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**Brief Report**

# Permeability of the fish intestinal membrane to bulky chemicals

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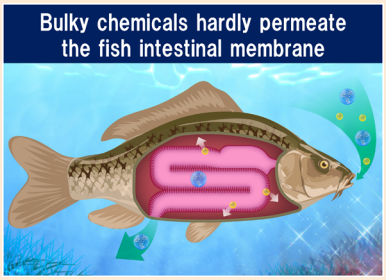
The ability to predict the environmental behavior of chemicals precisely is important for realizing more rational regulation. In this study, the bioaccumulation of nine chemicals of different molecular weights absorbed *via* the intestinal tract was evaluated in fish using the everted gut sac method. The amounts of chemicals that passed through the intestinal membrane after a 24-hr exposure were significantly decreased for chemicals with MW≥548 and $D_{max\ min}$≥15.8 Å (or $D_{max\ aver}$≥17.2 Å). These thresholds are consistent with those previously proposed in terms of MW (>800) and molecular size ($D_{max\ min}$>15.6 Å or $D_{max\ aver}$>17.1 Å) for the limit of permeable chemicals through the gill membrane. The results show that the same MW and $D_{max}$ criteria can be used to predict low bioaccumulation through both the gill membrane and the intestinal tract. These findings are helpful in reducing the need to conduct animal tests in environmental safety studies.




***Keywords:*** permeability, fish intestinal membrane, everted gut sac method, bioaccumulation.

## Introduction

Persistent, bioaccumulative, and toxic chemicals (PBTs), or persistent organic pollutants (POPs), are subject to control under various national and international regulatory frameworks, including the Stockholm Convention.[1,2] Bioaccumulation through food webs is the most critical issue for higher-order predators such as humans and large environmental organisms. Generally, the bioaccumulation potential of a chemical in fish has been evaluated by determining the ratio of the steady-state concentration of the test compound in fish to that in water (*i.e.*, bioconcentration factor, BCF) after the chemical has been absorbed *via* the gills over a specified period of time.[3,4] While a large number of such "bioconcentration tests by aqueous exposure" have already been conducted, based on the analysis of experimental physicochemical data, there have also been attempts to predict the bioaccumulation potential of chemicals without recourse to animal tests. One of the basic principles underlying this initiative is that bulky molecules cannot pass through the gill membrane.[5–8] For example, chemicals with a molecular weight (MW) >800 are regarded as having low bioaccumulation potential, and bioconcentration tests do not need to be conducted in Japan according to the Chemical Substances Control Law (CSCL).[2,9,10] Based on our previous results, we proposed that chemicals with $D_{max\ min}$>15.6 Å or $D_{max\ aver}$>17.1 Å have low bioaccumulation potential,[11] where $D_{max\ min}$ and $D_{max\ aver}$ are the minimum and average diameter, respectively, of the smallest spheres accommodating the locally stable conformers in water as calculated by molecular dynamic (MD) simulation.

Fish usually absorb chemicals contained in water through the gill membrane. However, in the environmental food web, aquatic and terrestrial organisms often absorb chemicals through the digestive organs from the food they ingest. In order to evaluate the latter, the dietary bioaccumulation test in the Organisation for Economic Co-operation and Development (OECD) 305 guideline revised in 2012, which was originally designed to test for chemicals that are poorly soluble in water, is applicable.[3] In this new *in vivo* test, a diet containing the test chemical is fed to fish over a specified period of time. Then the ratio of steady-state concentration of the test chemical in fish to that in the diet is determined as the biomagnification factor (BMF). However, after this new test was approved under the CSCL in 2018,[4] it was revealed that its implementation is accompanied by considerable

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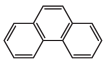
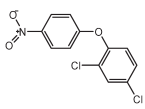
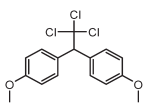
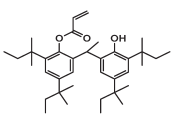
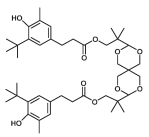
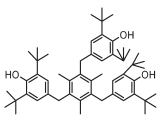
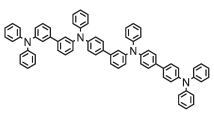
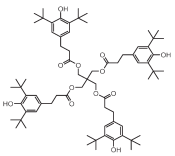
difficulty due to issues of accuracy. One of the most challenging of these is feeding a specified amount of the diet to each fish, because the available food is monopolized by stronger fish at the expense of weaker fish.[12] This variability limits the reliability of the test results, and hence, the effect of molecular properties on how readily molecules can pass through the intestinal membrane remains unclear.

Technically, it is difficult to accurately determine how much of a substance passes through the lumen of the intact intestinal tract into the bloodstream in an *in vivo* study. To overcome this problem, we adopted the *in vitro* everted gut sac method[13] for the current study to determine the MW or size threshold, thus making it possible to screen for chemicals that can pass through the intestinal membrane but have low bioaccumulation potential. The intestinal tract was turned inside out and immersed in a buffer solution containing the test chemical, and the amount of chemical that passed through the membrane into the tract was quantitated. This method has been used for rats, mice, rabbits, sheep, chickens, turtles, pigs, and frogs[14–17] but has been used hardly at all for fish.[18,19] Apart from the everted gut sac method, a vertical or horizontal glass-diffusion cell system can also be used to measure the permeability of biological membranes.[20,21]

**Table 1.** Test chemicals and their bioaccumulation potential

| Test chemical | | | Molecular weight | log $K_{ow}$ | Water solubility [mg/L] | Bioaccumulation potential | $D_{max\ min}$ [Å] | $D_{max\ aver}$ [Å] |
|---|---|---|---|---|---|---|---|---|
| No. | Name (CAS No.) | Structure | | | | | | |
| **1** | Phenanthrene (85-01-8) |  | 178 | 4.46[a] | 1.15[a] | — | 11.1 | 11.6 |
| **2** | o-Terphenyl (84-15-1) |  | 230 | 5.52[a] | 1.24[a] | BCF=1400[29] BMF[d]=0.0912[4] | 11.8 | 12.1 |
| **3** | Nitrofen (1836-75-5) |  | 284 | 4.64[a] | 1.0[a] | BCF=3400[29] BMF[d]=0.179[4] | 13.0 | 14.0 |
| **4** | Methoxychlor (72-43-5) |  | 345 | 5.08[a] | 0.1[a] | BCF=620[29] BMF[d]=0.0340[4] | 13.9 | 15.1 |
| **5** | Sumilizer GS (123968-25-2) |  | 548 | 12.4[b] | <0.005[c] | BCF<78[29] | 15.8 | 17.2 |
| **6** | Sumilizer GA-80 (90498-90-1) |  | 741 | 10.5[b] | <0.004[c] | BCF<6.6[29] | 16.8 | 23.3 |
| **7** | Irganox 1330 (1709-70-2) |  | 775 | 17.2[b] | 0.008[c] | — | 20.0 | 20.9 |
| **8** | *N*,*N*′-diphenyl-*N*,*N*′-bis[4′-(diphenylamino)-biphenyl-4-yl]benzidine (167218-46-4) |  | 975 | 19.5[b] | $9.75\times10^{-7}$[b] | — | 29.3 | 34.9 |
| **9** | Pentaerythritol tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (6683-19-8) |  | 1178 | 11.5[b] | <0.1[c] | — | 21.0 | 23.8 |

[a] Measured value from experimental database of log $K_{ow}$ or water solubility in EPI Suite™ ver. 4.11. [b] Estimated by EPI Suite™ ver. 4.11. [c] Measured value from REACH registered substances database. [d] Corrected by lipid content and growth rate

However, the surface area of the membranes in these systems was generally too small to allow the concentration of the membrane-permeating hydrophobic compounds to be quantified. Therefore, using the everted gut sac method, we investigated the intestinal permeation of nine chemicals with different MWs in the common carp and discussed the relationship between permeation and the MWs or sizes of the chemicals.

## Materials and methods

### 1. Chemicals

The nine chemicals tested in this study are shown in Table 1 along with information on their bioaccumulation potential. These chemicals are not proteins,[22,23] amino acids,[22,23] perfluoro alkyls,[24–26] or metals,[27,28] which are known to be taken up by an active mechanism or *via* a binding protein, but aromatic hydrocarbons including alcohols, halides, esters, and amides. According to the National Institute of Technology and Evaluation (NITE),[22,23] these aromatic hydrocarbons pass through the biomembrane primarily by passive diffusion. Some BMF values measured in the dietary bioaccumulation test are available in the CSCL guideline,[4] and several BCF values measured in bioconcentration studies are available on the Chemical Risk Information Platform developed by NITE.[29] When information on the measured BCF was not available, the BCF values were estimated using EPI Suite™ ver. 4.11, developed by the US Environmental Protection Agency and Syracuse Research Corp.

Phenanthrene (**1**) and nitrofen (**3**) were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan); *o*-terphenyl (**2**) and Irganox 1330 (**7**) were purchased from Sigma-Aldrich (St. Louis, MO, USA); and methoxychlor (**4**), *N*,*N*′-diphenyl-*N*,*N*′-bis[4′-(diphenylamino)biphenyl-4-yl]benzidine (**8**), and pentaerythritol tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-propionate] (**9**) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Sumilizer GS (**5**) and Sumilizer GA-80 (**6**) were provided by Sumitomo Chemical (Tokyo, Japan). Phosphate-buffered saline (PBS) tablets (pH 7.4) were purchased from Takara Bio (Shiga, Japan). Distilled water (HPLC grade), formic acid, acetonitrile (HPLC grade), tetrahydrofuran (THF, stabilizer free, special grade), and ammonium acetate (Guaranteed Reagent), which were used for chemical analysis, were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

### 2. Everted gut sac method

Common carp (*Cyprinus carpio*) provided by the Kitamura Fish Farm (Yashiro, Japan) were acclimated at 23°C in dechlorinated tap water under aeration for at least 1 week. After immersing the carp (weight: 300 to 500 g; body length: 27 to 32 cm) in ice water for about 1 hr, the intestinal tract was excised, and a section from 5 cm distal to the stomach to 1 cm proximal to the anus was removed. The length of the intestinal tract ranged from 24 to 39 cm. The inside and outside of the intestinal tract were gently washed with a phosphate buffer prepared using PBS tablets. Figure 1A shows how the gut sac was everted. The intestinal tract proximal to the anus was tightly fixed to a polyethylene tube (about 15 cm long, 4 mm outer diameter) with a Teflon thread (Fig. 1A-1). The gut sac was everted inside out by pushing the polyethylene tube in the direction of the stomach (Fig. 1A-2). After eversion of the gut sac, the portion of the intestinal tract fixed to the polyethylene tube with the thread was cut (Fig. 1A-3), and the tube was removed from the tract (Fig. 1A-4). Finally, the length of the everted gut sac was adjusted so that it was 20 to 25 cm long, and both ends were tightly bound to two plastic straws (10 cm long, 4 mm outer diameter) with Teflon threads.

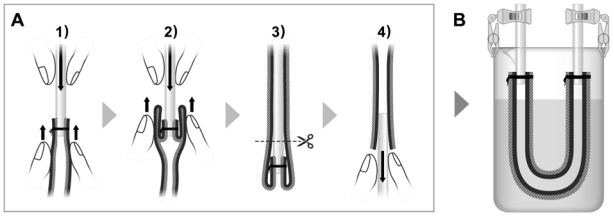



**Fig. 1.** Illustration of the everted gut sac method. Procedure for everting the intestinal membrane (A) and the experimental setup for measuring the permeability of the membrane by chemicals (B).

A tall beaker was filled with 500 mL of a phosphate buffer containing the test chemical at a concentration of 500 mg/L for **1** or 100 mg/L for **2** to **9** (*i.e.*, outer solution). As the water solubility of all compounds (Table 1) was below these nominal concentration values, the actual concentrations in the outer solutions were likely maintained at their saturated level. The two plastic straws from which the everted intestine was hanging were clipped to the edge of the tall beaker, and the inside of the intestinal tract was filled with a few milliliters of a phosphate buffer (*i.e.*, inner solution) (Fig. 1B). After gently stirring the outer solution at 23°C for 24 hr with a magnetic stirrer at 150 rpm, the test chemicals in the inner and outer solutions were determined. Prior to definitive studies, preliminary exposure to each chemical was conducted in singlicate. When no test chemical was detected in the inner solution, a definitive study was conducted in duplicate. Otherwise, the definitive study was conducted in quadruplicate.

### 3. Analytical method

To analyze the test chemicals in the outer solution, acetonitrile was added to 2 mL of the outer solution to make 5 mL for **1**. Insoluble materials in this solution (1 mL) were removed by centrifugal filtration (Millipore Ultrafree-MC, HV, 0.45 $\mu$m, Merck, NJ, USA), and the filtrate was further centrifuged at 5,000 rpm for 1 min. The supernatant was subject to HPLC analysis with fluorescence detection. For **2**, **3**, and **4**, all of the outer solutions were collected, and the inside wall of the beaker was washed three times with 150 mL of methanol. Methanol was added to the combined solution to make 1 L. Ten mL of this solution was extracted twice with 2 mL of *n*-hexane. The *n*-hexane layers were combined and brought up to 5 mL with *n*-hexane, which was submitted to GC-MS analysis. Compounds **5** to **9** were analyzed using the same procedure, except THF was used as the solvent without *n*-hexane extraction. Additional THF was added to the

combined THF solution to bring the volume up to 1 L, which was submitted to LC-MS/MS analysis. The spike and recovery tests were conducted in duplicate for each chemical. The average recovery rates ranged from 97.0% for **7** to 103% for **3**, and the lowest recovery rate was 95.7% for **7**.

To analyze **1**, **5**, or **6** in the inner solutions, the sample was first diluted 1.5-fold with acetonitrile, and about 1 mL of the diluted solution was centrifuged at 13,000 rpm for 5 min. Compound **1** was quantified by HPLC using fluorescence detection, and **5** and **6** were quantified by LC-MS/MS. For **2** to **4**, a portion of the inner solution was mixed with 1 mL of *n*-hexane. The mixed solution was shaken by a mechanical shaker (SR-2DW, TAITEC, Aichi, Japan) for 10 min and centrifuged at 3,000 rpm for 5 min. The supernatant was collected and the remaining residue extracted with 1 mL of *n*-hexane three times, and *n*-hexane was added to the combined *n*-hexane layers to make 5 mL for GC-MS analysis. For **7** to **9**, the same procedure was performed to prepare the combined *n*-hexane layers, in which 10 $\mu$L of diethylene glycol was added as a stabilizing agent. Then the *n*-hexane solution was evaporated, and the residue was dissolved in 1 mL of THF for LC-MS/MS analysis. For the inner solutions, the average recovery rates in duplicate ranged from 88.7% for **7** to 96.2% for **8**, and the lowest recovery rate was 87.3% for **7**.

The intestinal tract was cut into 1 cm squares with scissors and homogenized with 3 mL of a phosphate buffer by ShakeMaster®NEO (Bio Medical Science, Tokyo, Japan) at 1,500 rpm for 6 min. To extract the test chemicals, the homogenate was shaken with 5 mL of acetonitrile for **1**, *n*-hexane for **2** to **4**, and THF for **5** to **9** for 10 min, and the extract was centrifuged at 7,000 rpm for 10 min. The extraction procedures were repeated three times, and the same solvent was added to the combined extracts to make 20 mL. For **1** and **5** to **9**, about 1 mL of the solution was centrifuged at 5,000 rpm for 1 min, and the supernatant was submitted to HPLC analysis with fluorescence detection for **1** and LC-MS/MS analysis for **5** to **9**. For **2** to **4**, the solution was filtered through a C18 cartridge (InertSep C18 50 mg/1 mL, GL Sciences, Tokyo, Japan), and *n*-hexane was added to make 5 mL for GC-MS analysis. The average recovery rates in triplicate ranged from 78.7% for **9** to 95.3% for **7**, the lowest recovery rate was 75.1% for **9**, and the maximum standard deviation was 4.8% for **7**.

*4. Instrumental analysis*

HPLC was performed using a Shimadzu LC-10A system equipped with a fluorescence detector and LabSolutions software (Shimadzu, Kyoto, Japan). L-column ODS (150×4.6 mm, 5 $\mu$m; CERI, Fukuoka, Japan) was used and eluted with a mixture of distilled water (A) and acetonitrile (B) at a flow rate of 1.0 mL/min at 40°C. The gradient program for the mobile phase B in A was set as follows: 0 to 12 min, linear gradient from 20 to 100%; 12 to 20 min, 100%; 20 to 35 min, 20%. The injection volume and the excitation/fluorescence wavelengths were fixed at 10 $\mu$L and 250/350 nm, respectively.

GC-MS was performed using an Agilent 8890 GC and a 5977B GC/MSD System in positive ion electron impact mode. A J&W HP-5ms capillary column (30 m, 0.25 mm ID, film thickness 0.25 $\mu$m; Agilent Technologies, CA, USA) was used. The carrier gas was helium at a flow rate of 1 mL/min. The oven temperature was set at 50°C and raised to 300°C at a rate of 12.5°C/min. The injector was operated at 250°C in splitless mode. Analysis of **2** to **4** was carried out at *m*/*z* 230, 283, and 227, respectively, in selected ion monitoring mode.

LC-MS/MS was performed using a Q Exactive Focus system (Thermo Fisher Scientific, MA, USA) equipped with an electrospray ionization source in positive ionization mode. The source and vaporization temperatures were set at 350 and 300°C, respectively. L-column3 ODS (150 mm×2.1 mm, 5 $\mu$m; CERI, Japan) was used for LC, which was eluted with a mixture of distilled water containing 5 mM ammonium acetate (A) and a mixture of methanol, acetonitrile, and THF (1/2/2, v/v/v) containing 5 mM ammonium acetate (B) at a flow rate of 0.2 mL/min at 40°C. For **5** and **6**, the gradient program for the mobile phase B was set as follows: 0 to 20 min, linear gradient from 10 to 90%; 20 to 40 min, 90%; 40 to 60 min, 10%. For **7** to **9**, the gradient program for the mobile phase B was set as follows: 0 to 10 min, linear gradient from 10 to 80%; 10 to 20 min, from 80 to 95%; 20 to 35 min, 95%, 35 to 60 min, 10%. The injection volume was 10 $\mu$L. The analytical parameters for the mass module controlled by Xcalibur software (version 4.4) were as follows: sweep gas flow 0 arbitrary units (a.u.), sheath gas flow 40 a.u., and auxiliary gas flow 10 a.u. for **5** and **6**, and sweep gas flow 2 a.u., sheath gas flow 48 a.u., and auxiliary gas flow 10 a.u. for **7** to **9**. While **8** was analyzed in SIM mode with a target ion *m*/*z* of 974.4, the other chemicals were analyzed in SRM mode: precursor and product ions with their respective collision energies were *m*/*z* 548.0 and 233.2, 287.3, 494.0 with 30 eV for **5**; *m*/*z* 739.0 and 163.0, 521.1, 563.0 with 45 eV for **6**; *m*/*z* 792.6 and 569.4, 219.2 with 31 eV for **7**; *m*/*z* 1194.8 and 788.0, 731.0, 729.1, 219.2 with 40 eV for **9**.

*5. Molecular size in water*

The $D_{max}$ values of **1** to **9** were determined *via* MD simulation using Material Studio 2018 (BIOVIA, USA). Each chemical was set in a 39.1 Å virtual cube filled with 2,000 water molecules at a density of 1.0 g/cm$^3$. The MD simulation was performed for up to 500 ps with a time step of 1.0 fs using an NTP ensemble. After the simulation, the three-dimensional coordinates of conformers were sampled every 1 ps, and the diameters of the smallest spheres ($D_{max}$) accommodating the conformers were calculated in an original Excel program using Visual Basic Application. The $D_{max\ min}$ and $D_{max\ aver}$ were determined as the minimum value and arithmetic mean, respectively, of all $D_{max}$ values for each chemical. The calculation conditions have been described in detail in a previous report.[11)]

## Results

Figure 2 shows the concentrations of the nine test chemicals in the outer (black bar) and inner solutions (gray bar) of the gut sac measured after a 24-hr exposure, along with the MWs of the

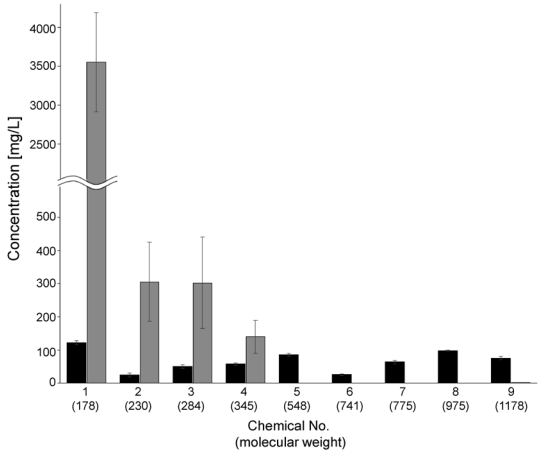



**Fig. 2.** Concentrations of test chemicals in the outer (black bar) and inner (gray bar) solutions in the everted gut sac system after 24-hr exposure with standard deviations (thin line). Numbers in parentheses are the molecular weights of the test chemicals.

chemicals (figures in parentheses). Each set of data was calculated from multiple replicates with the standard deviations (thin line) (*i.e.*, $n=4$ for **1** to **4** and **9**, and $n=2$ for **5** to **8**). The concentrations of **1** to **4**, with MW≤345, in the inner solutions were 3550±639, 305±120, 303±134, and 140±49 mg/L, respectively, being significantly higher than those in the outer solutions (122±5.8, 24.8±6.2, 50.4±5.4, and 57.6±4.0 mg/L, respectively). In contrast, all concentrations of **5** to **8**, with MW≥548, in the inner solutions were below the quantification limits—0.06, 0.1, 0.08, and 0.1 mg/L, respectively—and the concentration of **9**, with MW 1178, in the inner solution was 0.3±0.2 mg/L. The concentrations of **5** to **9** in the outer solutions were 85.8±4.2, 25.9±1.8, 63.7±5.1, 97.7±1.5, and 75.7±5.1 mg/L, respectively. These findings indicate that barely any of **5** to **9** with MW≥548 was able to pass through the intestinal membrane even though the concentrations in the outer solutions were high.

Figure 3 shows the average amount of each chemical as a percentage (%) of the total amount added to each experimental system in the outer solution (black bar), in the inner solution (gray bar), and in the intestinal membrane (white bar). Compounds **1** to **4** showed high permeation, and relatively large amounts were detected in the intestinal membrane, ranging from 21.7 to 34.3%. In contrast, less than 2.6% of compounds **5** to **9**, which are barely able to permeate the intestinal membrane, were detected in the intestinal membrane. The material balances of **1** to **9** respectively calculated based on the sum of the amounts of chemicals in the inner solutions, the outer solutions, and the intestinal tissues at the end of the experiments ranged from 47.3 to 100% of the amount applied. For **1**, **2**, and **6** in particular, the material balance was less than 60%.

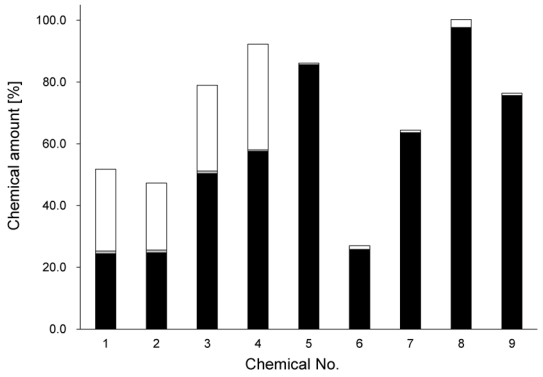



**Fig. 3.** Distribution of test chemicals in the outer solution (black bar), the inner solution (gray bar), and the intestinal membrane (white bar). The values are expressed as a percentage (%) of the total amount of each chemical applied to the experimental system.

## Discussion

*1. Permeation of the intestinal membrane*

The concentrations of **1** to **4** in the inner solutions were higher than those in the outer solutions (Fig. 2); relatively large amounts were detected in the intestinal membrane (Fig. 3). The high concentrations in the inner solutions may have been due to the presence of a large amount of tiny insoluble materials from the visceral tissues that could be visually observed. Tested compounds are not expected to be absorbed by active transport but by passive diffusion, as proposed by NITE.[22,23] Thus, it seems probable that four of the test chemicals (**1** to **4**) passed through into the inner solutions and were adsorbed in part onto the insoluble materials that were removed from the equilibrium system. Since the chemicals in the inner solutions were quantitated as the sum of freely dissolved chemicals and those adsorbed onto the insoluble materials, the apparent concentrations in the inner solutions could have become higher. The insoluble materials are also likely to cause the relatively large concentration fluctuations in the inner solutions because their amounts could not be controlled. Whatever the case, it was evident that a significant amount of **1** to **4** with MW≤345 passed through intestinal membrane. On the other hand, compounds **5** to **9** with MW≥548 were barely able to permeate the intestinal membrane. The low material balances of **1**, **2**, and **6** in Fig. 3 were likely caused by rapid metabolism, since their biotransformation half-lives in fish calculated by EPI Suite™ ver. 4.11 (Environmental Protection Agency and Syracuse Research Corp, USA) were less than 10 days (*i.e.*, 2.6, 6.4, and 0.28 days for **1**, **2**, and **6**, respectively). Although the everted gut sac method can be used to evaluate the permeation of biotransformed compounds in the system, the focus of this study was on the ability of untransformed compounds to permeate the intestinal membrane.

The relationship between MW and membrane permeation has also been examined using other *in vitro* experimental systems. In the case of a Caco-2 membrane, the molecular cutoff values were reported as MW 600 or 700 for a compound set consisting primarily of drugs.[25,26] In the case of a parallel artificial membrane permeability assay (PAMPA), it was reported that passive permeability began to reduce from MW>800 and was severely limited for cyclic peptides having MW>1,000.[27] These results are compatible with the permeability of the fish intestinal mem-

brane examined in this study. In addition to the everted gut sac method, the diffusion cell method is able to measure the permeability of biological membranes. The results obtained in this study for the fish intestinal membrane are comparable to those obtained using artificial membranes.

Finally, these results were compared with the experimentally determined BMF and BCF values. The BMF values of **2** to **4** (Table 1) suggest their potential for uptake through the intestinal tract. The results showed that the *in vitro* tests using the gut sac could provide the same prediction accuracy as *in vivo* studies. The trends in terms of intestinal permeability were also consistent with the bioaccumulative trends *via* the fish gill predicted by the BCF values. The measured BCF values of **2** to **4** ranging from 500 to 5,000 reflect moderate bioaccumulation potential, while BCF values of less than 100 for **5** and **6** suggest low bioaccumulation potential. The consistency between intestinal permeation and measured BMFs or BCFs shows, as far as metabolically untransformed compounds are concerned, that the everted gut sac method is suitable for evaluating the membrane permeation by these chemicals in fish. Thus, this method obviates the need to conduct laborious bioaccumulation tests.

*2. Implications for current legislation*

The threshold MW for uptake through the fish gill membrane has been independently proposed to be 500,[33] 600,[34] or 700,[5] based on the measured BCF values, and taking safety factors into consideration, the CSCL has adopted the threshold of 800.[10] Namely, a chemical with MW$>$800 is defined as having low bioaccumulation potential *via* the fish gill membrane, and a fish bioconcentration study is not required for chemicals having an MW of 800 or higher. As shown in Fig. 2, a chemical with MW$\geq$548 cannot pass through the intestinal wall, so the same uptake threshold of 800 can also likely be applied as a criterion for low bioaccumulation through the digestive organs *via* the food web.

To relate the steric property of a molecule to its environmental behavior, apart from MW, a 3-D molecular size indicator, $D_{max}$, has also attracted considerable attention. $D_{max}$ is the diameter of the smallest sphere accommodating the locally stable conformer of a chemical, and the arithmetic mean ($D_{max\ aver}$) or the minimum ($D_{max\ min}$) value of those obtained for a set of stable conformers is often used as a descriptor. In particular, $D_{max\ aver}$ has been linked with bioaccumulation potential: under the REACH regulation, $D_{max\ aver}>17.4$ Å is accepted as evidence of low bioaccumulation (BCF$<$5000).[35,36] While generally used $D_{max}$ values are derived based on the stable conformers in a vacuum, using a quantum chemical calculation, we recently developed a method for calculating these values in water, which is considered preferable when evaluating molecular behavior in an aquatic environment using an MD simulation.[11] Accordingly, we proposed $D_{max\ min}>15.6$ Å or $D_{max\ aver}>17.1$ Å in water as a new criterion for predicting the low bioaccumulation potential of a chemical (BCF$<$5,000). The $D_{max\ min}$ and $D_{max\ aver}$ values of **1** to **9** are shown in Table 1. Of these, the $D_{max\ min}$ values of **5** to **9** are higher than our proposed threshold of 15.6 Å, and the $D_{max\ aver}$ values are higher than 17.1 Å, so their bioaccumulation potential through the gill membrane is deemed to be low. The *in vitro* data in this study also showed that **5** to **9** have very low, if any, ability to pass through the fish intestine. Therefore, it is likely that the criterion for evaluating bioaccumulation through the gill membrane can also be applied to evaluating that through the intestinal membrane.

Finally, since it is known that the BCF and BMF values are strongly correlated with the *n*-octanol/water partition coefficient ($K_{ow}$). The relationship between $K_{ow}$ and the present results is also of interest. Arnot *et al.* found that the BMF values began to decline for chemicals when log $K_{ow}$ exceeds 7 and fell below 1 (*i.e.*, low bioaccumulation potential) when log $K_{ow}$ exceeds 9.74.[37] This result agrees with our findings that chemicals **5** to **9**, with low membrane permeation, are highly hydrophobic with log $K_{ow}>10.5$ (Table 1). However, because it is difficult to obtain reliable measured values for log $K_{ow}>6$, these higher $K_{ow}$ values are all estimates and have not been sufficiently validated with actual measurements. Therefore, MW or size is better for evaluating the bioaccumulation potential of hydrophobic chemicals.

## Conclusion

The permeability of the intestinal tract by nine test chemicals with various physicochemical properties was evaluated *in vitro* using the everted gut sac method. The relationship between intestinal permeability and the molecular weight of the chemicals showed that almost no permeation occurred for those having MW$\geq$548, supporting the threshold (*i.e.*, 800) defined by the CSCL. The molecular size parameter $D_{max}$ was also related to permeation. The same $D_{max}$ criterion can likely be used to predict the potential for accumulation through either the gill membrane or the intestinal tract. The findings obtained in this study will significantly contribute to establishing valid prediction of the bioaccumulation potential of chemicals, thus obviating the need to conduct animal tests in environmental safety studies.

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