Appendix to the UK-CA's report on the identification of PBT and vPvB substance results of evaluation of PBT/vPvB properties of D4

Appendix 2 ADDENDUM to D4 PBT Evaluation Fact Sheet of February 2013 (EA, 2013)

Environmental fate and behaviour studies are still being conducted by the producers of D4, academic research groups and other regulators. This addendum summarises relevant studies that have been produced or published since 2011, which was the cut-off date for the previous version of the PBT fact sheet. Most of these have been brought to the attention of the dossier submitter by the D4 producers, but a targeted literature search was also carried out using PUBMED covering the years 2012 and 2013. The focus of the search was on papers relevant to the PBT assessment (particularly bioaccumulation). The references summarised below are included in the main reference list. This appendix also briefly considers additional papers highlighted during the public consultation (PC) by the Member State Committee.

Biodegradation

As part of a study into the fate and behavior of D4 in a municipal waste water treatment plant in Beijing City, China, Xu *et al.* (2013) carried out an *in vitro* study on the anaerobic degradation of D4. The test used a batch system consisting of sealed glass vials containing 40 mL of an activated sludge-liquid mixture obtained from the anaerobic tank of the waste water treatment plant. The sludge mixture had a dry solids content of 10 g/L and a pH of 6.5-6.8. D4 was added to the vial at either 2, 5 or 10 µg/L and then incubated at 30°C with shaking for up to 60 hours under a nitrogen-carbon dioxide headspace (approximately 20 mL). The amount of D4 present in liquid phase and the headspace was determined at intervals (0, 10, 20, 40 and 60 hours). Sterile sludge was used as a control.

Degradation of D4 in this test system was around 9.1-32.7% after 10 hours and 44.4-62.8% after 60 hours (the figures refer to both D4 and D5 combined). D4 was found to be relatively stable in the sterile control. Xu *et al.* (2013) concluded that degradation of D4 during anaerobic waste water treatment would contribute to its removal. (This study is not mentioned in the October 2014 update of the CSRs.)

Bioaccumulation

Studies performed by the Japanese regulatory authorities

i) A GLP bioconcentration study with Common Carp (*Cyprinus carpio*) has been carried out using D4 according to the OECD TG 305 method (CERI, 2007). The test substance had a reported purity of 100.0 per cent. A pre-test with Japanese Medaka (*Oryzias latipes*) gave a 96-h LC_{50} for D4 of >5.6 mg/L at 24°C. The bioconcentration test was carried out using two nominal ^{14}C-D4 exposure concentrations (2.5 µg/L and 0.25 µg/L) in a continuous-flow system with a 60-day exposure period followed by a 15-day depuration period. The fish were between 6.6 and 10.9 cm in length at the start of the test and were fed at a rate of 2 per cent of total body weight per day (the fish were left unfed for 24 hours before sampling). The water used in the test was groundwater with a pH of 7.4-7.9. The temperature and dissolved oxygen content of the water during the test were 24.0-24.9 °C and 6.0-7.9 mg/L, respectively.

   The test solution was prepared by firstly dissolving D4 and the dispersant (hydrogenated castor oil) in N,N-dimethylformamide to give a D4 concentration of 2,500 mg/L (the dispersant was present at ten times the amount of D4). This

was then further diluted with N,N-dimethylformamide to give stock solutions with D4 concentrations of 250 mg/L and 25 mg/L. These stock solutions were then continuously fed to the exposure tanks at a rate to give nominal concentrations of D4 of 2.5 µg/L and 0.25 µg/L in the final exposure tanks. A control was also prepared containing the dispersant at a concentration corresponding to the highest exposure level. Although not given in the test report, the concentration of dispersant in the control (and highest test level) would have been 25 µg/L and the amount of N,N-dimethylformamide present in the exposure tanks would have been around 0.01 mL/L (in each case the dilution of the stock solution would be 100,000).

The concentration of D4 in the test water was determined by GC-MS analysis at various times during the uptake phase of the study. The mean concentrations (± standard deviation) determined (based on measurements on day 4, 11, 25, 39, 53 and 60) were 2.5±0.10 µg/L at the higher exposure level and 0.225±0.009 µg/L at the lower exposure level.

The fish were analysed during the uptake phase to the same time schedule as the water samples. At each sampling time four fish were taken from each exposure group and two samples (each consisting of two fish pooled) were analysed. The concentration in the fish was found to reach steady state within 39 days and the mean steady state BCF values were approximately 3,200 L/kg for the 2.5 µg/L exposure group and 3,000 L/kg for the 0.225 µg/L exposure group. Mean measured steady-state concentrations in whole fish were 7,898 µg/kg for the 2.5 µg/L treatment group and 668 µg/kg for the 0.22 µg/L treatment group. The mean measured concentration at day 15 of depuration ranged from 957-3,180 µg/kg for the 2.52 µg/L treatment group, and 108-168 µg/kg for the 0.22 µg/L treatment group.

As the fish were increasing in size during uptake (see below), the reported steady state may be misleading (as an increasing mass of substance in the fish might be accompanied by increased size, such that the overall concentration does not appear to change significantly; if the fish had not been growing, the concentration may have continued to increase).

Fish were also analysed on days 1, 2, 5 and 15 of depuration. This showed around 12-40 per cent of the steady state concentration of D4 remained in the fish after 15 days. Based on these data the CERI (2007) report estimated that the depuration half-life was between 6.5 and 8.8 days.

The lipid contents of the fish were determined to be 3.18 per cent at the start of the test and 4.22 per cent at the end of the test. Further measurements at depuration day 1 showed the lipid contents to be 5.36 per cent for the higher exposure group and 6.56 per cent for the lower exposure group. Given the variation in the data reported, it is difficult to calculate a meaningful average value for the experiment but it is clear that the lipid content was relatively close to the 5 per cent value considered in the REACH guidance document. Therefore, the data have not been normalised for the actual lipid contents in the experiment.

No further kinetic analysis of the data was reported in CERI (2007). It is evident from some of the raw data that growth of the fish may have been significant in this test (the mean fish weight increasing by a factor of two for both treatment groups during uptake) and so it is relevant to consider the effects of growth-correction on these results. Using the fish weights in the study report (presumably relating to the pooled samples of two fish that were analysed), the growth rate constant can be estimated (from the slope of a plot of ln [fish

weight] against time) to be around 0.0166 day^{-1} for the 2.5 µg/L exposure level and 0.0165 day^{-1} for the 0.225 µg/L exposure level during the uptake phase. However, the fish weights given during the depuration phase were more or less constant (when these are included, the overall growth rate constants reduce to around 0.010 day^{-1} for the 2.5 µg/L exposure level and 0.011 day^{-1} for the 0.225 µg/L exposure level over the entire experimental period (exposure plus depuration)).

The overall depuration rate constants (k_2) for the study have been estimated from a plot of ln [concentration in fish] against time for the depuration phase. This results in k_2 values of 0.0789 day^{-1} for the 2.5 µg/L exposure level and 0.107 day^{-1} for the 0.225 µg/L exposure level. The uptake rate constants (k_1) have been estimated by fitting (least squares) the uptake curve to the equation given in the OECD TG 305 method using these values for k_2 (sequential method). This results in k_1 values of 284 L/kg/day for the for the 2.5 µg/L exposure level and 443 L/kg/day for the 0.225 µg/L exposure level. A plot showing the fit to the data is given in Figure A1.1.

The kinetic BCF is the k_1/k_2 ratio. This was 3,600 L/kg for the 2.5 µg/L exposure level and 4,140 L/kg for the 0.225 µg/L exposure level.

As the fish were increasing in size during uptake, the estimated rate of uptake may be lower than if the fish were not growing (since the test substance concentration will be reduced by growth). Consequently the kinetic BCF might actually be higher if the test had been conducted on fish that were not growing. The approach recommended by OECD TG 305 to take this into account is to subtract the overall growth rate constant from the depuration rate constant. If this is done, the kinetic BCF at 2.5 µg/L becomes 4,120 L/kg (if the overall growth rate is used) or 4,560 L/kg (if only the growth rate during the uptake phase is used) (similar values of 4,610-4,890 L/kg are obtained at the lower exposure concentration). However, since the fish were not growing during depuration, this could be misleading.

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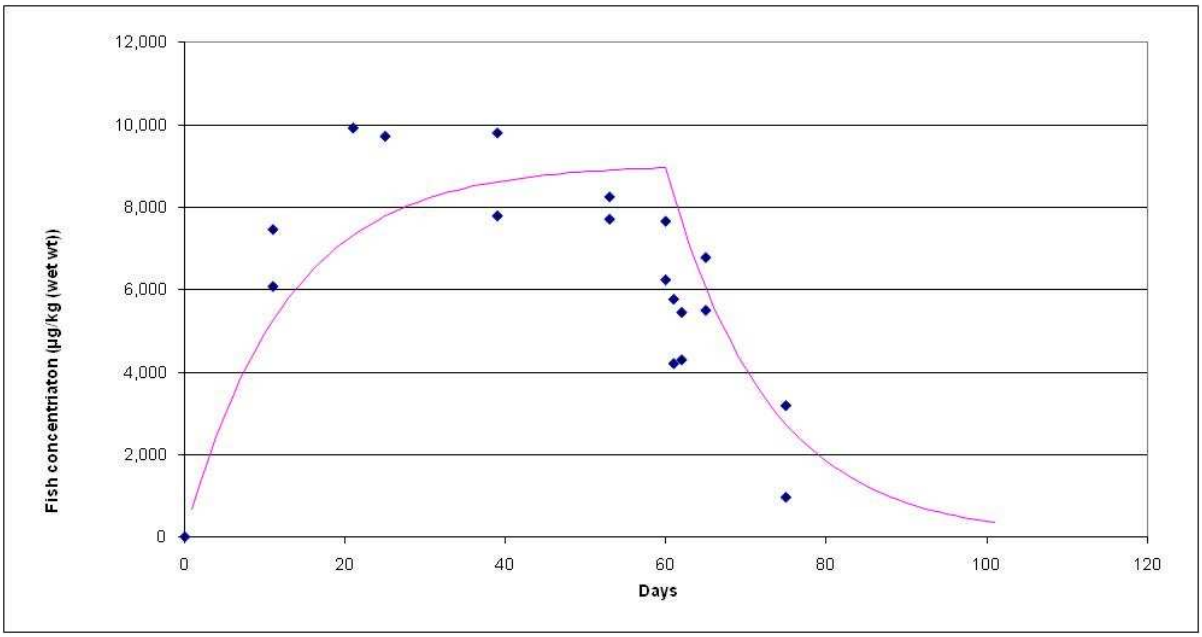
Figure A1.1 Plot showing fit to the experimental data for the CERI (2007) bioconcentration study for the 2.5 µg/L exposure level



Overall the CERI (2007) study appears to be well carried out and reliable. The results show that the growth-corrected BCF for D4 in carp is above 4,000 but less than 5,000 L/kg in this study. It should be noted that the reported fish weights suggest that little or no growth was occurring during the depuration phase and so the relevance of growth correction of the kinetic data in this case is questionable. The steady state BCF is around 3,000 L/kg without any correction for growth, although this could be too low as the fish were growing during the uptake phase. (This study is not mentioned in the October 2014 update of the CSRs.)

ii) A further bioconcentration study with D4 has been carried out in carp (*Cyprinus carpio*). The full study report (CERI, 2010) is currently available only in Japanese but the raw data presented allow the bioconcentration parameters reported to be verified. The study was carried out to GLP using the OECD TG 305 methodology.

The study was carried out using two nominal concentrations of ^{14}C-D4 (2.5 µg/L and 0.25 µg/L). A dispersant (hydrogenated castor oil) and a solvent (N,N-dimethylformamide) were used to prepare the test solutions. A control containing the dispersant/solvent was also prepared. The total duration of the test was 72 days, consisting of a 60-day uptake phase followed by a 12-day depuration phase. A flow-through system was used.

The fish had a length of between 6.2 and 10.3 cm at the start of the test and were fed at a rate of around 2 per cent of body weight per day over the duration of the study. The test was carried out at a temperature of 24.0-24.9 °C and test water had a pH between 7.6 and 7.8 and a dissolved oxygen concentration of between 5.7 and 7.3 mg/L throughout the test.

The concentrations in water were analysed on day 3, 7, 20, 32, 46, 54 and 60 of the uptake phase. The concentrations were found to be stable, with the mean concentrations (±standard deviation) at the two exposure levels being 2.39 (±0.047) µg/L and 0.24 (±0.013) µg/L, respectively. The concentration in fish was determined on the same days as above from day 7 onwards, and steady

state was found to have been reached by day 46. Mean measured steady-state concentrations in whole fish were 7,895 µg/kg for the 2.39 µg/L treatment group and 1,088 µg/kg for the 0.24 µg/L treatment group. The mean measured concentration at day 12 of depuration ranged from 2,050-2,170 µg/kg for the 2.40 µg/L treatment group, and 339-349 µg/kg for the 0.23 µg/L treatment group. The steady state BCFs determined for the last three sampling times were 3,353 L/kg (day 46), 3,132 L/kg (day 54) and 3,502 (day 60) at the 2.39 µg/L treatment level and 4,310 L/kg (day 46), 3,668 L/kg (day 54) and 3,924 (day 60) at the 0.235 µg/L treatment level. The mean BCF at steady state was 3,329 L/kg at the 2.39 µg/L treatment level and 3,967 L/kg at the 0.235 µg/L treatment level. The lipid content of the fish was 4.89 per cent at the start of the test and 4.15 per cent at the end of the test. Further lipid measurements (possibly taken on day 47 although this is not clear) were 6.43 per cent for the 2.39 µg/L treatment level and 5.84 per cent for the 0.235 µg/L treatment level. The average of these reported values is close to 5 per cent (mean is 5.3 per cent) and so it is not necessary to normalise the reported values to the "standard" lipid content of 5 per cent recommended in the REACH guidance.

The test substance was found to depurate relatively slowly, with the concentration declining to around 26-35 per cent of the steady state concentration by day 12 of depuration, and the depuration half-life was estimated to be between 7.0 and 8.2 days. No other kinetic parameters were determined in the CERI (2010) report, but the raw data allow a more detailed kinetic analysis to be undertaken. When this is done for the 2.39 µg/L exposure level, the uptake rate constant (k_1) can be estimated as 407 L/kg/day and the overall depuration rate constant can be estimated as 0.0991 day^{-1}, giving a kinetic BCF of 4,116 L/kg. Similarly, for the 0.235 µg/L group, the k_1 value determined is 467 L/kg/day, and the k_2 value is 0.0843 day^{-1}, giving a kinetic BCF of 5,540 L/kg. It should be noted that in both cases the concentrations in fish measured on uptake day 20 and 32 were higher than the steady state concentration. This was particularly noticeable for the 0.235 µg/L treatment group where the concentrations measured on uptake day 20 were 1,650 and 1,760 µg/kg (two replicates) compared with concentrations of 1,300 and 1,320 µg/kg at day 32, 1,050 and 1,030 µg/kg at day 46, 859 and 971 µg/kg at day 54 and 1,060 and 872 µg/kg at day 60. This means that the uptake curves for these tests (particularly at the lower concentration) were not well defined. Plots showing the fit of the derived kinetics to the experimental measurements are shown in Figure A2.2 and Figure A2.3.

The fish weights were reported in the CERI (2010) study at each time point for the exposed fish. These allow the growth rate constant to be determined from plots of ln [fish weight] against time. Such plots for the uptake phase yield growth rate constants of 0.0160 day^{-1} for the 2.39 µg/L treatment group and 0.0169 day^{-1} for 0.235 µg/L treatment group. Similar to the previous study, the mean fish weight increased by a factor of two for both treatment groups during uptake, whereas the fish weights reported during the depuration phase were more or less constant. When these are included in the plots the growth rate constants reduce to around 0.0126 day^{-1} for the 2.39 µg/L treatment group and 0.0128 day^{-1} for the 0.235 µg/L treatment group. Thus the growth-corrected kinetic BCF would be around 4,705-4,898 L/kg for the 2.39 µg/L treatment group and 6,530-6,930 L/kg for the 0.235 µg/L treatment group.

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Figure A2.2 Plot showing fit to the experimental data for the CERI (2010) bioconcentration study for the 2.39 µg/L exposure level

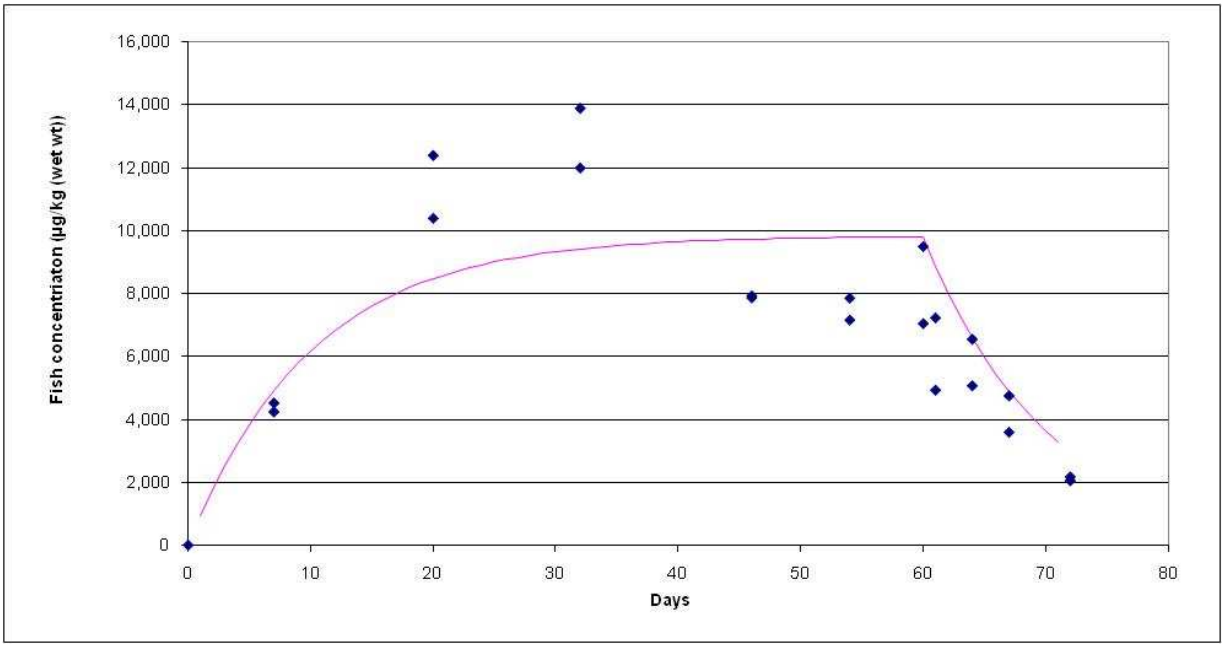
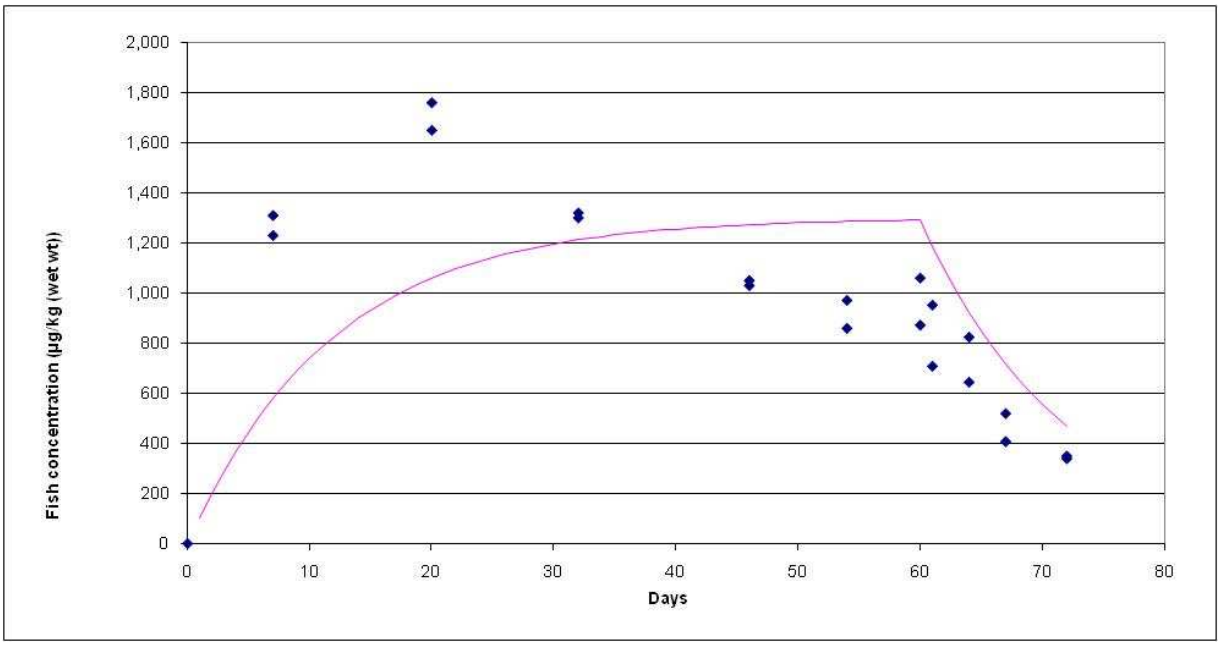


Figure A2.3 Plot showing fit to the experimental data for the CERI (2010) bioconcentration study for the 0.235 µg/L exposure level



Overall the CERI (2010) study appears to be well carried out. The results show that the growth corrected BCF for D4 in carp is above ca. 5,000 L/kg in this study. It should be noted, however, that the uptake curves are generally not as

well defined as in some of the other studies (see Figure A2.2 and Figure A2.3) and the reported fish weights would suggest that little or no growth was occurring during the depuration phase itself, making the relevance of growth correction questionable in this case. The kinetic BCF without growth correction was in the range 4,100 - 5,500 L/kg, although variations in the uptake curves complicate the interpretation of these values. The mean BCF at steady state was in the range 3,300 – 4,000 L/kg. (This study is not mentioned in the October 2014 update of the CSRs.)

iii) It is understood that two further bioconcentration tests with D4 in carp have been performed in Japan (CES, personal communication), but the data have not been made available to the DS (these are "older" data from "CERI/MSI").

In summary, the data show that the BCF for *C. carpio* is well above 2,000 L/kg, with a steady state BCF in the range 3,000 – 4,000 L/kg without any correction for growth (for comparison, growth-corrected kinetic BCFs were in the range 4,120-6,930 L/kg).

Dietary bioaccumulation in Rainbow Trout

Documents submitted during PC cite a study in Rainbow Trout *Oncorhynchus mykiss* by Woodburn *et al*. (2013). This is a formal publication of a study report already evaluated by the DS and summarised in the main report (Dow Corning, 2007; full details of this study are provided in EA, 2009). The DS has not evaluated the published article, but notes that although some of the derived BMF values are different to those quoted in this report, the overall conclusion is the same (i.e. the lipid-normalised steady state BMF is below 1, but the kinetic BMF is above 1 when growth is taken into account). The values cited in this report are consistent with those in the CSRs (October 2014 update).

BSAF in marine fish

Industry documents submitted during PC cite a study by Hong *et al*. (2014), which estimated a BSAF value for D4 of 0.716 ± 0.456 in a marine fish (*Hexagrammos otakii*) sampled from a site northeast of China. This has not been evaluated by the DS. However, the registrants suggest that whilst this species may feed on benthic organisms, it does not appear to live within the sediment. (This study is not mentioned in the October 2014 update of the CSRs.)

Study of allometric relationships for Atlantic Cod liver concentrations

A further study mentioned but not summarised in industry documents submitted during PC is that of Warner *et al*. (2014). This study has not been evaluated by the DS. However, it indicates that D4 concentrations in Atlantic Cod (*Gadus morhua*) livers (n=20) collected at two locations near Tromsø, Norway in November 2010 and April 2011 were negatively correlated with fish length and weight, indicating a greater elimination capacity compared to uptake processes with increasing fish size. D4 was detected in all livers but two (from Nipøya, n=8, considered a remote location in this study). The arithmetic mean liver concentration was 25 (range 9.3 – 45.6) µg/kg ww at the first location and 5.0 (range <2.4 – 9.9) µg/kg ww at the second. Stomach contents of fish collected at the two sites were similar, so the difference in concentration was not linked to dietary feeding (more likely it was linked to distance from the pollution source). This study suggests that relationships between allometric measurements and D4 concentrations should be taken into account in future field studies of bioaccumulation potential. (This study is not mentioned in the October 2014 update of the CSRs.)

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Lake Champlain food chain accumulation study

A further field study has been carried out at Lake Champlain, USA, highlighted during PC (Powell, 2014), but this has not been reviewed by the DS. The median TMF was calculated as 1.3 – 1.4, and the probability that the TMF exceeded 1.0 was in the region of 80%. However, the variability was high, and the median r^2 value was in the range 3-4%. Attempts were made to adjust the fish concentrations for likely exposure, and this gave a median TMF of 0.6 – 2.9. The report's conclusion was that a reliable TMF could not be obtained for D4. (This study is not mentioned in the October 2014 update of the CSRs.)

Norwegian lake food chain accumulation study

Borgå *et al.* (2013a and 2013b) carried out a study of the pelagic food web in Lake Mjøsa, Norway (to replicate the study reported by Borgå *et al*. (2012), which was summarised in EA, 2013) and extended it to include a similar lake in the same area (Lake Randsfjorden) and a lake thought to be remote from any known sources of emission (Lake Femunden[1]). All three lakes are deep and contain well-defined pelagic food webs including zooplankton, planktivorous fish and Brown Trout (*Salmo trutta*) as a top predator. (This study is included in the October 2014 update of the CSRs, and is indicated as 'reliable with restrictions'.)

Lake Mjøsa has a pelagic food web with Brown Trout (*Salmo trutta*) as the top predator, Smelt (*Osmerus eperlanus*) and Vendace (*Coregonus albula*) as primary planktivorous prey, and an invertebrate community consisting of cladocerans, copepods and *Mysis relicta*. Lake Randsfjorden has some similarities to Lake Mjøsa and has a well-defined pelagic food web with Brown Trout and Arctic Char (*Salvelinus alpinus*) as top predators, and Whitefish (*Coregonus lavaretus*) and Smelt as planktivorous prey. Lake Femunden has a pelagic fish community of Brown Trout, Arctic Char and Whitefish. The main food web difference between the lakes is that Lake Mjøsa includes *Mysis relicta* in the invertebrate community, Vendace among the planktivorous fish, and excludes Arctic Char as top predator. Whitefish is assumed to be a benthic feeding species in Lake Mjøsa but assumed to replace Vendace in the pelagic food web of Randsfjorden and Femunden.

The samples were collected between July and September 2012. Fish and invertebrates were sampled from the pelagic zone in all three lakes. In addition benthic fish (Whitefish, Perch (*Perca fluviatilis*) and Burbot (*Lota lota*)) were sampled from Lake Mjøsa. As well as biota samples, samples of surface sediments were also collected from all three lakes along with surface water and effluent samples from Lake Mjøsa and Lake Randsfjorden. The majority of biota samples in Lake Mjøsa (zooplankton, *Mysis reticta*, Vendace and Smelt) were collected mid-lake in an area south of the town of Helgøya. Brown Trout were collected from close to the town of Gjøvik but as this species uses the entire lake in search of food it was thought that these samples were representative of a larger geographical area. In Lake Randsfjorden the biota samples were all collected mid-lake from an area south of Brandu and in Lake Femunden the biota samples were collected from the southern basin.

The fish samples consisted of skinless fillets from one individual except for small Smelt where five or six skinless fillets were pooled for each sample. For Burbot, both fillets and liver were sampled. Pre-cleaned field blanks were handled in the same way as the biota samples. Sediment samples were taken from the surface layer (0-1 cm depth) in areas

[1] Although Lake Femunden was considered a remote lake with low human impact, the map given in the paper shows a small village close by and so point sources of emission cannot be totally ruled out.

close to the discharge from waste water treatment plants where this was possible. Each sample consisted of a pool of three cores from each sampling area. Deeper sediments (typically from 30 cm or deeper) were also collected to act as reference samples. Water samples from Lake Mjøsa were collected from a depth of 15 m[2]. Grab samples of effluent were collected from the outlets of three waste water treatment plants in each of Lake Mjøsa and Lake Randsfjorden. Precautions were taken during sampling to avoid inadvertent contamination of the samples (for example all personnel avoided the use of personal care products).

The trophic level of each species was assigned based on $\delta^{15}N$ measurements and the carbon source for the organism was determined based on $\delta^{13}C$ measurements. The zooplankton from the epilimnion was defined as the baseline consumer and assigned a trophic level of 2. The other trophic levels were assigned relative to this using an enrichment factor (ΔN) of 3.4‰ TL^{-1}.The number of samples collected and assigned trophic level are summarised in Table A2.1.

The samples were analysed for the presence of D4, D5 and D6 (cyclic volatile methylsiloxanes, cVMS). In addition known bioaccumulative substances (polychlorinated biphenyls (PCB-153 and PCB-180) and dichlorodiphenyldichloroethylene (p,p'-DDE) in Lake Mjøsa and Lake Randsfjorden, and polybrominated diphenyl ethers (PBDE-47 and PBDE-99) in Lake Mjøsa) were analysed in the sample to act as reference substances. Procedural blanks, field blanks and an internal matrix control (homogenate of herring from the Baltic sea for biota samples and a sediment sample from Lake Mjøsa for abiotic samples) were also analysed at intervals along with the samples. The limit of quantification (LOQ) for biota was set to the mean plus 10×standard deviation of the procedural blanks and the LOQ for sediment was set at 3×maximum quantity measured in the reference sediments. The levels found are summarised in Table A2.1. The levels were not blank-corrected[3].

The levels of cVMS found in Lakes Mjøsa and Randsfjorden were generally higher than found in Lake Femunden, reflecting the local sources of release into the lakes. The concentration of D4 was above the LOQ in only 23% of the biota samples (a total of 91 samples were analysed) and 0% of the sediment samples (a total of 18 samples were analysed). In Lake Femunden, all cVMS were below LOQ in all samples analysed[4] except for a few trout in which D5 was above the LOQ.

All of the effluent water samples contained all cVMS above the LOQ, with the exception of D6 in a sample from Lillehammer, Mjøsa. For the particulate samples of surface water, an error in the field resulted in no field blank being available. Since it could therefore not be excluded that these samples were contaminated, the measured concentrations were designated "<" values.

The sediment samples showed a high spatial variation in the concentration of cVMS in Lake Mjøsa and Lake Randsfjorden, with the highest concentrations near to the towns of Brandbu and Grjøvik respectively, reflecting the local sources of input (i.e. waste water treatment plants) in these areas.

[2] For the surface water samples the particulate phase was analysed for cVMS and the dissolved phase was analysed for the reference substances.

[3] The difference between total D4 content in field blanks and samples from Lake Mjøsa was lower than for other cyclic siloxanes. For Lake Randsfjorden, more samples were close to or below the LOQ. In Lake Femunden only D5 was quantified above the LOQ in trout.

[4] As low levels in this lake were foreseen, sediments and samples of the top predators Brown Trout and Arctic Char were analysed first. As only low levels were found, the remaining samples collected in Lake Femunden (zooplankton, Whitefish, Arctic Char) were not analysed.

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Table A2.1 Summary of levels of D4 in samples collected from Lakes Mjøsa, Randsfjorden and Femunden

Lake	Species	Food web	No. of samples analysed	Mean trophic level (±standard error)	Mean D4 concentration (ng/g lipid) (±standard error)
Lake Mjøsa	Zooplankton (epilimnion)	Pelagic	3	2.0±0.0	<46
	Zooplankton (hypolimnion)	Pelagic	4	2.6±0.2	36±3
	Mysis relicta	Pelagic	4	2.8±0.1	53±13
	Vendace (*Coregonus albula*)	Pelagic	7	3.9±0.0	81±8
	Smelt, small (*Osmerus eperlanus*)	Pelagic	5	3.8±0.1	<24±3
	Smelt, large (*Osmerus eperlanus*)	Pelagic	5	4.4±0.0	<17±3
	Brown Trout (*Salmo trutta*)	Pelagic	5	4.4±0.0	27±7
	Whitefish (*Coregonus lavaretus*)	Benthic	5	3.6±0.1	<38
	Perch (*Perca fluviatilis*)	Benthic	6	4.0±0.1	<29
	Burbot, liver (*Lota lota*)	Benthic	6		44±7
	Burbot, muscle (*Lota lota*)	Benthic	6	4.4±0.1	<61
Lake Rands-fjorden	Zooplankton (epilimnion)	Pelagic	4	2.0±0.0	<34
	Zooplankton (hypolimnion)	Pelagic	3	3.0±0.3	51±2
	Whitefish (*Coregonus lavaretus*)	Bentho-pelagic	9	3.2±0.1	<19
	Smelt (*Osmerus eperlanus*)	Pelagic	5	3.5±0.1	<11
	Brown Trout (*Salmo trutta*)	Pelagic	5	3.8±0.1	16±3
Lake Femunden	Arctic char (*Salvelinus alpinus*)	Pelagic	1	-[a]	<10
	Brown Trout (*Salmo trutta*)	Pelagic	6	-[a]	<40

Note: The trophic level of the fish from Lake Femunden was not reported.

The $\delta^{13}C$ measurements showed a clear separation of the pelagic feeding fish from the benthic feeding fish in Lake Mjøsa. In Lake Ransfjorden, a relatively high variation in the $\delta^{13}C$ value was found in Whitefish, suggesting that there was some variation in the diet of this species. Earlier investigations of stomach contents of whitefish from this lake had shown both purely pelagic feeding fish and fish feeding on benthic and terrestrial invertebrates. Therefore the TMFs for Lake Ransfjorden were calculated both including and excluding whitefish.

The TMF was estimated from the slope of a plot of the natural logarithm of lipid

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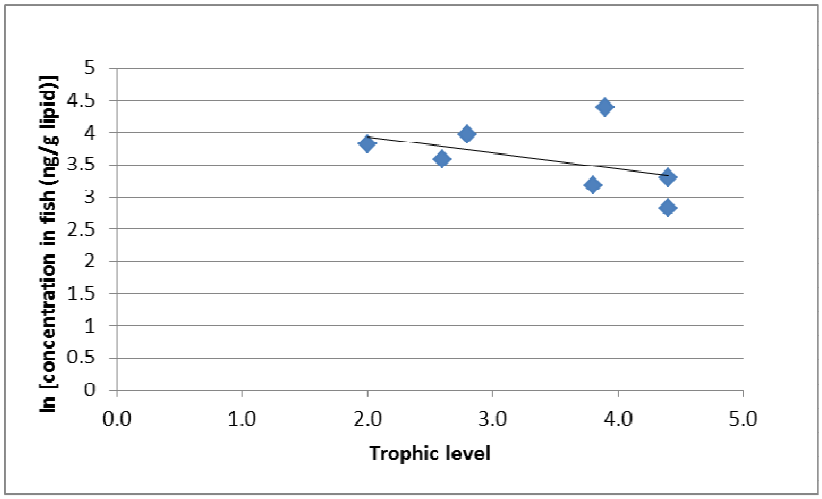
normalised concentration in biota versus trophic level. The benthic fish (from Lake Mjøsa) and sediment samples were not included in the analysis. Where the concentration of D4 was <LOQ but >LOD (limit of detection) the actual estimated concentration was used in the analysis (rather than replacing the <LOQ value with a fixed or random value). For Lake Randsfjorden, one hypolimnion zooplankton sample was identified as a multivariate outlier and so was excluded from the analysis. A plot showing the mean concentrations against the trophic level for Lake Mjøsa is shown in Table A2.2 (these values were derived in the actual publications from plots of the individual data points rather than the mean data points).

The TMF for D4 was found to be similar between Lakes Mjøsa and Randsfjorden regardless of whether whitefish were included or excluded. The TMF for D4 was in the range 0.58-0.76 (and in all but one case the TMF was statistically significantly <1 at the 95% confidence level; see Table A2.2) indicating that trophic dilution was occurring. However over half of the samples had concentrations below the limit of quantification in all derivations of the TMF which means that there is some uncertainty in the derived TMF for D4.

Figure A2.4 Plot of ln [mean concentration in biota (ng/g lipid)] versus trophic level for Lake Mjøsa



The levels of D4 in the pelagic food webs were also found to correlate with the levels of The levels of D4 in the pelagic food webs did not correlate with those of either D5 and D6 (for which TMFs >1 were derived for the same food web) nor with the levels of known biomagnifying substances, for example PCB-153 and p,p'-DDE. The TMFs for these reference substances were higher in Lake Mjøsa than Lake Randsfjorden but were above 1 in both lakes. This provides further support that D4 was not biomagnifying in these food webs.

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Table A2.2 Summary of TMFs derived by Borgå *et al*. (2013a & 2013b)

Lake	Number of data points	TMF	95% confidence interval	p-value[a]	R^2 of regression	Comment
Lake Mjøsa	33	0.76	0.57-1.01	0.062	0.11	Not including whitefish; over half of the data were <LoQ
Lake Randsfjorden	17	0.57	0.35-0.93	0.027	0.29	Not including whitefish; over half of the data were <LoQ
	26	0.58	0.38-0.87	0.011	0.24	Including whitefish; over half of the data were <LoQ
Combined Lake Mjøsa and Lake Randsfjorden	51	0.69	0.54-0.89	0.0045	0.29	Not including whitefish; over half of the data were <LoQ
	59	0.70	0.56-0.88	0.0031	0.30	Including whitefish for Lake Randsfjorden; over half of the data were <LoQ

Note: a) The p-value indicates the statistical significance of the regression. Statistically significant difference is usually taken as a value of $p \leq 0.05$.

As is the case with the previous study by this research group, there are a number of uncertainties associated with these results, including the following:

- The Brown Trout in Lake Mjøsa were sampled from a different area of the lake than the other biota samples. The trout were sampled near to Grjøvik and the sediment samples suggested that this area may have been more heavily contaminated than other parts of the lake. However, it was noted that this species use the entire lake for feeding and so the levels found are probably more reflective of the levels in the whole lake rather than the specific area sampled. In addition, a similar level of trophic magnification was evident in the food webs of both Lake Mjøsa and Lake Randsfjorden.
- The fish samples analysed were skinless fillets (with the exception of Burbot livers), so the reported concentrations do not necessarily reflect the levels present in whole fish.
- A number of species included in the regressions had levels of D4 below the limit of quantification in both Lake Mjøsa and Lake Randsfjorden. In addition, the total number of samples analysed for each species is low (3 – 9), so the representivity and variation of the concentrations is unclear.

Despite these limitations, this study provides some evidence that D4 was not biomagnifying in pelagic food webs of both Lake Mjøsa and Lake Randsfjorden. The TMF determined in both lakes was similar and the overall TMF from both lakes combined was determined to be 0.70 with a 95% confidence interval of 0.56-0.88. In addition, the levels of D4 did not correlate in the pelagic food chain with the reference substances that are known to biomagnify. This study provides more information than the previous study

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at Lake Mjøsa (Borgå *et al*., 2012), when the concentration of D4 was below the limit of quantification in the majority of samples, so it was not possible to estimate a TMF. It is, however, possibly relevant to note that the concentration in Brown Trout (occupying the highest trophic level) had a mean concentration of 190 µg/kg lipid in the previous study, which seems to be significantly higher than this study (27 µg/kg lipid).

Tokyo Bay food chain accumulation study

A further study of the bioaccumulation of D4 is currently in the process of being published (Powell *et al.*, 2014)[5]. (This study is partially reported in the October 2014 update of the CSRs, and is indicated as 'reliable without restriction'.) A pre-publication draft of the study has been made available to the dossier submitter. The study was of a pelagic marine food web in Tokyo Bay. The samples for the study included sediment and fish (see Table A2) collected between 4th and 15th November 2011 from a defined 500 km^2 area covering approximately 55% of inner Tokyo Bay. The area was defined using a two-dimensional probability design based on 25 km^2 square grids extending seaward from the head of the bay to the narrows between Cape Kannon and Cape Futtsu. Sediments were collected from 20 locations by systematically sampling each 25 km^2 grid and fish were collected within the northern part of the study area. Precautions were taken during sampling, storage and analysis to avoid unintentional contamination of the samples and loss from evaporation and degradation. As well as D4, the study included PCB-180 as a benchmark chemical and PCB-153 as a reference chemical.

The trophic positions of the organisms were determined based on $\delta^{15}N$ measurements and $\delta^{13}C$ measurements were used to assess the sources and flow of dietary carbon in the food web. The trophic levels assigned to the organisms (using a $\Delta^{15}N$ of 3.4‰ TL^{-1}) are shown in Table A2.3 along with the measured concentrations of D4. In all cases the concentration of D4 was above the method detection limit[6].

The concentration of D4 (and also PCB-153 and PCB-180) in sediment varied spatially across the area, generally decreasing with distance from the inner part of the estuary (close to the mouths of the Arakawa River and the Edogawa River). The $\delta^{15}N$ and $\delta^{13}C$ measurements in sediment also appeared to be related to the proximity of the rivers entering the bay but no significant trends were apparent. As a result of the existence of this concentration gradient in the sediment, the study area was stratified and mean concentrations in sediments were calculated using appropriate methods for a stratified experimental design.

Table A2.3 Summary of levels of D4 in samples collected from Tokyo Bay

Species	Number of samples analysed	Trophic level (based on a $\Delta^{15}N$ value of 3.4‰ TL^{-1})	Mean lipid content (%)	Mean D4 concentration (ng/g lipid) (±standard deviation)[a]
Dotted Gizzard Shad (juvenile) (*Konosirus punctatus*)	3 composites (each of 11 individuals)	3.0	8.0	178±4.4
Silver Croaker	3 composites (each of	3.1	5.9	198±41.3

[5] A further related report was highlighted during PC (ECC, 2013), but this has not been reviewed by the DS.

[6] The method detection limit (MDL) was the level in a sample matrix that could be measured and reported with >99% certainty as being greater than zero. The limit of quantification was defined as 3 times the MDL. The actual non-censored values were reported.

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Species	Number of samples analysed	Trophic level (based on a $\Delta^{15}N$ value of 3.4‰ TL^{-1})	Mean lipid content (%)	Mean D4 concentration (ng/g lipid) (±standard deviation)[a]
(*Pennahia argentata*)	13 individuals)			
Japanese Sardinella (*Sardinella zunasi*)	3 composites (each of 48 individuals)	3.1	4.5	488±31.1
Japanese Anchovy (*Engraulis japonicas*)	3 composites (each of 55 individuals)	3.5	3.9	229±38.9
Dotted Gizzard Shad (adult) (*Konosirus punctatus*)	1 composite (of 5 individuals)	3.8	17.0	55.6±(11.1)
Chub Mackerel (*Scomber japonicas*)	1 composite (of 4 individuals)	4.1	20.0	41.9±(8.4)
Red Barracuda (*Sphyraena pinguis*)	1 composite (of 5 individuals)	4.1	11.0	149±(29.9)
Japanese Sea Bass (*Lateolabrax japonicas*)	6 individuals	4.4	6.3	389±99.7

Notes: a) For the species where only one sample was analysed the standard deviation (given in brackets) was estimated using sampling variances from other studies conducted on cVMS.

The $\delta^{13}C$ measurements indicated that all fish species were feeding on a similar carbon source, and that this carbon source was different to that in the sediment. The $\delta^{15}N$ measurements suggested that the food web covered around 1.4 trophic steps with planktivorous forage species at the base of the food web (e.g. juvenile Dotted Gizzard Shad (*Konosirus punctatus*), Silver Croaker (*Pennahia argentata*) and Japanese Sardinella (*Sardinella zunasi*)) and piscivorous predatory species at the top of the food web (e.g. Red Barracuda (*Sphyraena pinguis*), Chub Mackerel (*Scomber japonicus*) and Japanese Sea Bass (*Lateolabrax japonicas*)). Examination of the gut contents indicated that the Japanese Sea Bass were feeding exclusively on Japanese Anchovy (*Engraulis japonicas*) and Japanese Sardinella at the time of sampling. With the exception of Japanese Sea Bass the species sampled were thought to actively migrate throughout the estuary (Japanese sea bass were not thought to migrate as actively as other species).

Several approaches were used to estimate the TMF, and the results are summarised in Table A2.

i) Trophic magnification factors were firstly estimated from the fish data from the slope of a plot of ln [concentration in fish (ng/g lipid)] versus trophic level. The TMF for D4 was 1.3 with a 95% confidence interval of 0.5 to 3.3 but was not statistically different from 1 (p=0.52). The TMFs derived for PCB-153 and PCB-180 were 2.7 and 2.8 respectively.

ii) The TMFs were also estimated from the same data using a multivariate

probabilistic method (to take account of bias resulting from experimental design). This resulted in a median TMF for D4 of 0.6 (95% confidence interval 0.5-0.8, probability of TMF >1 0.1%). The median TMFs derived for PCB-153 and PCB-180 were both 2.2 using this method.

Table A2.4 Summary of bioaccumulation parameters derived for Tokyo Bay

Parameter		**D4**	**PCB-153**	**PCB-180**
Biota-sediment accumulation factor (BSAF)	Dotted Gizzard Shad (juvenile)	1.6	1.0	0.44
	Silver Croaker	1.3	0.87	0.57
	Japanese Sardinella	2.2	1.3	0.65
	Japanese Anchovy	1.1	1.4	0.94
	Dotted Gizzard Shad (adult)	0.55	2.6	1.5
	Chub Mackerel	0.38	3.3	1.8
	Red Barracuda	0.60	2.5	1.6
	Japanese Sea Bass	2.0	5.4	3.3
TMF using the standard method; $\Delta^{15}N$ = 3.4‰ TL^{-1}	TMF	1.3	2.7	2.8
	95% Confidence Interval	0.5-3.3	1.4-5.3	1.4-5.6
	TMF statistically significantly different from 1	No (p=0.52)[a]	Yes (p=0.01)[a]	Yes (p=0.01)[a]
Probabilistic TMF; $\Delta^{15}N$ = 3.4‰ TL^{-1}	Median TMF	0.6	2.2	2.2
	95% Confidence Interval	0.5-0.8	1.7-2.9	1.7-3.0
	Probability TMF >1	0.1%	>99.9%	>99.9%
Benchmark TMF[b]; $\Delta^{15}N$ = 5.9‰ TL^{-1}	Median TMF	0.4	3.9	4.0
	95% Confidence Interval	0.3-0.7	2.4-6.3	2.4-6.9
	Probability TMF >1	0.1%	>99.9%	>99.9%
Corrected benchmark TMF; $\Delta^{15}N$ = 3.9‰ TL^{-1}	Median TMF	0.5	3.6	4.0
	95% Confidence Interval	0.4-0.7	2.6-4.9	2.9-5.7
	Probability TMF >1	<0.1%	>99.9%	>99.9%

Note: a) The p-value indicates the statistical significance of the regression. Statistically significant difference is usually taken as a value of $p \leq 0.05$.
b) PCB TMFs were the median values from log normal distributions of TMF values reported in the literature for PCB-180 (n=22) and PCB-153 (n=26).

iii) Next the data were analysed using a benchmarking approach combined with the probabilistic method, using PCB-180 as the benchmarking chemical. For this approach the TMF for PCB-180 was assumed to be 4.0 and this was used to calibrate the food web, resulting in a benchmarked $\Delta^{15}N$ value of 5.9‰ TL^{-1}. This value was then used to derive the TMF for D4 and PCB-153. Using this approach the median TMF for D4 was 0.4 (95% confidence interval: 0.3-0.7; probability of TMF >1: 0.1%). The median TMF for PCB-153 was 3.9. Although this approach resulted in a TMF value for PCB-153 that was in line with the expected value for this substance, the $\Delta^{15}N$ value derived was outside the accepted range for aquatic food webs (generally taken to be between 3.0‰ TL^{-1} and 5.0‰ TL^{-1}). Powell *et al.* (2014) suggested that this was indicative of variable exposure in the current food web.

iv) As the sediment data also indicated the existence of concentration gradients within the sampled area, and hence the possibility of variable exposure of the fish

sampled, an analysis was undertaken to correct for this based on estimated migration patterns for each species (based on their known ecology) and the concentrations in sediment (used as an indicator of exposure based on the assumption that the concentrations in water and sediment were in equilibrium over the long-term). This was carried out by estimating BSAF values for each species based on the mean concentration in each species (ng/g lipid) by the relative exposure concentration in sediment (ng/g total organic carbon) for that species. The BSAFs derived are summarised in Table A2. The BSAF for D4 was >1 in some cases but it was found to generally decrease (or not increase significantly) with increasing trophic level, which was in contrast to the BSAFs calculated for PCB-153 and PCB-180[7].

The BSAFs for PCB-180 were then used to apply an exposure correction to the food web. Using this approach an exposure-corrected $\Delta^{15}N$ value of 3.9‰ TL^{-1} was calculated using the benchmarking approach outlined above. This was then used to estimate the TMF for D4 and PCB-153 using the probabilistic approach. The exposure-corrected median TMF for D4 was 0.5 (95% confidence interval 0.4-0.7, probability of TMF >1 <0.1%). The median TMF for PCB-153 was estimated to be 3.6. This method was considered by Powell *et al*. (2014) to provide the best estimates of the TMFs for this food chain.

Overall the study is well carried out and the analysis of the data is comprehensive. As with other field studies there are some uncertainties associated with the study (including small sample size and possibility of variable exposure) but the analysis carried out has attempted to minimise these. Nevertheless, it is relevant to note the following points:

- The species sampled covered 1.4 trophic levels, which is smaller than in some of the other studies available, although similar when only fish are considered (for example the Lake Erie study (see below) sampled fish between trophic level 3.1 and 4.2, compared with fish samples between trophic level 3.0 and 4.4 in the Tokyo Bay study).
- The exposure correction was based on data for PCB-180. It is possible that the distribution of D5 throughout the estuary may have been different to that for PCB-180. No detailed analysis of this was given in the paper but, from visual inspection of the sediment data, it would appear that the concentrations of D4 followed a similar pattern to that of PCB-180.
- In principle, "correction" to take account of concentration gradients is a more reasonable approach than assuming homogeneous exposure in such a large water body. However, by necessity this involves data manipulation and further assumptions. For example, fish home range may not be simply related to body size (as was assumed in the study). It is possible that other factors could also have influenced exposure (D4 concentrations in the water column were not measured).
- The choice of a single TMF for the PCB benchmarks directly affects the magnitude of the TMF derived for the substance of interest when the correction is applied. Borgå & Starrfelt (2014) point out that adjusting the enrichment factor only scales the extent to which the estimated TMF deviates from 1; the larger the enrichment factor, the further the TMF will be 'pushed'

[7] Similar observations were made by Kierkegaard *et al*. (2011), who found that D5 was bioaccumulating to a greater extent than PCB-180 in ragworm and Flounder in a UK estuary.

away from 1[8]. The reliability of the selected benchmark TMFs has not been assessed, and the variability in the underlying datasets might be important (e.g. it is possible that other values would be derived if they were corrected for exposure). The apparent differences in bioaccumulation behaviour between D4 and PCB-180 at lower trophic levels cast some doubt as to whether it is an appropriate benchmark. It is not known whether other benchmarks would give different values.

- Borgå & Starrfelt (2014) also observed that the probabilistic method used in the report uses probability distributions for contaminant levels in the different species and uses samples drawn from these distributions to estimate TMFs (instead of using the actual observed data). This approach has the merit of correcting for sampling design (as the approach weights each species equally, which is not usually the case for studies with different number of samples from different species), but also introduces some complicating aspects. In particular, the choice of distribution ignores variability and underestimates uncertainty. Caution is therefore needed when interpreting the reported "confidence bounds", as they may give a false impression of the precision of the TMF estimates.

Overall, the results of this study suggest that the TMF for D4 in this marine pelagic food web was ≤1.

Lake Erie food chain accumulation study

McGoldrick *et al.* (2014) investigated the biomagnification of D4 in the western basin of Lake Erie, Canada. (This study is included in the October 2014 update of the CSRs, and is indicated as 'reliable with restrictions'.) The biota used in the study were collected in the summer/autumn of 2009[9] in the vicinity of Middle Sister Island and included zooplankton, mayflies (*Hexagenia* sp.), Common Shiner (*Luxilus cornutus*), Yellow Perch (*Perca flavescens*), Emerald Shiner (*Notropis atherinoides*), Trout Perch (*Percopsis omiscomaycus*), White Perch (*Morone americana*), Freshwater Drum (*Aplodinotus grunniens*) and Walleye (*Sander vitreus*). The fish were analysed as whole fish samples (Walleye and Freshwater Drum were analysed as individual fish, the other species were analysed as composite samples of between 2 and 60 individuals with each composite being divided into 5 subsamples). Precautions were taken during sampling and analysis to avoid inadvertent contamination of the samples.

The trophic level of each species was determined based on $\delta^{15}N$ measurements, and $\delta^{13}C$ measurements were used to establish the carbon source. The relative contribution of pelagic- and benthic-based carbon to the diet of each species was estimated using a single isotope-two source mixing model. This analysis showed that the fish in the study were predominantly feeding on benthic-based carbon sources but that two of the species, Emerald Shiner and Trout Perch, were feeding on benthic- and pelagic-based carbon sources.

The concentration of D4 measured in each species, along with the assigned trophic levels and lipid contents are summarised in Table A2.5. The TMFs were estimated from the data using the lipid equivalent concentrations and various assumptions over the food

[8] The choice of $\delta^{15}N$ value does not affect whether or not the TMF is above or below 1, because it only affects the size of the slope of the ln [concentration] versus trophic level plot, not whether the slope is positive (TMF >1) or negative (TMF <1).

[9] The samples were frozen immediately in the field and then stored at either -80 °C (zooplankton and benthos) or -20 °C (fish) in the laboratory until processing. The length of storage of the samples prior to processing and analysis is not given.

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web composition. The TMF for D4 was determined to be 0.74 (95% confidence interval 0.39-1.2; probability of TMF >1 15%) when all species were included, 0.73 (95% confidence interval 0.39-1.2; probability of TMF >1 15%) when the zooplankton were excluded and 1.1 (95% confidence interval 0.51-1.9; probability of TMF >1 49%) when both zooplankton and Walleye were excluded.

Table A2.5 Summary of levels of D4 in samples collected from Lake Erie

Species	Estimated diet composition	Number of samples analysed	Mean trophic level (±standard deviation)	Mean lipid content (%)	Mean concentration of D4 (ng/g wet weight) (±standard deviation)
Zooplankton		1	2.0±0.32	0.3	Not detected
Mayfly (*Hexagenia* sp.)		1	2.2±0.08	1.3	7.0
Common Shiner (*Luxilus cornutus*)	13% pelagic – 87% benthic	2	3.1±0.08	3.5	7.9±0.5
Yellow Perch (*Perca flavescens*)	15% pelagic – 85% benthic	5	3.4±0.1	1.6	8.9±1.6
Emerald Shiner (*Notropis atherinoides*)	40% pelagic – 60% benthic	5	3.6±0.07	2.1	12±2.3
Trout Perch (*Percopsis omiscomaycus*)	49% pelagic – 51% benthic	5	3.6±0.08	0.7	12±2.9
White Perch (*Morone americana*)	3% pelagic – 97% benthic	4	3.7±0.05	5.3	13±4.5
Freshwater Drum (*Aplodinotus grunniens*)	28% pelagic – 72% benthic	5	4.0±0.12	3.4	9.6±2.1
Walleye (*Sander vitreus*)	20% pelagic – 80% benthic	15	4.2±0.12	13	13±4.1

The study also included analysis of PCB-180 as a reference substance that is known to bioaccumulate. The TMF derived for this substance was 1.2 when all species were included, 1.7 when mayfly were excluded, 0.55 when zooplankton were excluded, 2.1 when both mayfly and Walleye were excluded and 0.58 when both zooplankton and Walleye were excluded. This suggests that the TMF is dependent on the food web structure.

There are some uncertainties with this study resulting, for example, from the relatively small sample sizes and the inclusion of species with a relatively high contribution from pelagic carbon sources in what was essentially a benthic food web. It is also relevant to note that the recoveries of the ^{13}C-D4 used as analytical standard range from 40% to 117%, were highest for the zooplankton samples and generally decreased as the lipid

content of the fish increased. This may have introduced some bias into the results as the fish at the higher trophic levels generally had higher lipid contents than the fish at lower trophic levels, e.g. the lipid contents for the fish in trophic levels between 3.7 and 4.2 were in the range 3.4 to 13% compared to lipid contents between 0.7% of 3.5% for fish at lower trophic levels. This could potentially lead to an underestimation of the concentrations in fish at the higher trophic levels compared with lower trophic levels.

Borgå & Starrfelt (2014) noted similar caveats about the use of the probabilistic method and estimating fish home range as for the Tokyo Bay study (see above). In addition, they noted that the study suffers from low sample size at base of the food web, with unknown variance. The TMF (both range and mean) is sensitive to the species included in the regression, and the study did not consider the impact of including/excluding species that have a benthipelagic feeding regime (rather than benthic only). Lack of information on lipid normalisation and associated statistics makes it impossible to evaluate the significance of the approach used to take lipid into account.

Overall the results of this study suggest that trophic magnification of D4 was not occurring in this predominantly benthic food chain, although a TMF above 1 was suggested from one of the food web configurations (with a 49% probability that the TMF is above 1 when both zooplankton and a top predator (Walleye) were excluded). PCB-180 (a known bioaccumulative substance) was also found to have a TMF below 1 for some food web configurations.

Lake Ontario study

CES (2014) report the results of a monitoring study for a benthipelagic food web from Lake Ontario, Canada/USA. (This study is not mentioned in the October 2014 update of the CSRs.) This lake has a surface area of 18,960 km^2 and an average depth of 86 meters (maximum depth: 244 meters). The overall aim was to conduct temporal trend analyses to determine if the D4 concentration was stable or changing over a five-year period. However, the report only presents results from one year of sample collection (2011).

Surface sediments were collected using a stainless steel box core from five locations, two within Lake Ontario (deepwater basin) in August 2011 and three within Hamilton Harbor (which receives direct wastewater treatment plant effluent) during December 2011. The upper 5-cm of surface sediment was removed from the box core and sectioned into 1-cm thick strata using a pre-cleaned stainless steel core tube. Each 1-cm stratum was placed into individual polyethylene bags, homogenized in the bag and then transferred to stoppered glass jars and stored at 4 °C until analysis in March 2012. Samples of four fish species and mysid shrimp were collected using a bottom trawl or gill net during August 2011, from eleven locations in an area of roughly 15 x 10 km (the two lake sediments were collected from different locations than the biota). All biota samples were stored at approximately -20 °C in the dark until analysis in July/August 2012. Fish samples were either pooled or analysed individually (Lake Trout only) on a whole fish basis.

D4 concentrations were analysed by GC-MS using solvent standards of hexanes and tetrahydrofuran spiked with various concentrations of D4 and ^{13}D4 as internal standards. The reported limit of detection (LoD) and method detection limit (MDL) for sediment were 1.0 ng and 0.25 ng/g ww, respectively. For biota, the LoD and MDL were 1.5 ng and 0.67 ng/g ww, respectively. Concentrations that were quantifiable but below the defined MDL were considered to be non-detects. Uncensored measured values were reported and used for all calculations.

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A rigorous quality control programme was implemented, including avoidance of personal care products known to contain siloxane materials by personnel during sampling and analysis. All equipment in direct contact with samples was decontaminated by washing with consecutive rinses of hot water (or on-site lake water if this was not feasible while in the field). To determine potential contamination and/or loss of D4 from sample collection, handling and transport to the laboratory, field blanks, reference and control samples were prepared for sediment and fish. For fish, a small but significant difference was observed between D4 concentrations in reference fillets deployed in the bottom trawls versus those that were retained in the laboratory (0.67 ng/g ww). The observed difference was not believed to indicate any significant contamination of samples collected using this methodology. No other quality control samples deployed in the field indicated either loss or contamination of D4. Quality control samples included as part of analytical determinations of D4 concentrations indicated good recoveries, precision, and no contamination. Mean background levels observed in procedural blanks during the study ranged from 2.93 to 10.0 ng. Concentrations of D4 in biota and sediment were subsequently corrected for mean background concentrations.

Sediments collected from within Hamilton Harbor had a mean concentration (3.02 ng/g ww, standard deviation: 2.02 ng/g ww) approximately ten times higher than that of the main lake (0.25 ng/g ww, range: 0.08-0.42 ng/g ww), reflecting the local source of exposure. When normalised to organic carbon content, the mean sediment concentration was 258.6 ng/g in Hamilton Harbor and 27.59 ng/g in the lake.

Concentrations of D4 were quantifiable in all species sampled, with mean fish concentrations ranging from 1.00 to 8.03 ng/g ww (28.4 to 54.2 ng/g lw) (summarised in Table A2.3).

Table A2.3 Summary of levels of D4 in samples collected from Lake Ontario

Species	**Feeding/ trophic guild**	**Number of samples analysed**	**$\delta^{13}C$ value (‰)**	**$\delta^{15}N$ value (‰)**	**Mean lipid content (%)**	**Mean D4 concentration (±standard deviation)[a]**	
						ng/g ww	**ng/g lipid**
Mysid shrimp (*Mysis relicta*)	Benthi-pelagic planktivore	4 pooled samples from 4 locations	-23.56	12.82	7.72	4.60± 3.23	57.8± 36.5
Round Goby (*Neogobius melano-stomus*)	Benthic insectivore	6 pooled samples of 300 small individuals from one location	-21.03	14.44	1.86	1.00± 0.08	54.15± 6.7
		6 pooled samples of 37 moderate-sized individuals from one location	-22.55	14.71	2.30	1.67± 1.64	44.98± 11.25
Rainbow Smelt (*Osmerus mordax*)	Pelagic omnivore	9 pooled samples of 54 individuals from 3 locations	-22.97	15.74	5.61	1.73± 0.25	32.09± 7.34
Alewife (*Alosa pseudo-*	Pelagic carnivore	5 pooled samples of 13 individuals from	-22.80	12.87	5.70	1.64± 0.89	28.35± 10.36

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Species	Feeding/ trophic guild	Number of samples analysed	$\delta^{13}C$ value (‰)	$\delta^{15}N$ value (‰)	Mean lipid content (%)	Mean D4 concentration (±standard deviation)[a]	
						ng/g ww	ng/g lipid
harengus)		4 locations					
Lake Trout (*Salvelinus namaycush*)	Benthi-pelagic piscivore	19 individuals from 4 locations	-21.71	18.10	18.38	8.03± 2.83	43.52± 13.69

Based on known life histories, the biota samples covered three separate feeding guilds and five separate trophic guilds. In addition, the mean $\delta^{15}N$ of each species (an indicator of trophic level) ranged from 12.82‰ to 18.10‰, suggesting that a range of trophic levels was sampled. Mean $\delta^{13}C$ values were -21.03‰ to -23.56‰, suggesting that all species were part of a related food web. The study authors therefore considered that the species sampled were representative of the aquatic food web of Lake Ontario.

The report does not provide any analysis of trophic magnification or other measures of bioaccumulation potential. The mean organic carbon-normalised sediment concentration from the main lake (27.59 ng/g) could be used to estimate BSAFs but as the biota were not sampled from the same locations and there was a large variation in concentration between the two sediment sampling sites, such a calculation is not useful. Individual feeding relationships could indicate BMFs above 1 (e.g. BMF = 1.4 and 1.5 for the Lake Trout-Rainbow Smelt and Lake Trout-Alewife feeding relationships, respectively, on a lipid weight basis), but there is significant overlap in concentration between species.

CES (personal communication, 25 April 2014) stated that the results of this study are confounded by variable exposure, concentration gradients and "dietary switches resulting from invasive species", which casts some doubt on the usefulness of the data. Nevertheless, the same source provides an assessment of TMF in the Lake Ontario food web based on samples collected in November 2011 (i.e. not those presented in Table A2.3), although the original data are not provided. Trophic level (TL) was calculated using an assumed $\Delta^{15}N$ enrichment factor of 3.40‰ TL^{-1}. The evaluated food web consisted of mysid shrimp (TL=3.0), Alewife (TL=3.1), small 'goby' (TL=3.5), large 'goby' (TL=3.7), Rainbow Smelt (TL=3.9), and Lake Trout (TL=4.6). The resulting TMF based on log (mean lipid weight concentrations) was stated to be 1.0 (r^2=0.5%; standard error=0.038; p=0.62). The positioning of Alewife below Round Goby is somewhat surprising given its expected diet of mysids and small fish (whereas Round Goby eats aquatic insects and molluscs). In addition, the influence of any overlap in concentration amongst the species is not discussed.

However, CES (personal communication, 25 April 2014) goes on to state that the TMF is "anomalous" if not corrected for exposure, presumably on the basis of lower than expected TMFs derived for PCB-180 and -153 in the same food chain (1.8 and 1.5, respectively). As for the Tokyo Bay study reported above, additional calculations were therefore performed, to both 'benchmark' against PCB-180 (median benchmark TMF=4.0, relative trophic levels based on a ^{15}N enrichment factor of 3.31) and "correct" for exposure across concentration gradients (by estimating BSAFs; the data are not presented, but it is stated that BSAFs for Lake Trout and 'goby' were based on measured concentrations in sediment collected from the Niagara Delta (mid-water, near shore location; TOC=0.95% ww), and BSAFs for the other species were based on measured concentrations in sediment collected from the Niagara Basin (deep-water, offshore location; TOC=0.86% ww)). Using the 'concentration gradient-correction' approach, the

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TMF remains as 1.0 (with a 95% confidence interval of 0.6-1.7, and a 49% probability that the TMF exceeds 1). However, benchmarking against PCB-180 gave a TMF of 0.7 (r^2=28%; standard error=0.039; $p<0.01$), or 0.6 (95% confidence interval of 0.3-1.1) when 'concentration gradient-correction' was applied. As noted under the commentary on the Tokyo Bay study above, the relevance of these corrections is questionable (and the BSAFs may or may not be appropriate, depending on the sampling locations, variation in sediment concentrations, and whether the biota concentrations are linked to sediment sampled at any one particular site, especially for widely foraging species).

Overall the CES (2014) study is well carried out, but as with other field studies there are a number of uncertainties that limit its usefulness for assessing bioaccumulation potential (including small sample sizes and possibility of variable exposure). The contamination of the control fish sample is also of some concern given the relatively low concentrations that were measured for some species. Nevertheless, the results of the follow-up study mentioned in CES (personal communication, 25 April 2014) suggest that the TMF for D4 in this freshwater benthi-pelagic food web was ≤1.

Comparison of field studies

Powell *et al.* (2014) carried out a comparison of the TMFs derived for cVMS from the various studies. (This study is not mentioned in the October 2014 update of the CSRs, although the broad principles are included.) This included recalculation of the TMF for the food chain using the probabilistic approach with a $\Delta^{15}N$ of 3.4‰ TL^{-1} and species-specific probability density functions for $\delta^{15}N$ and the lipid-normalised concentrations defined by the means and standard deviations reported in each study. The probabilistic approach was considered by Powell *et al*. (2014) to be the most appropriate method of analysing the data to minimise bias resulting from experimental design. The results of this analysis for D4 are summarised in Table A2.7. The analysis did not consider the data from Lake Opeongo, Lake Champlain or Lake Ontario.

Table A2.7 Summary of TMFs derived for D4 in field studies (based on Powell *et al*., 2014)

Location	Food web	Range of trophic levels covered by the food chain	Median TMF (95% confidence interval given in brackets)
Tokyo Bay	Pelagic – marine	3.0-4.4	0.6 (0.5-0.8)[a]
Inner Oslofjord	Benthic – marine	1.5-4.0	0.6 (0.5-0.8)
	Pelagic – marine		0.7 (0.4-1.0)
Outer Oslofjord	Benthic – marine	2.1-4.1	0.7 (0.5-1.0)
	Pelagic – marine		1.0 (0.6-1.4)
Lake Pepin	Benthic – freshwater	2.0-3.8	0.5 (0.3-0.7)
Lake Mjøsa	Pelagic – freshwater	2.0-4.2	1.3 (0.8-2.1)
	Pelagic – freshwater	2.0-4.4	0.8 (0.5-1.1)
Lake	Pelagic –	2.0-3.8	0.6 (0.3-1.1)

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Location	Food web	Range of trophic levels covered by the food chain	Median TMF (95% confidence interval given in brackets)
Ransfjord	freshwater		
Lake Erie	Benthic and pelagic – freshwater	2.0-4.2	0.6 (0.4-0.9)

Note: a) An earlier unpublished preliminary study of Tokyo Bay suggested a TMF of 0.4-0.6 for D4.

Based on this analysis, median TMFs for D4 are in the range 0.5-1.3, and a TMF >1 is only derived for one of the two studies in Lake Mjøsa (although a TMF of 1 is indicated in the Outer Oslofjord, and the confidence interval includes a TMF > 1 at this site, Lake Mjøsa and Lake Randsfjorden). A significant number of the measured concentrations in the Lake Mjøsa studies were below the limit of quantification which may have introduced some uncertainty into the analysis.

These findings were considered further by Powell *et al*. (2014). With the exception of the first study in Lake Mjøsa, the probabilistic TMFs were not significantly different in benthic food webs compared with pelagic food webs for D4. Powell *et al*. (2014) considered that the findings in the Norwegian lakes may be related to variable exposure resulting from non-uniform migration patterns of some species and food web dynamics. Powell *et al*. (2014) noted that the range of $\delta^{13}C$ across the food web was larger in both Lake Mjøsa and Lake Randsfjorden than in other study areas suggesting that omnivorous feeding by consumers may have occurred or that samples were inadvertently collected from trophically distinct food webs. In addition Powell *et al.* (2014) considered that variable exposure resulting from concentration gradients may be a confounding factor in these studies (as is potentially a case with most studies).

It is relevant to note that this paper was attempting to find scientific explanations for the difference between the TMF found in Lakes Mjøsa and Randsfjorden and the other studies, and so concentrated on the potential uncertainties in the Norwegian study. However, there are potential uncertainties with all of the other field studies and these were not discussed in the same level of detail. Overall, although the concerns raised by Powell *et al.* (2014) are reasonable, it is not currently possible to assess the significance of the various uncertainties on the TMFs derived in Lake Mjøsa and Lake Randsfjorden.

Comparison of laboratory bioconcentration data between substances

Table A2.8 compares the available fish laboratory bioconcentration data for D4 and D5 with substances that are agreed to meet the vB criterion following submission of Annex XV dossiers to the Member State Committee[10]. Wet weight whole fish concentrations have been estimated from the cited BCF and aqueous exposure concentrations (unless otherwise stated), and do not take account of lipid content. Polyaromatic hydrocarbons other than anthracene have not been considered for the purpose of this exercise.

Table A2.8 Summary of BCF data for vB substances

Substance	CAS No.	BCF, L/kg	Maximum fish conc., mg/kg ww	Comment	Reference

[10] Comparisons of concentrations actually measured in wildlife have not been included because of the size of the task and variability of use patterns and quantities leading to very different exposures.

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Substance	CAS No.	BCF, L/kg	Maximum fish conc., mg/kg ww	Comment	Reference
Anthracene	120-12-7	>6,000	-	Exposure concentrations are not stated so whole fish concentrations cannot be derived.	EC (2008b)
Alkanes, C_{10-13}, chloro (short chain chlorinated paraffins)	85535-84-8	ca. 7,273	ca. 240	Data are for a C_{10-12} 58% wt Cl substance based on parent compound analysis. Fish lipid content not stated.	ECHA (2008b)
2-(2H-Benzotriazol-2-yl)-4,6-di-*tert*-pentylphenol (UV-328)	25973-55-1	4,590	0.4	Based on average BCF at study end. Fish lipid content 4.2%.	UBA (2014a)
2-Benzotriazol-2-yl-4,6-di-*tert*-butylphenol (UV-320)	3846-71-7	9,265	0.9	Fish lipid content 3.6%.	UBA (2014b)
5-*tert*-Butyl-2,4,6-trinitro-m-xylene (musk xylene)	81-15-2	3,730 and 10,500	9.9 and 33 (estimated)	Steady state not reached – plateau fish concentrations were estimated using a one-compartment model. Fish lipid content 3.4%. Another study resulted in slightly lower fish concentrations (but still >1 mg/kg).	ECHA (2008c)
Hexabromocyclo-dodecane (HBCDD)	25637-99-4	18,100 and 13,085	110 and 4.4	Fish lipid content not specified.	ECHA (2008a)
Henicosafluoro-undecanoic acid	2058-94-8	ca. 2,700 and 3,700	ca. 1.3 and 0.4	BCF in first study based on carcass only. Lipid normalisation not appropriate.	ECHA (2012b)
Pentacosafluoro-tridecanoic acid	72629-94-8	ca. 18,000 and ca. 13,000	ca. 3.6 and ca. 1.3	BCF in first study based on carcass only; second study cited as a BCF range so estimated fish concentration is based on the average. Lipid normalisation not appropriate.	ECHA (2012c)
Heptacosafluoro-tetradecanoic acid	376-06-7	ca. 23,000 and ca. 16,500	ca. 0.3 and ca. 1.6	BCF in first study based on carcass only; second study cited as a BCF range so estimated fish concentration is based on the average. Lipid normalisation not appropriate.	ECHA (2012d)
Octamethylcyclo-tetrasiloxane (D4)	556-67-2	≥11,495	≥2.6	Fish lipid content 6.4%.	EA (2009a)
Decamethylcyclo-pentasiloxane (D5)	541-02-6	≥5,860 and ca. 12,600	≥24 and ≥13	In the first study, fish lipid content varied from 2.9 to 4.1% during the uptake phase. In the second study, the variation was less and the mean lipid content was 5.71%.	EA (2009b) and EA (2014)
Pentabromo-diphenyl ether	32534-81-9	PentaBDE ca. 17,700 HexaBDE ca. 5,640	PentaBDE ca. 42 HexaBDE ca. 1.4	The analysis is complicated because several congeners were tested at the same time, and some corrections have to be made to the data. The cited data are for one pentaBDE and one hexaBDE constituent, respectively. Fish lipid content was 4.8%.	EC (2001)

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Whole fish concentrations associated with a high BCF depend on the dissolved concentration achieved in the experiment as well as (usually) the size and lipid content of the test organisms, species-specific factors (such as metabolism, which may change with life stage), and growth dilution, etc. Comparisons between studies using the same substance can therefore be complicated, and comparisons between substances should be treated with caution. Nevertheless, it can be seen that substances with vB properties can generally achieve whole fish concentrations in the laboratory in the range of 0.9 – ca. 50 mg/kg ww, with only one substance below this range[11]. A benchmark of 1 mg/kg ww might therefore be suitable as an indicator of high bioaccumulation potential.

The maximum whole fish concentrations for both D4 and D5 exceed 1 mg/kg ww, and so are comparable to substances such as UV-328 and UV-320, long chain perfluorocarboxylic acids, musk xylene, hexaBDE and HBCDD. Molar concentration is inversely proportional to the molecular weight (MW). The MW of D4 (297 g/mole) and D5 (371 g/mole) are lower than some of these substances (e.g. henicosafluoroundecanoic acid, 564 g/mole; HBCDD, 642 g/mole), so there will be more D4/D5 molecules present in the fish compared to these substances when concentrations are the same.

A similar comparative exercise could be performed for dietary bioaccumulation tests, but this has not been done for the purposes of this evaluation.

[11] In terms of the PBT concept, bioaccumulation concerns are linked to the potential for a substance to reach a toxic threshold in species that have not been tested in the laboratory. It is perhaps open to question whether substances achieving concentrations at the lower end of this range should be considered to be as hazardous as those at the upper end (two orders of magnitude higher), but this will also depend on factors such as molecular weight (i.e. the number of molecules present in the fish) and mode of any toxic action. In addition, this brief analysis shows that additional studies might highlight higher concentrations.