The effects of humic substances on the intake of micro-organic pollutants into the aquatic biota

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Abstract Humic substances, naturally occurring highly polymerized organic compounds, exist widely in the water and soil environments. It has been known that the humic substances affect the fate of micro-organic pollutants (e.g. intake, accumulation, movement, degradation, toxicity, etc.). Of these, the effect of humic substances on the intake into biota (i.e. living cell) is one of the most important. In this research, the effects of co-existing humic substances on the intake of micro-organic pollutants into aquatic biota were experimentally evaluated. The humic acid filtrate using a 3,000 Da ultra-filtration membrane was used. Two PAHs (i.e. pyrene and phenanthrene) were used as micro-organic pollutants. Liposome for simulating living cell membrane was synthesized in the laboratory, and used for investigating the intake of micro-organic pollutants into aquatic biota precisely. The batch experiment results (PAHs onto humic acid, humic acid into liposome, and PAHs into liposome (K_{lipw})) led to the fact that the sorption of PAHs into liposome is suppressed apparently by binding with humic acid in the aqueous phase. This suggests that the accumulation and/or toxicity of micro-organic pollutants is retarded by humic substances in the actual aqueous environment. Moreover, the experimental results indicated that the sorption into liposome (i.e. liposome/water partition coefficient (K_{lipw})) could be a better parameter for estimating the intake of micro-organic pollutants into aquatic biota than *n*-octanol/water partition coefficient (K_{ow}) in the aqueous environment.

Keywords Humic substances; K_{lipw}; K_{ow}; liposome; micro-organic pollutants

Introduction

Humic substances, naturally occurring highly polymerized organic compounds, exist widely in the water and soil environments. It has been known that the humic substances affect the fate of micro-organic pollutants (e.g. intake, accumulation, movement, degradation, toxicity, etc.). Of these, the consideration of the effects of humic substances on the intake into biota (i.e. living cells) is one of the most important.

The *n*-octanol/water partition coefficient (K_{ow}) (OECD107, 1995) is the most popular for evaluating the intake of micro-organic pollutants, since the measurement using real biota is difficult and complicated. The K_{ow} is used as an index for describing hydrophobicity (QSAR, Quantitative Structure-Activity Relationships), which can estimate the bio-concentration factor (BCF). However, the K_{ow} has a few defects due to the use of *n*-octanol; the remarkable ones are pointed out as follows:

The characteristic of the water saturated with *n*-octanol, for measuring K_{ow}, is different from the actual aqueous environment. It is presumably difficult to evaluate the K_{ow} of micro-organic pollutants with organic matter (e.g. humic substances and NOM). The index of K_{ow} cannot evaluate and/or estimate the function of cell membrane, since it is

Water Science and Technology **Vol 47 No 9 pp 117–124** © IWA Publishing 2003

sophisticatedly structured (Brian *et al*., 1997; Opperhuizen *et al*., 1988), which possibly plays a significant role in the intake of micro-organic pollutants into real organisms such as fishes.

Liposome is an artificially synthesized cell membrane. Liposome consists of amphiphile (i.e. hydrophobicity and hydrophilicity) a phospholipid molecule with spontaneous vesicle structure formation. We used this liposome instead of *n*-octanol for simulating living cell membrane, investigating the intake of micro-organic pollutants into biota more precisely.

Polycyclic Aromatic Hydrocarbons (PAHs), one of the micro-organic pollutants, are now widespread and unexpectedly are produced when fuel and the other organic compounds are burnt. Some kinds of PAHs are of great concern due to their latent carcinogens or mutagens. Presently, the fate and toxicity of PAHs in the environment have been discussed scientifically.

The objectives of this work are to evaluate the intake of PAHs into liposome (i.e. the liposome/water partition coefficient (K_{lipw})) and to quantitatively investigate the effects of coexisting humic substances on the intake in the aqueous environment. In this research, we defined the sorption into liposome as penetration, adsorption and absorption onto liposome.

Methodology

Micro-organic pollutants

Two PAHs (i.e. pyrene and phenanthrene) as micro-organic pollutants were used in this research. The PAHs were dissolved in phosphate buffer (10 mmol l^{-1}, pH 6.9).

Humic substances

The filtrate of humic acid (Aldrich Chemical Co.) was used. The 3,000 Da ultra-filtration membrane (Amicon Co., CENTRIPLUS-3) was used for simulating the molecular weight of the humic acid in the aqueous environment (Ikeda *et al*., 2001; Yu-Ping Chin *et al*., 1994). The filter was pre-washed and rinsed with NaOH (0.1 mol l^{-1}, 10 mL) and phosphate buffer (10 mmol l^{-1}, pH 6.9) sufficiently, and then the purchased humic acid in the phosphate buffer was filtrated.

Preparation of the liposome

We synthesized liposome by combining existing two methods (i.e. evaporation and extrusion techniques) (Moscho *et al*., 1996; Hunter and Frisken, 1998). We chose DSPC (distearoyl phosphatidylcholine, C18:0) since it is a major phospholipid in bio-membrane and is stable in gel phase at 25°C. First, we made GUV (Giant Unilamellar Vesicle) by rotary evaporator (2 minutes). Then, we extruded the GUV, filtrating by polycarbonate-membrane (pore size = 1.2 μm) in order to make the diameter uniform around a bacterial size. This extrusion could make the homogeneous unilamellar vesicle, LUV (Large Unilamellar Vesicle) suited for the batch experiment.

Evaporation technique. The preparation of GUV was adapted from the method of Moscho *et al*. modified by Wilson (personal communication). 100 mg of DSPC (Sigma Chemical Co., 99%, synthetic) was dissolved in 20 mL of chloroform (Aldrich Chemical Co., 99.9%, HPLC grade), and 0.2 mL of this solution was placed in a 50 mL round-bottom flask. 3 mL of the aqueous phase (phosphate buffer: 10 mmol l^{-1}, pH 6.9) was then quickly added along the flask walls. Only the organic solvent was removed in a rotary evaporator (Büchi Co., Rotavapor R-124, Vac V-500, Vacuum Controller B-721) under reduced pressure at 40°C and 40 rpm. After evaporation for 2 minutes (the final pressure reached 10-12 hPa), the remaining aqueous solution inside the flask contains the GUV in high concentration.

Extrusion technique. The diameter of GUVs is possibly from 1 μm up to 50 μm and some of them are not unilamellar but multilamellar. Extrusion is a technique in which a lipid suspension is forced through a polycarbonate filter above their gel-to-liquid crystalline phase transition temperature (55°C for DSPC) with a defined pore size to yield particles having a diameter near the pore size of the filter used (Mayer, 1986). The liposome solution obtained by evaporation technique was passed subsequently 21 times through polycarbonate filters (Millipore Co., 25 mm, pore size = 1.2 μm) under elevated temperature (65°C). We examined the homogeneity of this liposome by Laser Diffraction Particle Size Analyzer (Shimadzu, Wing Sald-2100).

Equilibrium batch sorption experiments

Three systems (PAHs sorption onto humic acid, humic acid intake into liposome, and PAHs intake into liposome) were carried out and the effects of co-existing humic acid were evaluated.

PAHs sorption onto humic acid (K_{oc}). The PAHs sorption onto humic acid (K_{oc}) was measured by fluorescence quenching method (Shimizu and Liljestrand, 1991), which is the technique for quantifying the sorption without any intense physical separations. The K_{oc} is defined as follows:

$$\text{PAHs} + \text{HA} \leftrightarrow \text{PAHs-HA} \tag{1}$$

$$K_{oc} = [\text{PAHs-HA}]/([\text{PAHs}][\text{HA}]) \tag{2}$$

In Eq. (1), PAHs, HA, and PAHs-HA are respectively free PAHs in the aqueous phase, Humic Acid in the aqueous phase, and PAHs sorbed onto Humic Acid. In Eq. (2), [PAHs-HA] is the concentration of PAHs sorbed onto Humic Acid, [PAHs] is the concentration of free PAHs in the aqueous phase, and [HA] is the concentration of Humic Acid in the aqueous phase, respectively. With the mass balance on PAHs, the following Equation is obtained.

$$C_{PAHs} = [\text{PAHs}] + [\text{PAHs-HA}] \tag{3}$$

In Eq. (3), C_{PAHs} denotes the total concentration of PAHs in the solution. Considering the fluorescence yield in measurement is proportional to free PAHs concentration in the solution ([PAHs]), the following equation is derived.

$$F_0/F = 1 + K_{oc}[\text{HA}] \tag{4}$$

In Eq. (4) (i.e. Stern-Volmer equation), F_0 is the fluorescence of initial PAHs, and F is the fluorescence of free PAHs under co-existing humic acid.

Humic acid intake into liposome. The humic acid intake into liposome was measured by using dialysis-membrane equalizer (Spectrum Co.). The dialysis-membrane (pore size 10,000 Da, Diachema Co., SC-101-M10H), which can pass through humic acid but cannot pass liposome, was set at the center in a Teflon cell of the apparatus. Liposome was put into one side, and humic acid was put into both sides for curtailing the time to equilibrium. The prepared Teflon cell was rotated at constant speed (10 rpm) for 48 hours, and then the concentrations of humic acid on the both sides were measured and compared by fluorescence analysis, to evaluate the sorption coefficient.

PAHs intake into liposome (K_{lipw}). We measured the PAHs intake into liposome by using the fluorescence enhancement phenomenon (Netzel *et al.*, 1995; Wagner and MacDonald, 1998), which is observed when the PAHs are trapped in the unilamellar structure of liposome. The main principle used in this research for quantifying the PAHs sorption is that the sorption of PAHs into liposome never changes the equilibrium in the same glass vial even if any physical separation techniques are applied. In this methodology, several glass vials were prepared with identical initial concentrations of PAHs and liposome and mixed for 24 hours (25°C, 30 rpm by a rotary shaker). After mixing, these vials were mildly centrifuged at various speeds (1,000–2,000 rpm, 5–10 minutes), and then their fluorescence and absorbance were measured for PAHs and liposome, respectively. The K_{lipw} is defined as follows:

$$\text{PAHs} + \text{lip} \leftrightarrow \text{PAHs-lip} \tag{5}$$

$$K_{lipw} = [\text{PAHs-lip}]/([\text{PAHs}][\text{lip}]) \tag{6}$$

In Eq. (5), PAHs, lip, and PAHs-lip are respectively free PAHs in the aqueous phase, liposome in the aqueous phase, and PAHs sorbed into liposome. In Eq. (6), [PAHs-lip] is the concentration of PAHs sorbed into liposome, [PAHs] is the concentration of free PAHs in the aqueous phase, and [lip] is the concentration of liposome in the aqueous phase, respectively. Then the following equation is derived.

$$F_0/F = 1 + K_{lipw}[\text{lip}] \tag{7}$$

In Eq. (7) (i.e. the form of the Stern-Volmer equation), F_0 is the fluorescence of initial PAHs, and F is the fluorescence of free PAHs in the aqueous phase after the equilibrium. F was calculated from the correlation of the fluorescence enhancement yield on each identical solution, and K_{lipw} was obtained consequently.

PAHs intake into liposome under co-existing humic substances. For estimating the characteristic of PAHs intake into liposome under co-existing humic acid, we calculated from the fore-mentioned results (PAHs sorption onto humic acid, humic acid intake into liposome, and PAHs intake into liposome). In the calculation, we assumed the three systems' reactions were independent of one another. Additionally, we also measured practically the PAHs intake into liposome under co-existing humic acid by fluorescence enhancement techniques. In this experiment, PAHs, liposome and humic acid were put into a glass vial, and then mixed for 24 hours.

Results and discussion

Particle size of liposome

The particle size distribution of liposome that is refined in this research is shown in Figure 1. The x-axis is logarithm of particle size, and the y-axis is existence ratio on the particle size. From Figure 1, we can confirm the extrusion effect, which leads to shift of the particle size into the vicinity of 1 μm. The liposome was made with the uniform diameter around a bacterial size, and suited for the batch equilibrium experiment.

Equilibrium batch sorption results

PAHs sorption onto humic acid (K_{oc}). The sorption of pyrene and phenanthrene onto humic acid were obtained respectively from the Stern-Volmer plots using Eq. (4) (Figure 2). In both lines, the intercepts were not significantly different from one, and the slopes become K_{oc}. The values of K_{oc} are shown in Table 1.

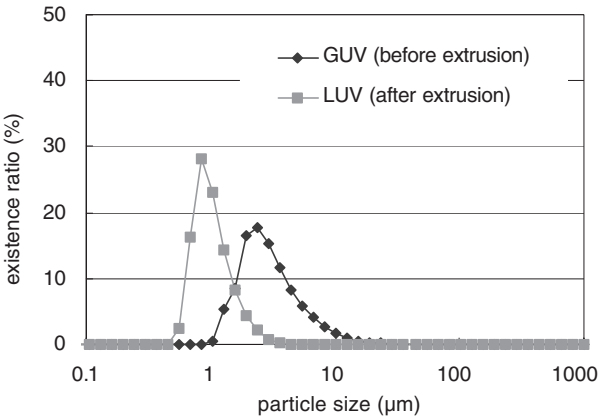

Figure 1 Extrusion effect

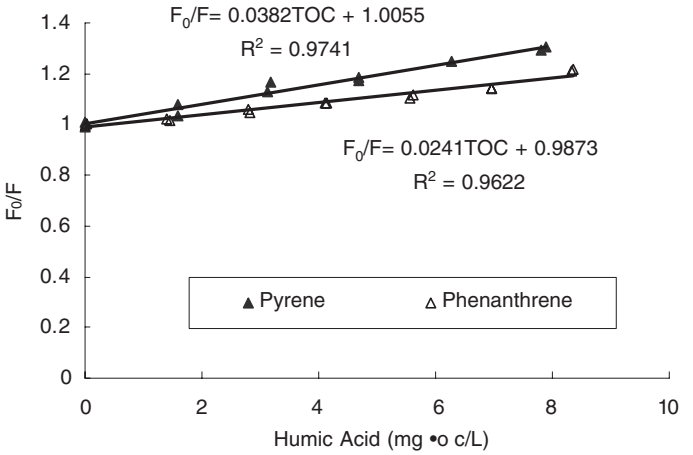

Figure 2 The sorption coefficient of PAHs onto humic acid (3,000 Da filtrate)

Table 1 The values of K_{oc} of two PAHs onto humic acid (3,000 Da filtrate)

	Pyrene	Phenanthrene
K_{oc} (L/kg)	38,200	24,100

Humic acid intake into liposome. It was confirmed that there is no noticeable intake of the humic acid into liposome, since no concentration gradient occurred in the batch sorption experiment by using dialysis-membrane equalizer. Therefore, we considered that its sorption is negligible, i.e. the intake coefficient of humic acid into liposome is zero.

PAHs intake into liposome (K_{lipw}). The sorption of pyrene and phenanthrene into liposome were obtained respectively from Eq. (7) based on the fluorescence enhancement phenomenon, in the batch sorption experiment (Figure 3). The values of K_{lipw} are shown in Table 2.

PAHs intake into liposome under co-existing humic substances. The results of the previous three systems, which are measured independently, demonstrate that the movement of hydrophobic micro-organic pollutants (e.g. PAHs) into liposome is suppressed by humic substances; since PAHs are sorbed onto humic acid in the aqueous environment, and the

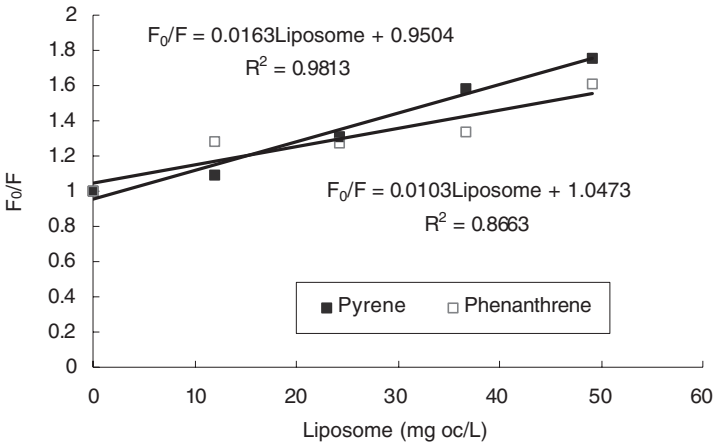

Figure 3 The sorption coefficient of PAHs into liposome

Table 2 The values of K_{lipw} of two PAHs into liposome

	Pyrene	Phenanthrene
K_{lipw} (L/kg)	16,300	10,300

humic acid hardly sorbed into liposome (i.e. the sorption coefficient = 0). This suggests that the accumulation or toxicity of micro-organic pollutants is retarded under co-existing humic substances.

The effects of humic substances on the fate of PAHs in environment. Now, we discuss the effects of humic substances on the intake of PAHs into liposome quantitatively from the obtained results, for evaluating the effects of humic substances on the fate of PAHs in the aqueous environment. In the case of no humic substances in the aqueous environment, the concentration ratio of PAHs ([PAHs]) to PAHs sorbed into liposome ([PAHs-lip]) is expressed as the concentration of liposome ([lip]) multiplied by the sorption coefficient into liposome (K_{lipw}) (= [lip]•K_{lipw}) from Eq. (6). On the other hand, in the case of co-existing humic substances, the ratio is expressed as follows:

$$[lip]\bullet K_p = [PAHs\text{-}lip]/([PAHs] + [PAHs\text{-}HA]) \quad (8)$$

In Eq. (8), K_p is given as the sorption coefficient of the free PAHs into liposome, i.e. apparent intake coefficient. Since, [PAHs-HA] is the concentration of PAHs sorbed onto humic acid, the Eq. (8) is developed by using K_{oc} and K_{lipw} (Eqs (2) and (6)) as follows:

$$[lip]\bullet K_p = ([PAHs\text{-}lip]/[PAHs])\bullet(1/(1 + K_{oc}\,[HA])) \quad (9)$$

$$K_p = K_{lipw} \bullet (1/(1 + K_{oc}\,[HA])) \quad (10)$$

The simulation by the Eq. (10) from experimental results is illustrated in Figure 4.

The horizontal straight line in Figure 4 is the case with no humic acid. Figure 4 demonstrates that the intake of two PAHs into liposome is suppressed by the sorption of PAHs onto humic acid, depending on humic substance concentration. It was also confirmed that the calculated results are consistent with the experimental results which are measured prac-

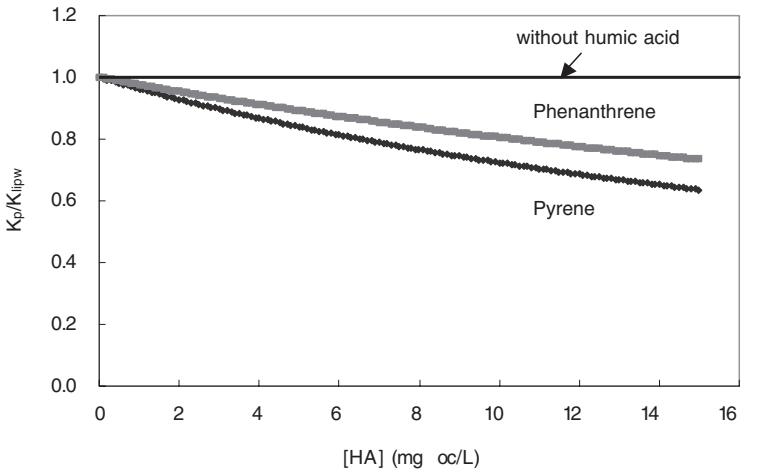

Figure 4 The effects of humic acids on the PAHs sorption into liposome

tically the PAHs intake into liposome under co-existing humic acid by fluorescence enhancement technique. Therefore, we concluded that the co-existing humic substances suppress the intake of micro-organic pollutants into biota in the actual aqueous environment.

Conclusion

We could synthesize liposome (i.e. artificial cell membrane), and evaluate the K_{lipw} by fluorescence enhancement technique. The sorption coefficient of humic acid into liposome was negligible. From these experimental results, it was confirmed that the sorption of micro-organic pollutants (e.g. PAHs) into liposome is suppressed apparently under co-existing humic substances. This suggests that the accumulation or toxicity of micro-organic pollutants is retarded in the actual aqueous environment.

The K_{lipw} adopted in this research could be a better parameter for estimating the intake of micro-organic pollutants into living cell than K_{ow} in actual aqueous environment. Further research is expected to investigate the validity of K_{lipw}.

Acknowledgements

We are grateful to Dr. Wilson C. of Stanford University for advice to synthesize liposome. Moreover, we wish to express our thanks to Mr. Eiji Kinoshita and Mr. Kenji Kinoshita of Shimadzu Co., for measuring the particle size distribution of liposome.

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