



Comparative transmembrane transports of four typical lipophilic organic chemicals

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ABSTRACT

Transmembrane transports of four kinds of lipophilic organic chemicals (LOCs) on suspending multilamellar liposomes (SML) and *Escherichia coli* (*E. coli*) were investigated, where both anthracene and phenanthrene were accorded to the lipid–water partition law and Sudan I and III to the Langmuir isothermal adsorption. Less than half of phenanthrene is transported into *E. coli*, where more than 60% are located in the cytoplasm. About 60% of anthracene entered the *E. coli* where only 10% was released into the cytoplasm. The partition coefficients of phenanthrene and anthracene partitioning from the extracellular liquid into membrane are 502 and 1190 L/kg but their inverse partition coefficients are only 0.180 and 0.018 kg/L. Over 60% of Sudan I and less than 40% of Sudan III accumulated on *E. coli* where most of them remained on the membrane. The transmembrane impedance effect (TMIE) is proposed for evaluating the cell-transport of polar LOCs.

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1. Introduction

Most of lipophilic organic chemicals (LOCs) widely distribute in the atmosphere, water bodies, sediments and soils in microcontaminant. Their exposures have brought some serious ecological problems and human health risk. Representative non-polar LOCs, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment (Covino et al., 2010) and comprise over 100 organic compounds (Reddy et al., 2010), which as the typical candidate carcinogens (Mahadevan et al., 2005) are produced in forest fires, paroxysmal eruption, solid fuel combustion, garbage incineration and motor exhaust gases (Crisafulli et al., 2008). In addition to non-polar organic compounds, many polar LOCs, e.g. diverse endocrine disrupting chemicals (EDCs), azo compounds and colour additives are discharged from chemical plants or used extensively in daily life. For example, Sudan compounds, one kind of azo dyes, which are aromatic compounds with one or more –N=N– groups (Li et al., 2010), have been employed as additives in foods, e.g. chilli powders, commercial sauces, salami, olive oil and many other frequently eaten foodstuffs (Mazzetti et al., 2004). The associated health problems have recently attracted much attention and concern because these compounds may induce urinary bladder

carcinoma, mutations and endocrine disturbances (Suryavathi et al., 2005). Sudan I–IV have been classed as category 3 carcinogens in humans by the International Agency for Research on Cancer (Tateo and Bononi, 2004).

The effects of lipophilic compounds on the ecological system and human health have drawn considerable attention. Data on the toxic effects of these xenobiotics, ranging from the molecular level to the whole organism, together with knowledge of physico-chemical characteristics of compounds, can be useful for establishing structure–activity relationships. Microorganisms are very powerful tools to provide information of membrane-mediated toxic effects of LOCs (Monteiro et al., 2005). Membrane-active drugs often impair bacterial growth (Neugebauer et al., 2007). The inhibitory effects are often directly related to the partitioning of the compounds, which suggests that the membrane is the primary site of toxic actions (Zhulin et al., 2003). Since the cell membrane of bacteria is directly involved in most vital functions e.g. signal and energy transduction and transport of solutes, it is not surprising that the perturbations promoted by LOCs in membrane structure and function are reflected on cell growth and viability (Martins et al., 2003). So the inhibition of growth, the effects on respiration and other vital functions have been used to assess the toxicity *in vitro* of several environmental pollutants (Monteiro et al., 2005). *Escherichia coli* was chosen as the model microorganism and exposed to four different kinds of LOCs: anthracene, phenanthrene and Sudan I and III, which differ markedly in chemical structure and polarity.

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LOCs accumulate in lipid compartments of the body, in which the phospholipids bilayer membranes are the primary permeability barriers (Flaten et al., 2007). A number of investigations have indicated that the octanol/water partition coefficient (K_{OW}) (Levitt, 2010) is a good parameter for the estimation of biopartitioning especially for non-polar organic chemicals e.g. PAHs (Crisafulli et al., 2008). However, some researchers have indicated that K_{OW} has only limited potential for estimating bioconcentration for a wide range of organic chemicals e.g. chlorinated benzenes in fish and the recent studies have shown that K_{OW} for diverse estrogenic chemicals does not correlate well with the partition coefficient between water and lipid membrane vesicles (Kwon et al., 2006). As a result, the interaction of lipophilic chemicals particularly with azo, nitro, hydroxyl, hydrogenated and other polar groups may involve the additional partition mechanisms. The aim of this work was to reveal the comparative transmembrane transports of typical LOCs by investigating their interactions with *E. coli*.

2. Methods

2.1. Chemicals and materials

Lecithin was used to prepare the suspending multilamellar liposomes (SML). Two PAHs (anthracene and phenanthrene) and Sudan compounds (Sudan I and III) were used to investigate transmembrane transport in order to estimate the difference of their membrane toxicities quantitatively.

Stock solutions of PAHs were prepared by dissolving the two compounds at 1000 mg/L in ethanol and used to investigate the transmembrane transport of non-polar LOCs; Solutions of 100 mg/L Sudan I and 100 mg/L Sudan III were prepared in 0.2% ethanol and used to investigate the transmembrane transport of polar LOCs. A series of Britton–Robinson (BR) buffers from pH 2.18 to 11.02 were prepared with acetic acid, phosphoric acid, boric acid and sodium hydroxide. Phosphate buffered saline (PBS), pH 7.4, was prepared by mixing 0.8% NaCl, 0.02% KCl, 0.014% Na_2HPO_4 and 0.024% KH_2PO_4 . It was used to re-suspend the bacterial bodies. Sodium chloride (1.0 mol/L) was used to examine the effects of electrolytes on the interactions of PAHs with SML. A cellular matrix-simulating was prepared by mixing 2% ovalbumin, 0.2% glucose and nine amino acids (each 0.05%): glycine, L-alanine, L-arginine, L-cysteine, L-histidine, L-glutamic acid, L-lysine, L-proline and L-threonine in PBS, all of which were purchased from Sinopharm Chemical Reagents. It was used for *in vitro* experiments instead of the *E. coli* matrix. An L–B nutrient medium containing 1.0% peptone, 0.5% yeast extract and 1.0% NaCl was prepared and used to culture *E. coli*. Prior to use, the L–B medium was disinfected under 1034×10^5 Pa for 20 min.

The aqueous solubility of anthracene and phenanthrene are only 0.04 and 1.15 mg/L (Kuramochi et al., 2006) and those of Sudan I and III are less than 100 mg/L. In order to reach the enough measurement sensitivity, the concentrations of phenanthrene and anthracene must be more than 0.02 mg/L and those of Sudan compounds over 0.30 mg/L. As well known, such four compounds are alcohol soluble and the presence of ethanol will increase their solubility greatly. The existence of little ethanol has not affected the formation of SML (Li et al., 2007). The *E. coli* bacteria were extracted from a brewing sewage which contained approximately 0.2% ethanol. Thus, the bacteria are resistant to ethanol.

2.2. Preparation of SML

Lecithin (Sinopharm Chemical Reagents) was used to prepare liposomes suspension as follows: 20 mg/ml lecithin was suspended in deionized water and then dispersed with an ultrasonica-

tor at maximal amplitude at 4 °C for 300 s total, comprising 15 s intervals of ultrasonic irradiation followed by 45 s of rest (Saar et al., 2005). Phospholipids in suspensions are expected to exist as mixtures of multilamellar liposomes and amorphous liposome coagulates. The average hydrodynamic diameter of the lipid aggregates is less than 0.54 μm when the phospholipids/solution ratio is over 2 g/L (Qu et al., 2007). Therefore, the SML could be formed, which be accumulated for the phospholipids multilayer via lipophilic aryl tails and ion-pair heads interactions (Fig. S1 A).

2.3. Interactions of PAHs and Sudan compounds with SML

As an example, the partitioning of phenanthrene is described below. A series of the mixtures containing SML and 2.00 mg/L phenanthrene were prepared and centrifuged. Over 90% of the SML can be separated by a high-speed centrifuge. After centrifugation, the fluorescence intensity of the supernatant was measured at $\lambda_{\text{ex}} = 270$ nm and $\lambda_{\text{em}} = 367$ nm with a Model F-4500 fluorescence spectrometer (HITACHI, Japan). The SML deposit was dissolved in ethanol and measured by spectrofluorophotometry using the same method. Thus, the equilibrium concentrations (C_L) of phenanthrene remaining in the supernatant were calculated and those in SML (C_L^{SML}) were calculated by $C_L^{\text{SML}} = M_L^{\text{SML}}/M_{\text{lip}}$, where M_{lip} and M_L^{SML} are the mass of lecithin added and the mole number of LOCs accumulated in SML. The Freundlich empirical formula $\gamma = kC_L^m$ was used to investigate the interactions of LOCs with SML where k and m are the constants and m is often between 0 and 1 for surface adsorption. The same procedure was performed for partitioning of anthracene in SML as that of phenanthrene but the measurement is at $\lambda_{\text{ex}} = 250$ nm and $\lambda_{\text{em}} = 405$ nm, and the same procedure was performed for the study of the interaction of Sudan I and III. The solutions were measured at 477 nm for Sudan I and at 504 nm for Sudan III with a Model Lambda 25 spectrophotometer (Perkin–Elmer Instruments, USA). A dual-wavelength spectral method was used to correct the light-absorption interference of the SML–ethanol background.

2.4. Inverse partition of anthracene and phenanthrene

Though the “partitioning” contains “equilibrium” in itself, the compositions in the practical cytoplasm are much more complicated than those in a simple aqueous solution. It is necessary that the “inverse partitioning” experiments were carried out. Following the above procedure, the phenanthrene (or anthracene) – SML deposit was re-suspended with the cellular matrix-simulating and mixed. The equilibrium liquid was centrifuged and separated according to the above procedures. The concentrations of phenanthrene or anthracene in water solution and SML deposit were determined by spectrofluorophotometry. Thus, the inversely partitioning of phenanthrene or anthracene from SML to the cellular matrix-simulating was calculated.

2.5. Culture of *E. coli* and determination of membrane phospholipids species

E. coli strain was used for the living cell experiments. *E. coli* was cultured in the L–B nutrient media at 37 °C. The *E. coli* turbidity light-absorption of the liquid was measured by spectrophotometry and the activity of *E. coli* bacteria was determined with methylthiazolyldiphenyltetrazolium. When the growth equilibrium is at 20 h, the number and size of living *E. coli* bacteria were counted and observed with a light microscopy (Leica, Germany) by the conventional plate count method after Gram's staining. 5 ml of *E. coli* liquid was centrifuged at 2600 \times g when the *E. coli* reached the growth equilibrium at approximately 20 h (Li et al., 2007). The *E. coli* was collected and re-suspended with PBS and then the

cytolysis was performed with a Model JY92-II Ultrasonic Cell Disruptor (Ningbo Xinzhi Instruments, China). The deposit was dissolved in dichloromethane and the phosphorus content of membrane phospholipids was determined with a Model Optima 2100 DV inductively coupled plasma–optical emission spectrometry (ICP–OES) (Perkin–Elmer Instruments, USA) (Ruiz et al., 2005). It was used to calculate the mole number of membrane phospholipids of *E. coli* bacteria.

2.6. Interactions of LOCs with *E. coli* bacteria

In order to investigate the distribution of LOCs around *E. coli* cell, one of the LOCs was added into *E. coli* bacteria liquid. According to the operation procedure (Fig. S2): mixing of LOCs with bacteria bodies, centrifuging, re-suspending, cytolysis and dissolving of bacteria bodies, and measuring of the LOC solutions (Li et al., 2007), the concentration of the LOCs distribution in different parts of *E. coli* cell (free in the extracellular liquid, entering into *E. coli* cytoplasm and accumulated on cell membrane) were determined. Thus, the transmembrane distribution of LOCs was analysed.

2.7. Toxicological testing of *E. coli*

The four LOCs were added respectively into *E. coli* culture liquid to estimate their toxicity to *E. coli*. According to Section 2.5, into an *E. coli* growing equilibrium at 20 h, one of LOCs (2.0 mg/L anthracene and phenanthrene, 100 mg/L Sudan I and III) was added, mixed and cultivated continuously. The *E. coli* activity was determined with methylthiazolyldiphenyltetrazolium (MTT) at 555 nm by spectrophotometry (Wei et al., 2002).

3. Results and discussion

3.1. Interactions of phenanthrene and anthracene with SML

From the measurement results of two non-polar LOCs: phenanthrene and anthracene distributed in the SML and freeing in the equilibrium liquid (Fig. S3 A) combining with the standard equations Table S1. From plots C_L^{SML} vs. C_L of phenanthrene and

anthracene (Fig. 1A), both m are greater than 1. Therefore, this phenomenon does not correspond to surface adsorption. Phenanthrene and anthracene may enter the internal of SML and accumulate by the hydrophobic π – π stacking as illustrated in Fig. S1 B. The good linearity between C_L^{SML} and C_L demonstrated that both phenanthrene and anthracene bound to SML according to the lipid–water partition law. Their partition coefficients ($K_{l/w}$) were calculated as shown in Table 1. Because $K_{l/w}$ of phenanthrene is less than that of anthracene, anthracene penetrates the SML more readily. Anthracene has a less steric effect than phenanthrene with a bay region so it can enter cell membrane more easily. By comparison of the $K_{l/w}$ values (Table 1) with their phospholipid–water partition coefficients (Qu et al., 2007), the lipotropy of phenanthrene and anthracene in 0.2% ethanol media is much weaker than that in a completely aqueous solution.

3.2. Interaction of Sudan compounds with SML

Some EDCs with hydroxyl or carboxyl group partitions into the gel-phase phospholipids to produce over more than 10 kJ/mol of the enthalpy change (ΔH) (Kwon et al., 2007). It is impossible for only the hydrophobic interaction occurs between the EDCs with phospholipids. The hydrogen bond should take part in the partition interaction. Similar to such EDCs, Sudan compounds contain the hydroxyl and azo groups, which could form the hydrogen bond interaction with the polar charge heads of phospholipids.

The light absorption of Sudan I and II–SML was measured (Fig. S3 B) and plots C_L^{SML} vs. C_L are shown in Fig. 1B. The adsorption of Sudan I and III on SML tends towards saturation as the initial concentration increased. Both m of Sudan I and III are around 0.6 by fitting the Freundlich empirical formula. Plots γ^{-1} vs. C_L^{-1} (Fig. S4) indicated that their interactions with SML obeyed the Langmuir isothermal adsorption. From their chemical structures, the hydrogen bond would form between the lecithin polar head and the azo and hydroxyl groups of Sudan compounds, and the aryl groups could interact hydrophobically with the lecithin fatty acyl tails. Like the other water-soluble azo compounds (Li et al., 2008), Sudan compounds may be adsorbed on the surfaces of SML but their aryl groups imbedded into the fatty chains of

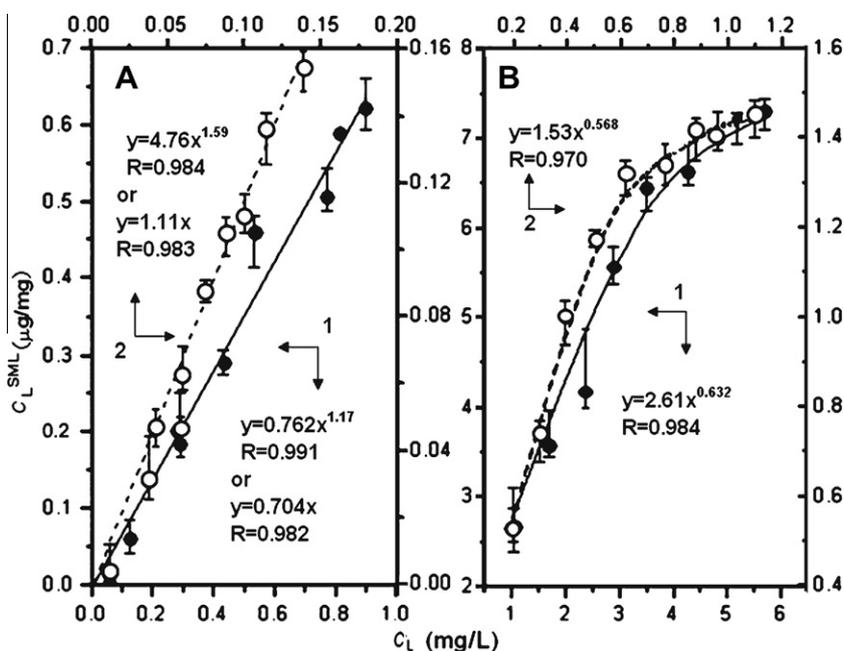


Fig. 1. Plots C_L^{SML} vs. C_L for four LOCs: A-1: phenanthrene; A-2: anthracene; B-1: Sudan I and B-2: Sudan III. C_L^{SML} is the concentration of LOC in SML, equal to the mass ratio of LOC accumulated in the SML to lecithin and C_L is the equilibrium concentration of LOC free in the aqueous phase.

Table 1
Determination of the interactions of four LOCs with SML and *E. coli* bacteria.

Parameters	LOCs	SML	<i>E. coli</i>
<i>Adsorption constant of Sudan compounds</i>			
N^a	Sudan I	1/23	1/14
	Sudan II ^b	1/31	1/29
	Sudan III	1/137	1/46
	Sudan IV ^b	1/314	1/114
$K, \times 10^4$	Sudan I	5.25	2.11
	Sudan II ^b	1.75	1.86
	Sudan III	35.1	7.92
	Sudan IV ^b	29.2	6.02
<i>Partition coefficient of PAHs</i>			
$K_{l/w}$	Phenanthrene	704	502
	Anthracene	1110	1190
$K_{cyt/l}$	Phenanthrene	4.52×10^{-3}	0.180
	Anthracene	5.54×10^{-4}	0.018
$K_{cyt/w}$	Phenanthrene	3.18	90
	Anthracene	0.61	21

^a The molar ratio of LOCs binding to phospholipids.

^b From Ref. Li et al. (2007).

phospholipids (Fig. S1 C), where the multi-non-covalent bonds e.g. hydrogen bond, hydrophobic interaction and van der Waals force act simultaneously (Gao et al., 2008). As a result, it is more reasonable to clarify that the “partition” of Sudan compounds in gel-phase phospholipids is the “surface adsorption”. Using the Langmuir adsorption formula: $\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KN C_L}$, the adsorption constant (K) and the mole number (N) of Sudan compounds at saturation were calculated (Table 1) from the gradient and intercept of lines (Fig. S4). Twenty-three and 137 lecithin molecules bind to each molecule of Sudan I and III, respectively. For convenience of comparison, the binding parameters of Sudan II and IV (Li et al., 2007) are listed in Table 1 too. The N value decreases with an increase in the molecular weight of Sudan compounds. Like the other diazo-conjugate dye, the adsorption efficiency was determined not only by the chemical and textural characteristics of the adsorbent, but also by the properties of the dyes: size, ionic species and polarity (Davila-Jimenez et al., 2009). Both Sudan I and II contain two polar groups (hydroxyl and azo ones), but Sudan II differs from Sudan I containing two non-polar methyl groups. Sudan II is therefore insufficiently hydrophilic to weaken the polar interaction, so it has lower N and K values. In Sudan III, a polar phenylazo group replaces an $-H$ of Sudan I, so the K of Sudan III is higher. However, it is a significantly larger molecule than Sudan I, so a single molecule would occupy more lecithin. Thus, N of Sudan III is lower than that of Sudan I. The similar explanation applies to the comparison of Sudan II with IV.

In addition, the effects of pH, electrolyte and temperature on the interactions of four LOCs with SML were determined (Fig. S5) and the mechanism analysed (ESM Text).

3.3. Inverse partition of phenanthrene and anthracene

A cellular matrix-simulating was prepared and used to study the inverse partition of LOCs. Fluorescence measurements (Fig. S6 A) showed that phenanthrene and anthracene partitioned inversely into this solution. The mass concentrations (C_L^{SML}) remaining in the SML were calculated. From the variation (Fig. S6 B), the inverse partition coefficients ($K_{cyt/l}$) were calculated from the linear gradients (Table 1): a higher $K_{cyt/l}$ indicates more favoured release from the SML into the cellular matrix-simulating. From Table 1, phenanthrene has a much higher $K_{cyt/l}$ than anthracene, so phenanthrene is released more easily into the solution. Comparison of the $K_{cyt/l}$ and $K_{l/w}$ values for PAHs shows that $K_{cyt/l}^{-1}$ is not equal to $K_{l/w}$, so the cellular matrix-simulating affected

PAH partition. In order to describe the transport of PAHs from the extracellular medium to the cytoplasm, a new term, the cytoplasm concentration factor ($K_{cyto/w}$) was proposed: $K_{cyto/w} = K_{l/w} \times K_{cyt/l}$. A higher $K_{cyto/w}$ indicates easier entry into the cytoplasm from the extracellular aqueous medium through the membrane phospholipids. The $K_{cyto/w}$ values were calculated for phenanthrene and anthracene (Table 1) and it is five times greater for phenanthrene than for anthracene. Thus, phenanthrene partitions inversely more easily than anthracene, i.e. phenanthrene may disperse readily into the cytoplasm from membrane when they interact with cells.

3.4. Distributions of LOCs

The above analyses showed that phenanthrene and anthracene are enriched within SML. They may be released into the cytoplasm by the passive diffusion when they interacted with cells. By contrast, Sudan I and III may not enter the cytoplasm because they did not penetrate the SML. Transport of any LOC into the cell is a dynamic non-equilibrium process because cell growth is continuous. Chemicals in the extracellular medium will enter the cells rapidly by passive diffusion (Miyata et al., 2004) and accumulate until cell death. According to the operation procedure (Fig. S2), the fluorescence intensity and light-absorption of the various solutions containing anthracene, phenanthrene, and Sudan I and III were measured (Fig. S7), and their masses or mole amounts in the extracellular medium, membrane and cytoplasm were calculated by combining with the standard equations (Table S1). As the mass balance calculation, the total amount of LOCs in the three parts of *E. coli* cell (in the extracellular medium, cytoplasm and membrane) is about 90–110% of the initial added amount. Therefore, the measured and calculated data are valid and credible. The distribution of LOCs is shown in Fig. 2. Less than half of phenanthrene is transported into the *E. coli* cell, where more than 60% is located in the cytoplasm (Fig. 2A). 60% of anthracene entered the cell in which only about 10% was released into the cytoplasm (Fig. 2B). Therefore, both phenanthrene and anthracene can enter the *E. coli* cytoplasm through the membrane phospholipids. The difference may be attributed to the further interaction of phenanthrene with suspended substances and proteins in the cytoplasm. Thus, phenanthrene may lead to more serious cytoplasm toxicity than anthracene, which was confirmed in the above *in vitro* experiments. And their K_{ow} values being 37,200 and 47,900 L/kg (Qu et al., 2007) also indicate that anthracene rather stays in the membrane phospholipids. Over 60% Sudan I accumulated on *E. coli* in which more than 90% remained in the membrane (Fig. 2C). Less than 40% Sudan III accumulated on *E. coli* where most of them remained in the membrane bilayer (Fig. 2D). Without doubt, Sudan compounds can be caught strongly by the cell membrane, i.e. Sudan I and III will hardly enter the cytoplasm. As a result, the transmembrane impedance effect (TMIE) is proposed for evaluating the cell-transport of polar LOCs, i.e. in the passive diffusion process, polar LOCs will bind on the polar structure of the outer membrane molecules via hydrogen bonds, intermolecular forces and electrostatic capture and remain in the outer membrane surface. Such an effect is negligible for non-polar LOCs.

3.5. Interactions of PAHs with *E. coli* bacteria

From the mass concentration of membrane phospholipids species, the *E. coli* cytoplasm volume and the mass data of LOCs (Fig. 2, ESM Text), plots of C_L^{mem} vs. C_L (Fig. 3A) and plots C_L^{SML} vs. C_L^{cyto} (Fig. 3B) were obtained for phenanthrene and anthracene. The partition and inverse partition of phenanthrene and anthracene in the *E. coli* bacteria corresponded to the lipid–water partition law. From the determination of phospholipids’ mole

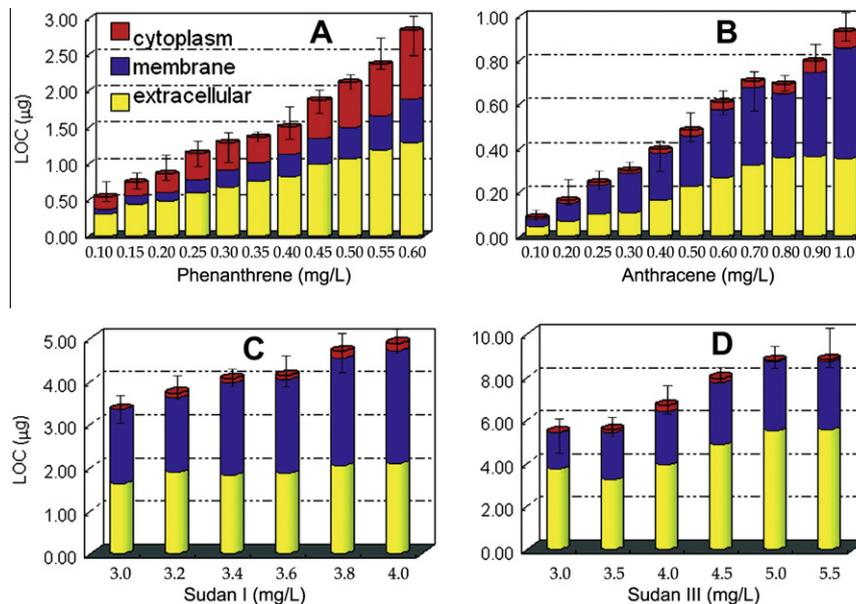


Fig. 2. Distribution of phenanthrene (A), anthracene (B), Sudan I (C) and Sudan III (D) in extracellular water, membrane and cytosol, in 5.00 ml of *E. coli* culture liquid for 0.5 h from mixing with LOCs.

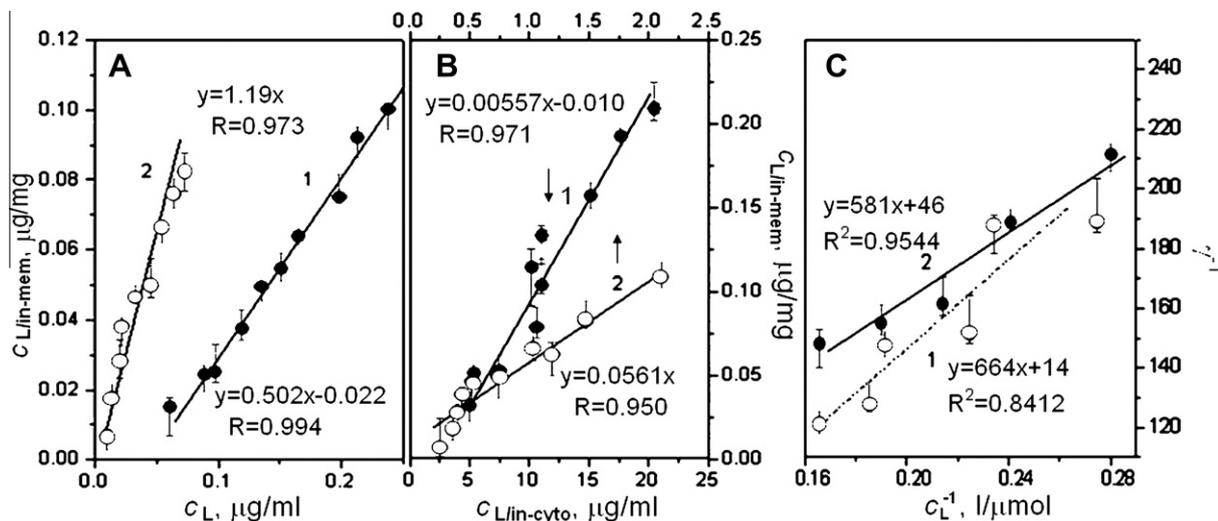


Fig. 3. Linear regression for the interactions of LOCs with *E. coli*. (A) Plots C_L^{mem} vs. C_L and (B) Plots C_L^{mem} vs. C_L^{cyto} of phenanthrene (1) and anthracene (2); (C) Plots γ^{-1} vs. C_L^{-1} of Sudan I (1) and III (2).

amounts of *E. coli* membrane (ESM Text) and data in Fig. 2, their partition coefficients ($K_{I/w}$ and $K_{\text{Cyt}/I}$) were calculated as shown in Table 1. Comparison of the $K_{I/w}$ values with the *in vitro* experimental data revealed no remarkable difference. However, the $K_{\text{Cyt}/I}$ values in *E. coli* were much more than those obtained by the *in vitro* experiments. In fact, the cytoplasm contains the cellular matrix, organelles and inclusions, in which much more complicated substances (e.g. enzymes, carbohydrates, proteins, chromosomes, mitochondrion, lipid aggregates and so on) co-exist together than the cellular matrix-simulating. Some of these substances e.g. protein (Glushkov et al., 2002), DNA (Platt et al., 2008) and lipids may interact with PAHs by various bonds (Xia and Pignatello, 2001). This means that $K_{\text{Cyt}/I}$ in the live bacteria is not only a simple inverse partition coefficient. The possible transport pathway of PAHs may be divided into three steps, as follows: The first step, PAHs enter the membrane phospholipids bilayer from the extracel-

lular liquid by partitioning. Comparison of the $K_{I/w}$ in *E. coli* with those obtained by *in vitro* experiments shows that step 1 proceeds rapidly to equilibrium. Afterwards, the membrane's crawling drives the inverse release of PAHs from the membrane into the cytoplasm. Successively, the dynamic metabolic mixing occurring in the cytoplasm promotes the immediate binding of PAHs to the intracellular macromolecules, as indicated above. Without doubt, such the interactions will cause a rapid decrease in PAHs concentration free in cytoplasm, i.e. PAHs will enter cytoplasm ceaselessly from the extracellular liquid by the partition – inverse release – binding pathway. The binding and metabolic process of PAHs at step 3 may control the speed of cellular transport of PAHs. Such a transmembrane transport pathway just caused the accumulation of non-polar LOCs in organism. Comparison of the $K_{\text{Cyt}/w}$ values for *E. coli* in Table 1 indicates that the phenanthrene and anthracene concentrations in the cytoplasm respectively approach 90 and 21

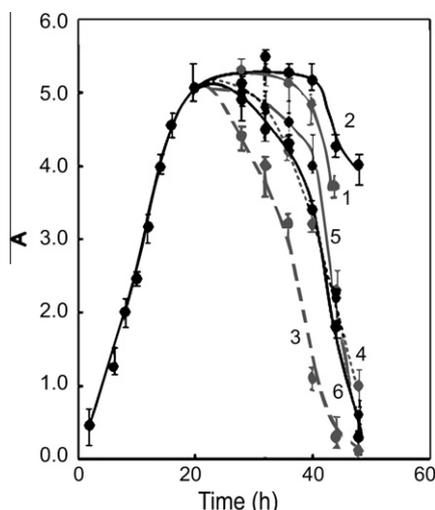


Fig. 4. Growth curves of *E. coli* at 37 °C where the bacteria were extracted from a brewing sewage. All foreign substances were added at 20 h of *E. coli* culture. 1: 0.2% ethanol, 2: control, 3: 2.0 mg/L phenanthrene, 4: 2.0 mg/L anthracene, 5: 100 mg/L Sudan I and 6: 100 mg/L Sudan III. All from 3 to 6 were in 0.2% ethanol media.

times those in the extracellular medium within 0.5 h after mixing. As indicated by the *in vitro* data, phenanthrene is released more easily from the membrane into the cytoplasm than anthracene and it has a much higher affinity with the inclusions of the cytoplasm. In addition to the Di-region, K-region (Sharma and Amin, 2005), Bay- and Fjord-region (Borosky and Laali, 2009) theories, the $K_{\text{cyto/w}}$ value provided a powerful experimental evidence to explain why phenanthrene is more readily toxic than anthracene.

3.6. Interactions of Sudan compounds with *E. coli* bacteria

From the measurement data of Sudan I and III (Fig. S7 B) and the determination of phospholipids mole amounts of *E. coli* membrane (ESM Text), γ was calculated. Plots γ^{-1} vs. C_L^{-1} of these compounds in the *E. coli* media fitted the Langmuir adsorption isotherm (Fig. 3C). From the *in vitro* Sudan compound – SML interaction illustration (Fig. S1), the feeding of Sudan compounds would accumulate on the *E. coli* membrane outside, i.e. Sudan I and III were prevented from entering the cytoplasm by the polar heads of the outer membrane phospholipids. For the convenience of comparison, the *E. coli* adsorption constants of all Sudan compounds are listed in Table 1. As in the *in vitro* experiments, N decreases with increasing molecular weight of Sudan compounds. The smallest, Sudan I, has the highest binding number because its binding area is the smallest. By contrast, N of Sudan IV is the lowest. Sudan III has more polar groups than Sudan I so its K value is higher in *E. coli*. The two non-polar methyl groups instead of the two –H groups of Sudan III structure changes into Sudan IV. The K value for Sudan IV is less than that for Sudan III. From Table 1, the *in vitro* N values of Sudan compounds are less than those in *E. coli* while their *in vitro* K values more than those in *E. coli*. One possible reason is that cellular movement may adjust the surface-binding position/state of Sudan compounds to decrease the steric hindrance effect. Also, the cell wall peptidoglycan, polyoses and exo-protein could bind Sudan compounds during transit. However, the membrane proteins could weaken the stability of Sudan compounds – the polar phospholipids heads interaction, resulting in decrease of K in *E. coli*. From the above analyses, Sudan compounds may lead to the membrane toxicity e.g. transport of nutrients and inhibition of ion pumps. Though the Sudan compounds is at trace level e.g. from 0.5 to 40 $\mu\text{g}/\text{kg}$ in chilli products (Ertas et al., 2007), the long-term uptake will make Sudan compounds continu-

ously enrich on the cell membrane afterwards to cause the health risk.

3.7. Inhibition of *E. coli* activity

In the presence of LOCs, the variation of *E. coli* activity was determined. By comparison of *E. coli* growth curves (Fig. 4), all the LOCs inhibited the growth of *E. coli* bacteria more or less. Phenanthrene has the most remarkable toxicity. This result is consistent with the $K_{\text{cyto/w}}$ values of phenanthrene and anthracene (Table 1) and the above analysis about the distributions of these two PAHs. Similarly, Fig. 4 also exhibited that the toxicity of Sudan III is more than that of Sudan I. From the K values of Sudan compounds (Table 1), Sudan III bound to the outside surface of *E. coli* membrane firmerly than Sudan I. Sudan III has a higher toxicity to *E. coli* than Sudan I. Therefore, the variation of *E. coli* activity is consistent with the determination data during the transmembrane distribution of LOCs.

4. Conclusions

Four LOCs: anthracene, phenanthrene, Sudan I and III can accumulate in SML and *E. coli* and persist in organisms. The polar LOCs, e.g. Sudan I and III were consistent with the general Langmuir adsorption isotherm while non-polar PAHs e.g. phenanthrene and anthracene conformed to a general model of partitioning behaviour. TMIE was proposed for identifying the membrane transport of LOCs. Anthracene and phenanthrene may enter the cytoplasm through the membrane phospholipids bilayer but Sudan compounds adsorb on the membrane surface. The present work is helpful in understanding the comparative membrane transport of diverse LOCs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biortech.2010.06.121](https://doi.org/10.1016/j.biortech.2010.06.121).

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