification. Sequencing data were deposited in the DNA Databank of Japan (DDBJ accession no. PRJDB16891). The quality of sequencing was as follows; the minimum number of valid pair reads was 3.2×10^4 (average 4.8×10^4) and the minimum Q20 and Q30 scores were 83.1 and 70.4% (average 89.6 and 79.7%), respectively.

To investigate bacterial families that increased due to the intrinsic biodegradation of the additives, activated sludge from the control+additive and control vessels was collected after 28 days of the cultivation. All families whose relative abundance increased more than 5% compared to the those in the control vessel are shown in Table 2.

To accurately identify the degraders of a test substance, our previous study[20] showed that it was effective to examine the microbiota when the test substance was undergoing active biodegradation. Thus, activated sludge in the test or test+additive vessel was collected when each test substance was actively biodegraded, while activated sludge in the control vessel was collected at around the time it was collected in the test+additive vessel. Figs. S1–S4 and S10 show the time points of activated sludge collection (closed diamond: ◆).

To investigate the microorganisms that were relevant to the biodegradation of a test substance and/or additive, the dominant 10 families degrading each test substance were detected by the following procedures: 1) subtracting the relative abundance of each family in the control vessel from that of each family in the test vessel, the test+additive vessel, or the additive vessel, as determined by the ready biodegradability test, 2) choosing the top ten families on the basis of the highest subtracted relative abundance values obtained from testing biodegradability in the test vessels, the test+additive vessels, and the additive vessels used to investigate the effectiveness of additives as facilitators of OB or AQ degradation. The top ten families are shown in Tables 3 and 4 for OB and AQ, respectively.

5. Analysis of dispersion state of the test substance after adding the modified CD

5.1. Dispersibility of the test substance

To learn how the dispersibility of the test substance AQ in the test medium was improved by adding the modified CD, the following experiment was conducted. A 200 mL beaker (diameter 65 mm, height 90 mm) was filled with 200 mL of the test medium. A dialysis membrane device, Spectra/Por® Micro Float-A-Lyzer®, was floated in the center of the beaker. The test medium in the beaker was stirred for 1 hr. Then, AQ itself or AQ dispersed with trimethylated β-CD was added to the test medium at a concentration of 100 mg L^{-1}. The test medium was stirred at a temperature of 22±1°C. After 1, 3, 6, 18, and 24 hr, 100 μL of the medium in the dialysis membrane device was collected using a microsyringe after the microsyringe was pumped three times in the device to homogenize the medium. Samples of the medium were collected in triplicate.

After adding 100 μL of acetonitrile to the collected medium, the concentration of AQ was measured by a TSQ Quantis LC-MS system (Thermo Fisher Scientific) equipped with an Accucore™ Vanquish C18+ UHPLC column (50×2.1 mm, particle size 1.5 μm, Thermo Fisher Scientific) run at 40°C. Mobile phase A containing 0.1% formic acid in distilled water and mobile phase B containing 0.1% formic acid in methanol were pumped at a flow rate of 0.2 mL^{-1}. The percentage of mobile phase B changed in a linear gradient from 70 to 80% over 3 min. The injection volume was 10 μL. MS/MS measurement was performed in selected reaction monitoring (SRM) mode, with heated electrospray ionization (ESI) 4554.55 V, sheath gas flow 16.1 Arb, aux gas flow 22.8 Arb, sweep gas flow 0.4 Arb, ion transfer tube temperature 350°C, vaporizer temperature 300°C, precursor ion 210.14 m/z, product ion 181.04 m/z, and collision energy 30 V. The quantification lower limit of AQ was 1 μg L^{-1}.

5.2. Equilibrium state between the test substance and the modified CD

To learn the equilibrium state between the test substance and the modified CD in the test medium, the following experiment was conducted with OB or AQ as a test substance and trimethylated α-, β-, or γ-CD as an additive. When the test substance is dispersed with the CD, the test substance and the CD form an inclusion complex in the test medium.[13] The stability constant ($K_{m:n}$) is a useful index of physicochemical change of the test substance when forming the inclusion complex.[22,23] When the CD, test substance (TS), and inclusion complex ($CD_m \cdot TS_n$) establish equilibrium (Eq. (5)), the stability constant ($K_{m:n}$) for $CD_m \cdot TS_n$ is given by Eq. (6).[22]

$$m\mathrm{CD} + n\mathrm{TS} \underset{}{\overset{K_{m:n}}{\rightleftarrows}} \mathrm{CD}_m \cdot \mathrm{TS}_n \tag{5}$$

$$K_{m:n} = \frac{(x)}{(a-mx)^m \cdot (b-nx)^n} \tag{6}$$

where m and n represent the mole number of CD and TS, respectively. a, b, and x represent the total concentrations of CD,

TS, and $CD_m \cdot TS_n$, respectively.[22] Several methods are known to determine the stability constant. We selected the HPLC method[22] because of its convenience and the good agreement of its stability constants with those determined by other methods such as spectroscopy.[22,23] The principle of measurement is as follows: the retention time of the test substance on the HPLC is shortened by adding the CD to the mobile phase. It is assumed that this change of the retention time is caused by formation of complexes in the HPLC column. Based on the assumption, a relationship between the retention time and the stability constant $K_{m:n}$ is given by Eq. (7).[22]

$$T_{obs} = \frac{T_o + T_c K_{m:n}[CD]}{1 + K_{m:n}[CD]} \quad (7)$$

where [CD] is concentration of the CD in the mobile phase. T_o is the retention time (measured value, min) of TS in the mobile phase without CD. T_{obs} is the retention time (measured value, min) of TS in the mobile phase with CD, and the T_{obs} changes with [CD]. T_c is the retention time of $CD_m \cdot TS_n$, which is the retention time approached as [CD] increases. When the relationship between T_{obs} and [CD] values are plotted, $K_{m:n}$ and T_c are determined by nonlinear least-squares fitting.

T_o and T_{obs} values for OB or AQ at 0, 0.02, 0.1, 0.2, and 0.4 mM trimethylated α-, β-, or γ-CD were determined in triplicate on a Vanquish Core HPLC instrument (Thermo Fisher Scientific). An L-column3 ODS column (150 mm×2.1 mm, 3 μm, Chemicals Evaluation and Research Institute, Japan, Saitama, Japan) was used at 22°C. Mobile phase A (distilled water), mobile phase B (acetonitrile), and mobile phase C (2 mM trimethylated α-, β-, or γ-CD in distilled water) were pumped isocratically at a flow rate of 0.2 mL min^{-1}. The percentage of mobile phase B was fixed at 70% and percentages of mobile phases A and C were (30%, 0%), (29%, 1%), (25%, 5%), (20%, 10%), and (10%, 20%) for 0, 0.02, 0.1, 0.2, and 0.4 mM of trimethylated α-, β-, or γ-CD, respectively. The injection volume and detection wavelength were fixed at 1 μL and 254 nm, respectively.

5.3. Particle size of complexes between the test substance and the modified CD

To establish the particle size of complexes formed by the test substance and modified CD in the test medium, the following experiment was conducted with OB or AQ as a test substance and trimethylated α-, β-, or γ-CD as an additive. The test substance was dispersed in the test medium with the CD in accordance with the method described in section 3. The particle size of the complex was measured with a dynamic light scattering (DLS) method using a Zetasizer Ultra RED instrument (Malvern Panalytical, Malvern, UK). The temperature was set to 22.0°C. The time to reach the temperature equilibrium was set to 120 sec. The measurement was conducted in triplicate.

5.4. Distribution of the test substance, the modified CD, and microorganisms

To ascertain the distribution of the test substance, the modified CD, and microorganisms in the test medium, the following experiment was conducted with trimethylated α-, β-, or γ-CD as an additive. As to the test substance, anthracene was used because it has a similar structure and molecular weight to AQ and emits fluorescence. First, anthracene was dispersed with the CD using the method described in section 3, and dissolved in the test medium. A portion of the test medium was deposited on a glass slide and observed under a fluorescence microscope (Axio Observer 3, Carl Zeiss AG, Oberkochen, Germany). The presence of anthracene was measured using an excitation wavelength of 385 nm and observation wavelengths in the range 370–400 nm. Then, activated sludge was added to the test medium and the sludge-medium mixture was cultivated under OECD 301F test conditions. After 5 and 41 days of the cultivation, 1 mL of the mixture was collected, mixed with SYTO™ 9 solution (2× concentration), and incubated at room temperature for 15 min to stain the DNA of bacteria in the activated sludge.[24] After gently dispersing the stained liquid, a portion of the staining solution was placed onto a glass slide and observed under a fluorescence microscope. The presence of anthracene only was detected using excitation and observation wavelengths of 385 nm and 370–400 nm, respectively. The presence of anthracene and activated sludge was detected using excitation wavelengths of 385 nm and 475 nm and observation wavelengths of 370–400 nm and 450–488 nm.

Results and discussion

1. Intrinsic biodegradability test of the additives

The intrinsic biodegradability of 22 additives was investigated. Table 2 shows the molecular weight, concentration of each additive at the initiation of cultivation, the biodegradation rate calculated using Eq. (4) after 28 days of the cultivation, and the bacterial families with abundances that prominently increased (*i.e.*, by at least 5%) compared to those in the control vessel. For the ISO reference materials, the EO ratio (if there is one) and the HLB value are also provided in Table 2. Accordingly, the rate of Tween® 85 biodegradation was 73% and Tween® 85 seemed to be almost completely biodegraded. However, there were no families with abundances that prominently increased beyond 5% due to biodegradation of Tween® 85. Since the Pluronic® surfactants with HLB values of 9 and 13, having an EO–PO–EO structure, exemplified in the ISO 10634 were not commercially available, we used available Pluronic® surfactants covering a wide range of HLB values to confirm the intrinsic biodegradability. The 12 types of Pluronic® surfactants having the EO–PO–EO structure were as follows: L-31, L-35, L-61, L-64, L-81, and L-121 (liquid form at room temperature), F-68, F-108, and F-127 (flake form at room temperature), P-105, P-123, and P-124 (paste form at room temperature). The three types having a PO–EO–PO structure were as follows: 10R5, 17R4, 31R1. The intrinsic biodegradation rates varied widely from 4.2 to 91%. None of them were non-biodegradable in 28 days. For the nonylphenols (IGEPAL® CO-520, CO-630, and 4-NP) having a hydrophilic structure with repeating EO units, the biodegradation rates were 54%, 63%, and 56%, respectively. It meant that

the nonylphenols were almost completely biodegraded. The biodegradation of the Pluronic® materials and the nonylphenols led to the proliferation of *Sphingomonadaceae* and increase in *Sphingomonadaceae* abundance by more than 5% compared to the control, though biodegradation of Pluronic® 10R5 resulted in only a 3% increase. These additives have a repeated structure of EO. *Sphingomonadaceae* could biodegrade the structure because members of the family are known biodegraders of polyethylene glycol.[25] IGEPAL® and 4-NP have nonylphenyl ether in the hydrophobic group. *Sphingomonadaceae* is known to include biodegraders of polycyclic aromatic hydrocarbon[26–28] as well. *Sphingomonadaceae* was considered to have contributed to the biodegradation of these aromatics. *Chitinophagaceae* dominated as biodegraders of Pluronic® P-124 and IGEPAL® CO-520, and *Xanthomonadaceae* dominated as biodegraders of Pluronic® L-121, IGEPAL® CO-630, and 4-NP. The abundances of both families are reported to be increased by the addition of polyoxyethylene (4) lauryl ether.[29] The families were considered to be contributors to the biodegradation of the repeated EO structures included in Pluronic® and nonylphenols.

For PFAS, the intrinsic biodegradation rates of PFPA, perfluorohexanoic acid, and perfluoroheptanoic acid ranged from −3.8 to 0.88%. PFAS were hardly biodegraded in 28 days. Based on the metagenomic analysis, no families increased in abundance more than 5%, and PFAS had no impact on the microbial community.

For the modified CDs, the intrinsic biodegradability of trimethylated *β*-CD, partially methylated *β*-CD, 2-hydroxypropylated *β*-CD, and *β*-CD sulfated sodium salt, which were commercially available and are modified with hydroxyl groups, were investigated. Next, trimethylated *α*- and *γ*-CD were synthesized and their intrinsic biodegradability was examined. The intrinsic biodegradation rates of 2-hydroxypropylated *β*-CD and *β*-CD sulfated sodium salt were 19% and 9.1%, respectively. For 2-hydroxypropylated CD, the abundances of *Caldilineaceae* and *Devosia_f* increased by 14.6% and 13.0%, respectively. For sulfated CD, the abundance of *HQ014634_f* increased by 6.2%. These families were considered to be contributors to the degradation of their respective CDs. In contrast, the intrinsic biodegradation rates of trimethylated *α*-, *β*-, and *γ*-CDs, as well as partially methylated *β*-CD, were almost 0%. Metagenomic analysis was not able to detect a prominent increase in abundance in any bacterial family.

2. *Biodegradability test using modified CDs*

Trimethylated *α*-, *β*-, and *γ*-CDs and partially methylated *β*-CD were chosen from additives shown in Table 2, because these CDs were not biodegradable in the test period and did not influence the relative abundances of specific bacterial families. The effect of these CDs on accelerating the biodegradation of OB and AQ, which are poorly water-soluble substances, was investigated, along with the analysis of dispersion state of the test substances with the addition of CDs.

2.1. *Trimethylated α-, β-, and γ-CDs*

First, we used trimethylated *α*-, *β*-, or *γ*-CD (composed of six, seven, and eight glucose units, respectively), since the affinity of the CD for a guest substance varies with the difference in inner diameter of the cavity.[30] Figure 1A and 1B shows the time-dependent biodegradation rates of OB and AQ, respectively, incubated with trimethylated *α*-, *β*-, or *γ*-CD for 42 days. The biodegradation rates of each replicate are shown in Figs. S1 and S2 for OB and AQ, respectively. As a result, OB without these CDs was not biodegraded in 42 days (1 in Fig. 1A). The biodegradation of OB dispersed with these CDs occurred remarkably after Day 16, and reached 40±4%, 43±7%, or 60±10%, respectively, at Day 42 (2*α*, 2*β*, and 2*γ* in Fig. 1A). Thus, all of these CDs accelerated the biodegradation of OB. The biodegradation rate of AQ itself at Day 42 was 60±10% (1 in Fig. 1B), while those of AQ dispersed with these CDs were 78±6%, 80±5%, and 86±5%, respectively (2*α*, 2*β*, or 2*γ* in Fig. 1B). All of these CDs accelerated the biodegradation of AQ. For both of the test substances, the biodegradation rate of the *γ*-CD was highest among the three CDs, and the rate of the *α*-CD was approximately similar to that of the *β*-CD.

The biodegradation rate of trimethylated *α*-, *β*-, and *γ*-CD at

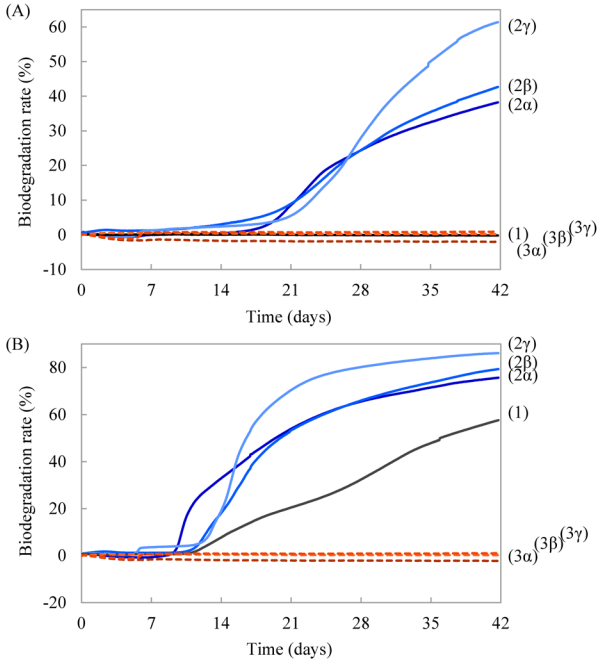

Fig. 1. (A) Time-dependent biodegradation rate for octabenzone (OB) (1, black solid line), OB with trimethylated *α*-cyclodextrin (CD) (2*α*, deep blue solid line), OB with trimethylated *β*-CD (2*β*, blue solid line), OB with trimethylated *γ*-CD (2*γ*, light blue solid line), trimethylated *α*-CD (3*α*, brown dashed line), trimethylated *β*-CD (3*β*, orange dashed line), and trimethylated *γ*-CD (3*γ*, light orange dashed line). (B) Time-dependent biodegradation rate for anthraquinone (AQ) (1, black solid line), AQ with trimethylated *α*-CD (2*α*, deep blue solid line), AQ with trimethylated *β*-CD (2*β*, blue solid line), AQ with trimethylated *γ*-CD (2*γ*, light blue solid line), trimethylated *α*-CD (3*α*, brown dashed line), trimethylated *β*-CD (3*β*, orange dashed line), and trimethylated *γ*-CD (3*γ*, light orange dashed line).

Table 3. Relative abundances of the top ten families for octabenzone

Taxonomy				Relative abundance* (%)											
Phylum	Class	Order	Family	+Trimethylated CD						+Emulsifier					
				2α	(3α)	2β	(3β)	2γ	(3γ)	2t	(3t)	2p	(3p)	2pf	(3pf)
Actinobacteria	*Actinobacteria_c*	*Corynebacteriales*	*Mycobacteriaceae*	57.6	(1.2)	38.0	(0.0)	18.6	(1.2)	10.5	(0.1)	20.2	(−0.7)	16.2	(−0.2)
Actinobacteria	*Actinobacteria_c*	*Corynebacteriales*	*Nocardiaceae*	0.4	(0.0)	0.9	(−0.0)	6.9	(0.4)	0.0	(0.0)	0.0	(−0.0)	11.7	(−0.0)
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	0.0	(−0.0)	0.0	(−0.0)	0.0	(−0.0)	−1.8	(−0.4)	−3.0	(−1.6)	−2.9	(1.5)
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	1.3	(−0.5)	1.7	(−0.2)	4.2	(−0.3)	1.4	(−0.5)	1.3	(−1.2)	0.1	(−0.6)
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	1.5	(0.8)	2.2	(0.5)	3.1	(0.4)	2.5	(0.3)	1.0	(0.3)	−0.1	(−0.1)
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*PAC002126_f*	−1.3	(−1.1)	−1.4	(−0.3)	−1.3	(−1.1)	−0.1	(0.2)	1.0	(−0.1)	0.9	(0.1)
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	0.0	(−0.0)	2.1	(−0.1)	8.6	(0.0)	3.5	(0.2)	21.4	(22.3)	1.9	(−0.0)
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	0.5	(−0.4)	0.2	(−0.2)	1.6	(0.7)	0.4	(−1.0)	0.0	(−0.1)	6.4	(−0.9)
Saccharibacteria_TM7	*Saccharimonas_c*	*Saccharimonas_o*	*PAC000016_f*	−0.7	(0.5)	−0.6	(0.7)	−0.7	(0.5)	0.3	(0.4)	0.4	(0.9)	0.0	(0.1)
Saccharibacteria_TM7	*Saccharimonas_c*	*Saccharimonas_o*	*Saccharimonas_f*	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)	1.8	(0.0)

CD, cyclodextrin. * Relative abundance on average after subtracting the abundance in the control of each test.

Day 42, which was calculated with Eq. (3) and averaged, was −2.0%, 0.22%, and 0.89%, respectively, for OB (3α, 3β, and 3γ in Fig. 1A) and −2.2%, 0.24%, and 0.99%, respectively, for AQ (3α, 3β, and 3γ in Fig. 1B). In addition, a quantification analysis with HPLC-CAD (charged aerosol detector, Corona Veo, Thermo Fisher Scientific) showed that 101%, 99%, and 105% remained on average at Day 42 for trimethylated α-, β-, and γ-CDs, respectively. Thus, none of these CDs was biodegraded but all of them met both the realistic standard of the ISO 10634 and the ideal standard of CSCL or the OECD 301. In addition, the CDs were not toxic to microorganisms. If the CDs had been toxic, BOD would have been lower than that of the control vessel. However, the BOD in the vessel containing each additive was similar to that in the control vessel throughout the test period. This is illustrated by the nearly flat biodegradation curves of 3α, 3β, and 3γ in Fig. 1A and 1B.

Tables 3 and 4 show the relative abundances of the top ten families for OB and AQ, respectively, being calculated by subtracting the abundances of families in the control vessel and then averaging the corresponding replicates. The original abundances of the top ten families for the test+additive, additive, and control vessels as well as Day 0 vessel for OB and AQ are shown in Tables S2 and S3, respectively. For OB, *Mycobacteriaceae* highly dominated in the respective presence of all three CDs (2α, 2β, and 2γ in Table 3), and *Nocardiaceae* and *Sphingomonadaceae* increased more than 5% in the presence of 2γ in Table 3. All of the families were increased more than 5% when OB adsorbed on silica gel was biodegraded.[31] Thus, these families were considered to have contributed to the degradation of OB. In the case of AQ, the relative abundances of *Mycobacteriaceae*, *Sphingomonadaceae*, and *Comamonadaceae* increased more than 5%, and these families were considered to have contributed to the biodegradation of AQ (1, 2α, 2β, and 2γ in Table 4). The additive vessels for 3α, 3β, and 3γ in Tables 3 and 4 showed that no families varied prominently (*i.e.*, <2%) in their relative abundance during the test period. Thus, it appeared that trimethylated α-, β-, and γ-CDs hardly affected microorganisms and accelerated the biodegradation for both OB and AQ.

Table 4. Relative abundance of the top ten families for anthraquinone

Taxonomy				Relative abundance* (%)						
Phylum	Class	Order	Family	AQ	+ Trimethylated CD					
				1	2α	(3α)	2β	(3β)	2γ	(3γ)
Actinobacteria	*Actinobacteria_c*	*Corynebacteriales*	*Mycobacteriaceae*	28.5	7.8	(1.2)	16.2	(0.0)	3.5	(1.2)
Actinobacteria	*Actinobacteria_c*	*Corynebacteriales*	*Nocardiaceae*	0.0	0.1	(0.0)	0.0	(−0.0)	0.0	(0.4)
Actinobacteria	*Actinobacteria_c*	*Propionibacteriales*	*Nocardioidaceae*	4.7	1.7	(−0.2)	0.2	(0.2)	1.1	(0.4)
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	0.1	0.1	(−0.0)	0.3	(−0.0)	0.1	(−0.0)
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Beijerinckiaceae*	0.4	0.0	(0.0)	4.3	(0.0)	0.1	(0.0)
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*PAC002126_f*	2.8	−0.4	(−1.1)	0.6	(−0.3)	−0.8	(−1.1)
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	4.4	0.2	(−0.0)	7.8	(−0.1)	5.7	(0.0)
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	0.0	1.7	(−0.4)	5.5	(−0.2)	5.8	(0.7)
Saccharibacteria_TM7	*Saccharimonas_c*	*Saccharimonas_o*	*PAC000016_f*	−0.8	−0.2	(0.5)	−0.5	(0.7)	0.1	(0.5)
Saccharibacteria_TM7	*Saccharimonas_c*	*Saccharimonas_o*	*Saccharimonas_f*	0.0	0.0	(0.0)	0.0	(0.0)	0.0	(0.0)

AQ, anthraquinone; CD, cyclodextrin. * Relative abundance on average subtracting the control of each test.

2.2. *Partially methylated β-CD*

To investigate whether the degree of methylation affects the enhancement of biodegradability of a test substance, the biodegradability test for OB or AQ was conducted with trimethylated β-CD (100% methylation) or partially methylated β-CD (60% methylation), which was commercially available. Figure 2A and 2B for OB and AQ, respectively, show the time-dependent biodegradation rates on average of OB or AQ (1), OB or AQ dispersed with trimethylated β-CD (2β) or partially methylated β-CD (2β'), and trimethylated β-CD (3β) or partially methylated β-CD (3β'). The degradation rates of each replicate for OB and AQ are shown in Figs. S3 and S4, respectively. Accordingly, OB without the CDs was not biodegraded in 42 days (1 in Fig. 2A), which is similar to 1 in Fig. 1A. The biodegradation rate of OB dispersed with trimethylated β-CD or partially methylated β-CD reached 59±5% (2β) or 56±3% (2β'), respectively, at 42 days (Fig. 2A). The biodegradation rate of AQ after 28 days was 44±9% (1), while that of AQ dispersed with trimethylated β-CD or partially methylated β-CD was 70±2% (2β) or 66±7% (2β') at 28 days (Fig. 2B). Thus, partially methylated CD accelerated the biodegradation of OB and AQ similarly to trimethylated β-CD. The intrinsic biodegradation rates of trimethylated β-CD (3β) and partially methylated β-CD (3β') at Day 42, which were

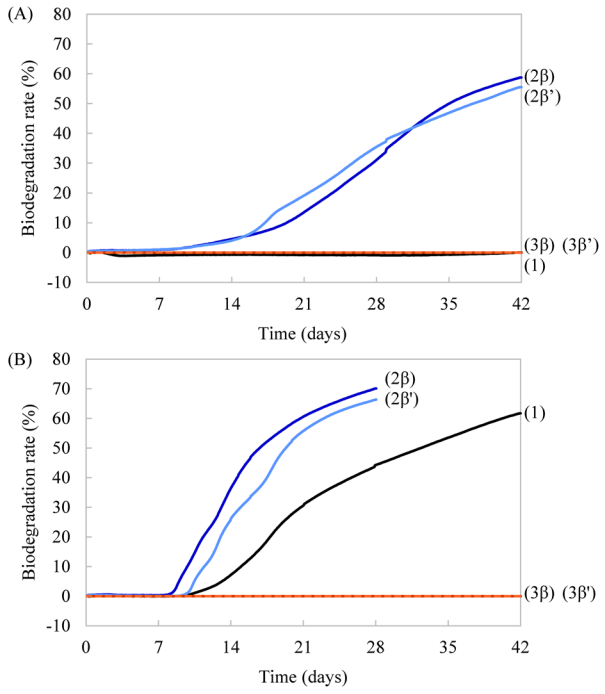

Fig. 2. (A) Time-dependent biodegradation rate for octabenzone (OB) (1, black solid line), OB with trimethylated β-cyclodextrin (CD) (2β, blue solid line), OB with partially methylated β-CD (2β', light blue solid line), trimethylated β-CD (3β, orange dash line), and partially methylated β-CD (3β', light orange dash line). (B) Time-dependent biodegradation rate for anthraquinone (AQ) (1, black solid line), AQ with trimethylated β-CD (2β, blue solid line), AQ with partially methylated β-CD (2β', light blue solid line), trimethylatedβ-CD (3β, orange dashed line), and partially methylated β-CD (3β', light orange dashed line).

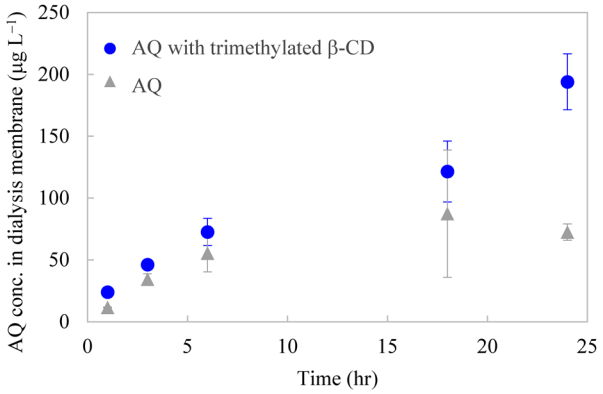

Fig. 3. Time-dependent concentration of anthraquinone (AQ) passing through the dialysis membrane when AQ itself (gray triangle) or AQ dispersed with trimethylated β-cyclodextrin (blue circle) was added to the test medium. Gray or blue and Error bars indicate the standard deviation for AQ itself or AQ dispersed with trimethylated β-cyclodextrin.

calculated with Eq. (3) and averaged, were −0.1% and 0.0%, respectively, for both OB and AQ (3β, and 3β' in Fig. 2A and 2B), indicating that neither trimethylated nor partially methylated β-CD was biodegraded.

2.3. *Analysis of the dispersion state of the test substance after adding a modified CD*

2.3.1. *Dispersibility of the test substance*

AQ itself or AQ dispersed with trimethylated β-CD was added to the test medium in which the dialysis membrane device was floated. After the test medium was stirred, aliquots of the test medium in the dialysis membrane device were collected for 24 hr to determine the concentration of AQ. In the case of AQ itself, the concentration of AQ reached a plateau after 18 hr and did not exceed 90 μg L^{-1} (Fig. 3). On the other hand, in the case of AQ dispersed with trimethylated β-CD, the concentration of AQ continued to increase over time, reaching approximately 200 μg L^{-1} at 24 hr. Considering that the MWCO of the dialysis membrane is 8–10 kDa and the molecular mass of trimethylated β-CD is 1.4 kDa, it appeared that not only AQ but also inclusion complexes of AQ and trimethylated β-CD passed through the dialysis membrane. These results clearly indicated that trimethylated β-CD enhanced the dispersibility of AQ.

2.3.2. *Equilibrium state between the test substance and the modified CD*

When OB or AQ is dispersed with trimethylated α-, β-, or γ-CD, an equilibrium is established between the test substance, the CD, and the inclusion complex consisting of these two components (see Eq. (5)). The stability constant ($K_{m:n}$) given by Eq. (7) was determined based on the relationship between T_{obs} and [CD] values (Fig. S5). As a result, $K_{m:n}$ (±standard error) for OB was 1.6±0.2, 1.0±0.2, or 1.5±0.1 ($\times 10^5$ M^{-1}) for trimethylated α-, β-, or γ-CD, respectively. $K_{m:n}$ for AQ was 1.5±0.2, 0.9±0.1, or 1.16±0.07 ($\times 10^5$ M^{-1}) for trimethylated α-, β-, or γ-CD, respectively. These high $K_{m:n}$ values for OB and AQ indicated that the equilibrium in Eq. (5) was heavily inclined to the right (*i.e.*, strongly favored the formation of the inclusion complex). It

meant that the majority of OB or AQ molecules dispersed with the CD existed in the test medium as inclusion complexes with CD.

2.3.3. Particle size of complexes consisting of the test substance and the modified CD

Since the test medium containing OB or AQ dispersed with the trimethylated CD were uniformly turbid, the particle sizes of the OB- or AQ-CD complexes were measured using a DLS method. As a result, *Z*-average (±standard deviation) for OB dispersed with trimethylated *α*-, *β*-, or *γ*-CD was 4.7±0.2, 2.7±0.4, or 1.3±0.2 *μ*m, respectively. *Z*-average for AQ with trimethylated *α*-, *β*-, or *γ*-CD was 5±1, 1.4±0.2, or 1.2±0.1 *μ*m, respectively. The outer diameter of non-methylated *α*-, *β*-, or *γ*-CD was reported as 1.37, 1.53, or 1.69 nm, respectively.[32] Trimethylated *α*-, *β*-, or *γ*-CD is considered to be slightly larger than that of the non-methylated *α*-, *β*-, or *γ*-CD, respectively. Therefore, it appeared that the inclusion complex consisting of the test substance and the trimethylated CD was aggregated and dispersed in the test medium for both OB and AQ.

2.3.4. Distributions of the test substance, the modified CDs, and microorganisms

To assess the distributions of a test substance, trimethylated *α*-, *β*-, or *γ*-CD, and microorganisms in the test medium, anthracene was used as the test substance because it is fluorescent and similar in structure and molecular weight to AQ. The microorganisms were also fluorescence stained. Figure 4 shows the fluorescence image of anthracene and microorganisms. In the test medium without activated sludge, blue fluorescence derived from anthracene was observed uniformly (Fig. 4A). It meant that the aggregates of anthracene and trimethylated *β*-CD were dispersed uniformly in the test medium. In contrast, after the test medium was cultivated with activated sludge for 5 days, the aggregates were clumped rather than dispersed, and the clumped aggregates were emitting blue fluorescence (Fig. 4B). When the stained microorganisms were also excited to emit a green fluorescent color, the distribution of the aggregates corresponded exactly with that of the activated sludge floc (*i.e.*, aggregates of microorganisms, Fig. 4C). Thus, the aggregates seemed to adhere to the floc. After 41 days of the cultivation, hardly any anthracene-derived blue fluorescence was observed on the floc (Fig. 4D). That trend was similarly observed at a lower magnification of the microscope at the same angle shown in Fig. S6. At this time point, the biodegradation rate of anthracene exceeded 40% based on the BOD, suggesting that the biodegradation was

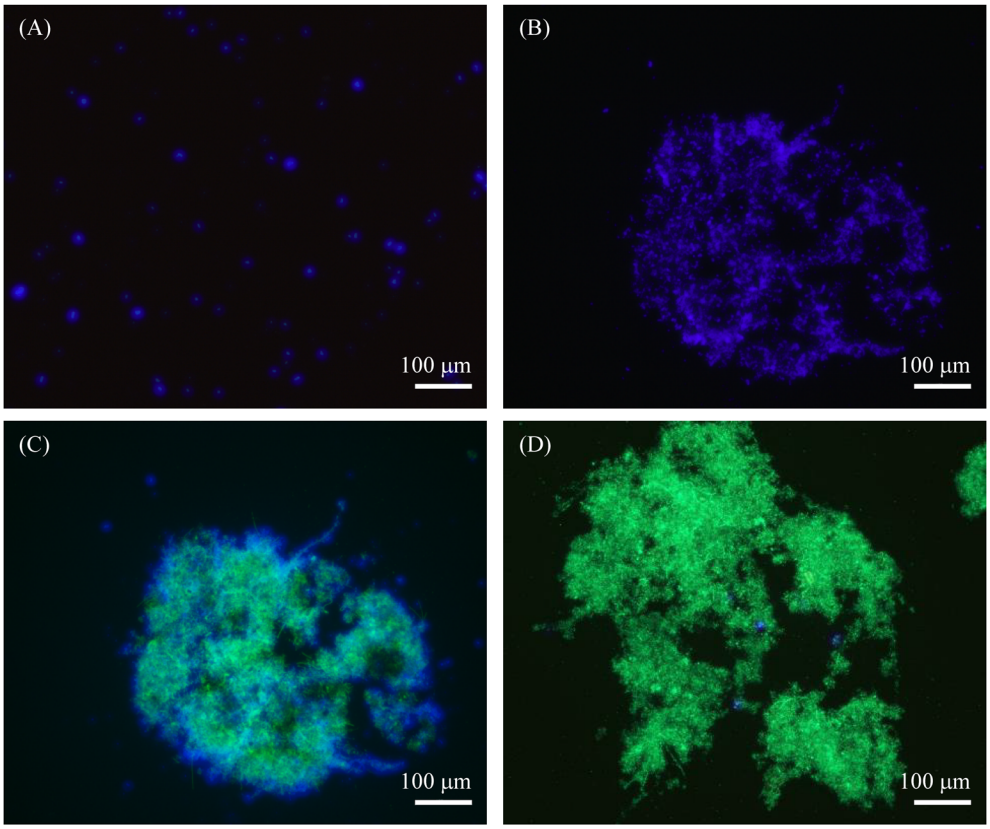

Fig. 4. Anthracene (blue fluorescence) dispersed with trimethylated *β*-cyclodextrin (CD) in the test medium, observed with the excitation wavelength of 385 nm (A). Anthracene (blue fluorescence) dispersed with CD in the test medium containing activated sludge (green fluorescence) and cultivated for 5 days, observed with the excitation wavelength 385 nm (B), and with both excitation wavelengths 385 nm and 475 nm (C). Anthracene (blue fluorescence) dispersed with CD in the test medium containing activated sludge (green fluorescence), cultivated for 41 days, and observed with both excitation wavelengths 385 nm and 475 nm (D).

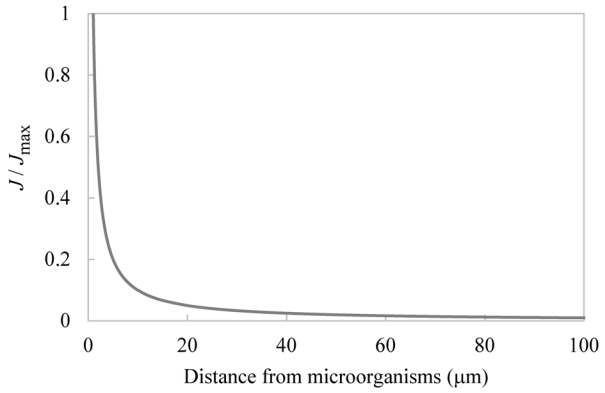

Fig. 5. Distance-dependent flux of a test substance calculated using the one-dimensional diffusion model.

reaching its final stage, and most anthracene in the aggregates on the floc was biodegraded by microorganisms. On the other hand, without addition of trimethylated β-CD, anthracene was barely detectable on the activated sludge flocs after 5 days with activated sludge (Fig. S7). Similar to aggregates of anthracene with trimethylated β-CD, the aggregates of anthracene with trimethylated α- and γ-CD were dispersed in the test medium without activated sludge, but adhered to the activated sludge floc after five days cultivation with activated sludge (Fig. S8). Figure 5 shows the flux of a test substance calculated based on the one-dimensional diffusion model, with the diffusion coefficient of anthracene[33] as an example. Details of the model and the calculation process are summarized in Fig. S9. The data clearly show that the supply of the test substance improves by orders of magnitude as the distance from microorganisms is decreased. In three-dimensional spaces, this increase is even more pronounced. The test substance attached to activated sludge flocs is more effectively biodegraded by microorganisms than when it is dispersed in the test medium. Therefore, it appeared that the addition of CD increased the mobility of the test substance, making it easier for the CD-test substance aggregates to adhere to the microorganisms, thereby enhancing the biodegradability of the test substance.

To summarize the above, the dispersion state of the test substance in the presence of modified CDs was analyzed as follows: When a floating dialysis membrane device was placed in test medium containing the test substance, the concentration of the substance in the device reached a constant value. In contrast, when a floating dialysis membrane device was placed in test medium containing inclusion complexes of the substance and trimethylated CDs, the concentration of the substance in the device exceeded the constant value and showed an upward trend. It appeared that the formation of the complex improved the fluidity of the substance in the test medium. Based on the stability constant for the test substance, the CD, and the inclusion complex, it was found that most of the substance reacted to form the inclusion complex. When the particle size in the test medium was measured, the particle was significantly larger than the outer diameter of one CD molecule. This suggested that the complexes were aggregated and present in the test medium. When the test substance and microorganisms were observed under a fluorescence microscope, the aggregates were found to adhere to microorganisms. From all these observations, it was considered that the test substance and the CD formed aggregates, the aggregates increased fluidity and the opportunity for contact with microorganisms, and the degradation of the test substance was enhanced.

3. Biodegradability test using Tween® 85, Pluronic®P-123, or PFAS

We searched for suitable additives, other than the CDs, that not only were non-toxic to microorganisms but also promoted the biodegradation of the test substance. The ISO 10634 exemplified Tween® and Pluronic® with HLB value of 9. Thus, we selected Tween® 85 and Pluronic® P-123 with HLB of 8, which is closest to the HLB among commercially available Pluronic® substances. Among the PFAS in Table 2, PFPA was selected since it was a low viscosity liquid and had good miscibility with OB. Figure 6 shows time-dependent biodegradation rates on average for OB (1), OB dispersed with Tween® 85, Pluronic® P-123, or PFPA (2t, 2p, or 2pf, respectively), and Tween® 85, Pluronic® P-123, PFPA (3t, 3p, or 3pf, respectively) for 42 days. The biodegradation rates of each replicate are shown in Fig. S10. As a result, OB without the additive was not biodegraded for 42 days (1 in Fig. 6). The biodegradation rate of 2t, 2p, or 2pf at Day 42 was 83±5%, 50±10%, or 34±8%, respectively. Thus, all of the three additives accelerated the biodegradation of OB.

As for Tween® 85 and Pluronic® P-123, BOD in the additive vessel tended to increase compared to that in the control vessel (3t and 3p in Fig. 5 were positive). In addition, the fact that the biodegradation of OB was accelerated showed that the two additives were not toxic to microorganisms. As for PFPA, the procedure control tests were conducted to confirm the occurrence of microbial activity with aniline as a reference substance. In the tests, the biodegradation rate of aniline after seven days was 61%

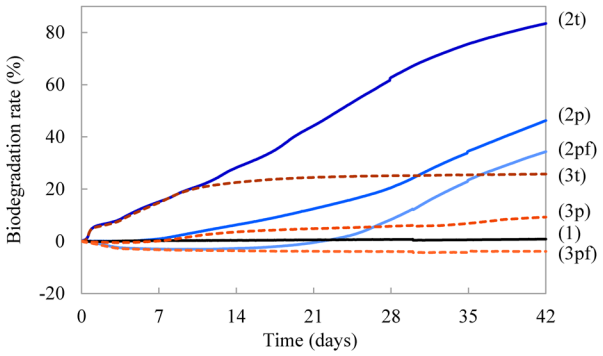

Fig. 6. Time-dependent biodegradation rate for octabenzone (OB) (1, black solid line), OB with Tween® 85 (2t, deep blue solid line), OB with Pluronic® P-123 (2p, blue solid line), OB with perfluoropentanoic acid (PFPA) (2pf, light blue solid line), Tween® 85 (3t, brown dashed line), Pluronic® P-123 (3p, orange dashed line), and PFPA (3pf, light orange dashed line).

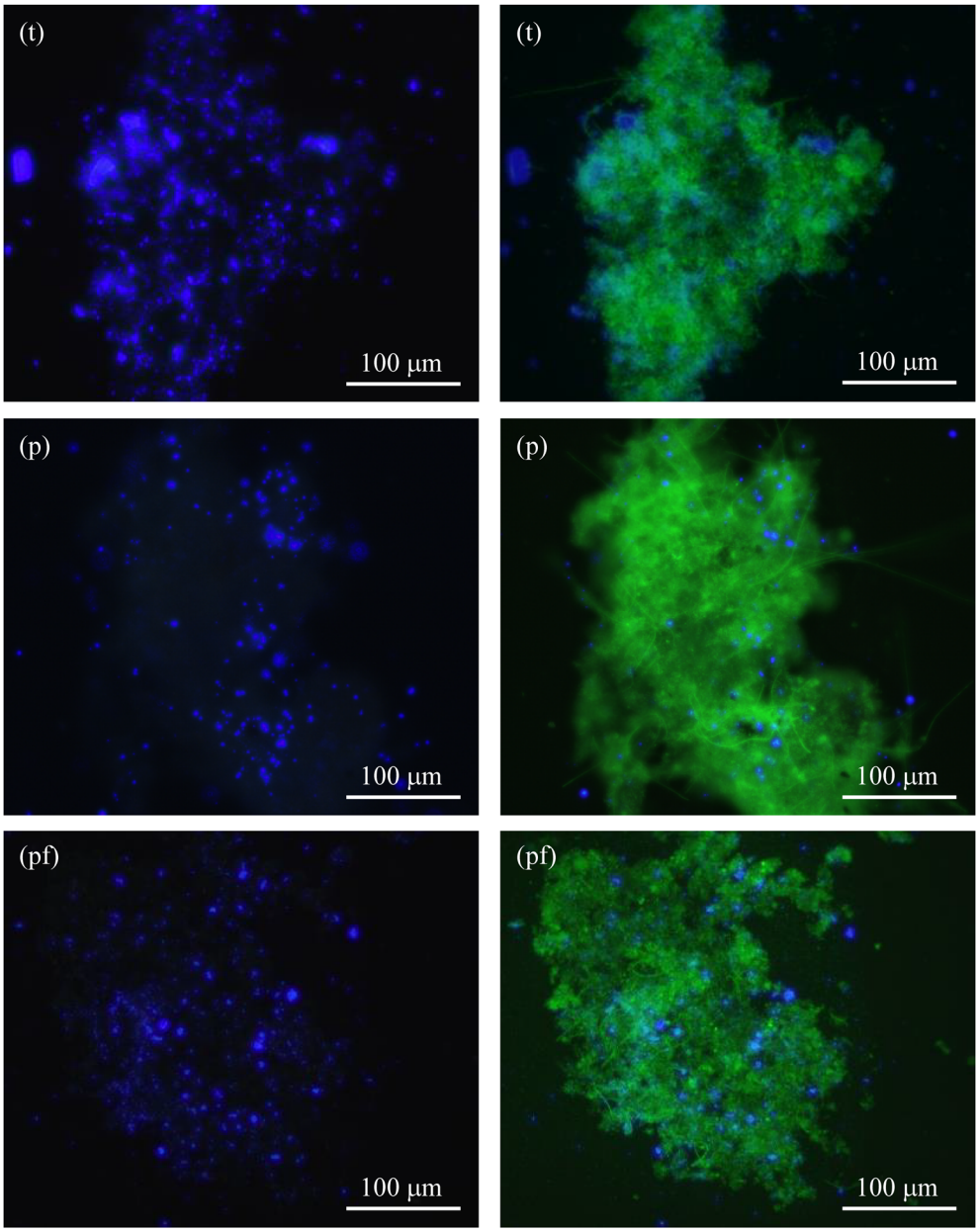

Fig. 7. Anthracene (blue fluorescence) dispersed with Tween® 85, Pluronic® P-123, perfluoropentanoic acid (t, p, pf, respectively), in the test medium containing activated sludge (green fluorescence), cultivated for 5 days, observed with the excitation wavelength 385 nm (three photos on the left), and with both excitation wavelengths 385 nm and 475 nm (three photos on the right).

with PFPA and 62% without PFPA. There was little difference in the biodegradation rate, indicating that no toxicity to microorganisms was observed for PFPA. The biodegradation rate of 3t, 3p, or 3pf at Day 42, which was calculated with Eq. (3) and averaged, was 26%, 9.3%, or −3.9%, respectively. Pluronic® P-123 and PFPA but not Tween® 85 met the realistic standard of the ISO 10634. Moreover, PFPA met the ideal standard of CSCL and the OECD 301. From not only the measured BODs but also analysis with LC-MS (TSQ Quantis) showing that 98% of PFPA remained, on average, at Day 42, it was concluded that PFPA was not biodegraded.

Table 3 shows the relative abundances of the top ten families for OB biodegradation. The original relative abundances of the top ten families are shown in Table S4. The families whose relative abundances increased more than 5% in any test+additive vessels compared to that in the control vessel were *Mycobacteriaceae*, *Nocardiaceae*, *Sphingomonadaceae*, and *Comamonadaceae*. The relative abundances of all the families were increased more than 5% when OB adsorbed onto silica gel was biodegraded.[31] Thus, these families were considered to contribute to the degradation of OB. Although Tween® 85 did not meet the realistic standard of the ISO 10634, neither the abundances of

the families that were considered to contribute the degradation of OB nor those of the other families increased remarkably (3t in Table 3). In contrast, even though Pluronic® P-123 met the realistic standard of the ISO 10634, the relative abundance of *Sphingomonadaceae*, which was considered to have contributed to the degradation of OB, increased up to 22.3% (3p in Table 3). *Sphingomonadaceae* is reported to include major species that biodegrade polyethylene glycol.[25] Pluronic® P-123 has a similar structure to that of polyethylene glycol. The increase in bacterial abundance stemming from the biodegradation of Pluronic® P-123 might promote the biodegradation of OB, and this could not be denied. In the case of PFPA, which met the ideal standard of CSCL or the OECD 301, relative abundance did not increase remarkably in any of the families (3pf in Table 3).

Figure 7 shows fluorescence images of anthracene dispersed with Tween® 85 (t), Pluronic® P-123 (p), or PFPA (pf), with microorganisms in the test medium after five days cultivation. Similar to the trimethylated CDs, the blue fluorescence derived from anthracene dispersed with Tween® 85, Pluronic® P-123, or PFPA existed in clumps in the test medium (three photos on the left in Fig. 7). When the stained microorganisms were also excited to emit green fluorescence, the distribution of anthracene matched perfectly the distribution of the activated sludge flocs (three photos on the right in Fig. 7). When using the emulsifiers, the test substance was dispersed finely and adhered to the activated sludge flocs, which increased the opportunity for contact with microorganisms, thereby enhancing the biodegradation of the test substance.

Conclusion

In this study, we primarily investigated the enhancement of the biodegradation of poorly water-soluble substances using modified cyclodextrins as dispersing agents. Trimethylated α-, β-, and γ-CDs, and partially methylated β-CD not only accelerated the biodegradation of OB and AQ but also met the ideal standard of the CSCL or the OECD 301. Trimethylated α-, β-, and γ-CDs hardly affected microorganisms on the basis of the biodegradation rate and relative abundance. Furthermore, we elucidated a part of the mechanism whereby trimethylated α-, β-, and γ-CDs promote the biodegradation of the test substances: the CDs formed inclusion complexes with hydrophobic substances. The complexes further aggregated and became dispersed in the test medium. These aggregates adhered locally to the activated sludge floc and accelerated the biodegradation of the hydrophobic test substances. In nature, the role of CDs in forming aggregates and enhancing biodegradation of the test substance is carried out by organic matter, and this mechanism is helpful for understanding the biodegradability of substances in natural environments.

Besides CDs, Tween® 85, Pluronic® P-123, and PFPA accelerated the biodegradation of OB, and the mechanism of biodegradation promotion by Tween® 85, Pluronic® P-123, and PFPA seemed similar to that by CDs. Although Pluronic® P-123 fulfilled the realistic standard of the ISO 10634, it might not be an appropriate additive for OB because the growth of *Sphingomonadaceae*, which is induced by the biodegradation of Pluronic® P-123, could facilitate the biodegradation of OB. PFPA fulfilled the ideal standard. However, PFPA persists for a long time once released into the environment. Thus, we believe that the above CDs are more useful additives for the ready biodegradability tests.

Electronic supplementary materials

The online version of this article contains supplementary material, which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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