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Effects of Organic Solvents in Luminescent *umu* Test using *S. typhimurium* TL210

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Summary

We evaluated the effects of 15 different organic solvents in the luminescent *umu* test, a simplified version of the conventional *umu* test, by determining the amount of luminescence induced by each solvent and considering its toxicity and stability of the solvents with regard to the following 15 types of solvents: methanol, dimethyl sulfoxide, acetonitrile, 1,4-dioxane, tetrahydrofuran, acetone, cyclohexane, ethyl alcohol, N,N-dimethylformamide, ethylene glycol monophenyl ether, ethylene glycol dimethyl ether, glycerol formal, 1-methyl-2-pyrolidone, tetrahydrofurfuryl alcohol, and formamide. We found that cyclohexane, methanol, acetonitrile, ethanol, and acetone could be used in the test. Methanol, in particular, was effective for determining the mutagenicity of environmental samples.

Key words: luminescent *umu* test, SOS reaction, DMSO, DNA damage

INTRODUCTION

Many substances in our environment contain trace amounts of toxic chemicals, such as mutagenic substances that damage DNA, thus inducing mutation. The *umu* test is one method of rapidly determining the mutagenicity of substances. The test uses the SOS reaction, which is induced by DNA damage. Because of the advantages of the *umu* test include rapidity and simplicity,[1,2] it likely will be applied to samples obtained from the environment. For example, we previously reviewed the possibility of applying the luminescent *umu* test, which is a simplified form of the conventional *umu* test, to sediment and soil samples[3].

In the mutagenicity test that uses bacteria (Ames test), organic solvents such as dimethylsulfoxide (DMSO) are used if the test substance is insoluble in water. DMSO is used widely because it is minimally toxic to bacteria and has a relatively small effect on metabolism by S9mix. Maron and coworkers[4] examined the applicability of 14 types of organic solvents to the Ames test. They reported that 12 types out of the 14 tested solvents were found to be applicable to the test under certain conditions. However, although DMSO is known to induce the SOS reaction[5], no similar survey of solvents has been made regarding luminescent *umu* test. In the present study, we

characterized methanol and the 14 solvents tested by Maron and colleagues with regard to the solvents' potential use in the luminescent *umu* test. Solvent characteristics evaluated included the amount of luminescence induced by the solvents, toxicity to tester bacteria growth, and chemical stability.

MATERIALS AND METHODS

Reagents

In the present study, we used the following 15 commercially available reagents: methanol (for testing of residual agricultural chemicals; manufactured by Wako Pure Chemical Industries Ltd.), dimethyl sulfoxide (DMSO, for fluorescence measurement; Dojin Kagaku Co., Ltd.), acetonitrile (for testing of residual agricultural chemicals; Wako), 1,4-dioxane (for spectrum measurement; Nacalai Tesque, Inc.), tetrahydrofuran (THF; Nacalai Tesque), acetone (for testing of residual agricultural chemicals; Wako), cyclohexane (for testing of residual agricultural chemicals; Wako), ethyl alcohol (highest grade; Nacalai Tesque), N,N-dimethylformamide (DMF, highest grade; Wako), ethylene glycol monophenyl ether (EGPE, highest grade; Nacalai Tesque), ethylene glycol dimethyl ether (EGME, highest grade; Nacalai Tesque), glycerol formal (GF; Tokyo Kasei Kogyo Co., Ltd.), 1-methyl-2-pyrrolidone (1M2P; Tokyo Kasei), tetrahydrofurfuryl alcohol (THFA; Tokyo Kasei), and formamide (for molecular biology applications; Nacalai Tesque). In addition, dichloromethane (extracting solvent for testing of residual agricultural chemicals) and ampicillin were manufactured by Wako; Bacto-tryptone by Difco; culture reagents (e.g., glucose, NaCl) and sodium azide (a mutagen), by Nacalai Tesque.

Preparation of vehicle exhaust particles for extraction

Vehicle exhaust particles (NIES No. 8, provided by the National Institute for Environmental Studies) underwent ultrasonic extraction[6]. First, 160 mg of particles were ultrasonically extracted in 10 mℓ dichloromethane for 10 min. The extract was then filtered through a glass-fiber filter (GF75, Advantec), after which it underwent ultrasonic extraction in 5 mℓ dichloromethane for 10 min. Finally the filtrates were convined and dried under nitrogen. The resulting solid was dissolved in 4 mℓ dichloromethane, aliquoted into separate vials (500 μℓ each), concentrated, and dried under a nitrogen stream. To obtain the samples for testing, we added 500 μℓ of each solvent to individual vials of prepared vehicle exhaust particles.

Luminescent umu test

The luminescent *umu* test uses *Salmonella typhimurium* TL210, which was created by incorporating gene cluster luxA-E derived from luminescent bacterium *Vibrio fischeri* in pSK1002 plasmid (drug tolerance factor). This simple test does not require substrates for visualization of results. In the present study, we used a slight modification of the test procedure of Tanada et al.[3] Briefly, frozen bacteria (600 μℓ) were defrosted quickly, and 500 μℓ thawed cells was inoculated into 24 mℓ TGA culture medium (1 % Bacto-tryptone, 0.5 % NaCl, 0.2 % glucose, and 20 (g/mℓ ampicillin) in an Erlenmeyer flask. The culture was incubated at 37 ℃ for 2 h with shaking at 120 rpm, after which the optical density at 600 nm (OD_{600}) of the culture was measured (usually ~0.2), and the solution was diluted with enough TGA medium to decrease the absorbance reading to 0.1. We then transferred 167 μℓ culture to each well of a 96-well clear-bottomed black plate (manufactured by ScreenMates) and added up to 10 μℓ of each solvent to each well. Enough sodium phosphate buffer (pH 7.0, 20 to 37 μℓ) was added to bring the volume in each well to 204 μℓ. Plates were agitated thoroughly on a mixer and then left at rest in an incubator kept at 30 ℃. The amount of luminescence and optical density was measured every 20 min from 120 to 240 min after the start of the reaction by using a luminescence reader (JNR AB2100, ATTO) and a Wallac 1420 ARVOsx multilabel counter (Perkin-Elmer), respectively. The integrated luminescence value in 1 sec was determined for each well. The amount of luminescence at 220 min, which was the maximal value in many tests, was adopted for evaluation.

Conventional umu test

Conventional *umu* testing with *S. typhimurium* TA1535/pSK1002[2] was done as follows. A frozen stock of bacteria was defrosted quickly, inoculated into LB culture medium (1 % Bacto-tryptone, 0.5 % NaCl, 0.5 % yeast extract), and incubated at 37 ℃ for 12 h with shaking (120 rpm). The culture was then diluted with a 100-fold volume of TGA medium, and incubation at 37 ℃ with shaking continued for another 2 h. The resulting bacterial solution and the test solvents were

placed in the wells of a microplate, which then was sealed. The plate was agitated thoroughly with a plate mixer and incubated at 37 ℃ for 4 h with shaking (900 rpm), after which the viable cell count in each well was estimated by measuring the OD_{595}. A 10-μℓ aliquot of each bacterial suspension was transferred to the well of a new 96-well microplate and diluted with 140 μℓ Z buffer. Z buffer solution was completed adding 2-mercaptoethanol immediately before using it. We then added 10 μℓ chlorophenol red-β-D-galactopyranoside (CPRG; 4 mg/mℓ) to each sample well, and samples were incubated at 37 ℃ for 30 min. Finally, 100 μℓ 1 M Na_2CO_3 was added to stop color development, and relative (β-galactosidase activity at OD_{570} was measured and used to determine the number of *umu* unit (*umu* unit$=OD_{570}/OD_{595}$) of the test substance.

RESULTS AND DISCUSSION

Effect of solvents on response of the luminescent umu test

We tested methanol and the 14 solvents evaluated for their effects in the Ames test[4] to determine their effects on the response of the luminescent *umu* test (Fig. 1). In particular, DMSO and EGDE exhibited marked dose-response effects, which disqualifies them for use as solvents in the luminescent *umu* test. Even when we added the minimal amount of solvent (1 μℓ /well), the amounts of luminescence of EGPE and formamide were lower than that of bacterial solution without solvent. This decreased the amount of luminescence was due to the marked toxicity of these solvents to the bacterial suspension; therefore EGPE and formamide are not suitable for use in the luminescent *umu* test.

Although the amount of luminescence of THF was small, no toxicity occurred when it was used immediately after the bottle was opened. However, as reported by Maron and coworkers[4], noteworthy toxicity occurred when the solvent was used about 170 days after the bottle was opened (Fig. 1); this difference in observed toxicity suggests that THF is unstable and therefore unsuitable for use in the luminescent *umu* test. Among the remaining 10 solvents, increasing the volume of solvent added tended to decrease the amount of luminescence. The maximal luminescence achieved by each solvent (difference from bacteria-only well) and the amounts of solvent added are as follows: DMSO, 5000 counts/sec (7 μℓ); methanol, 300 counts/sec (2 μℓ); acetonitrile, 200 counts/sec (4 μℓ); dioxane, 2000 counts/sec (2 μℓ); THF, 600 counts/sec (2 μℓ); acetone, 1300 counts/sec (7 μℓ); cyclohexane, 200 counts/sec (1 μℓ); ethanol, 1000 counts/sec (7 μℓ); DMF, 1700 counts/sec (2 μℓ); EGME, 7500 counts/sec (4 μℓ); GF, 3500 counts/sec (4 μℓ); 1M2P, 1800 counts/sec (1 μℓ); and THFA, 3000 counts/sec (1 μℓ). Because the amount of luminescence obtained in the absence of solvent (bacterial solution and buffer solution only) was 1000 to 2000 counts/sec, we conclude that cyclohexane, methanol, acetonitrile, ethanol, and acetone can be used as solvents without compromising the results of the luminescent *umu* test.

Conventional umu test using solvents with little luminescence response

The solvents that did not cause much change in the amount of luminescence even at maximal volumes were cyclohexane, methanol, acetonitrile, ethanol, and acetone. The balance between the effect of these solvents on the luminescence mechanism and their toxicity may account for this result, causing the change in the amount of luminescence to appear smaller than in actuality. To assess this possibility, we evaluated the SOS response of these 5 solvents in the conventional *umu* test, which measures light absorbance. In addition, we estimated the viable cell count at 4 h after the start of the reaction by measuring the OD_{595}.

As shown in Fig. 2, none of the tested solvents produced an intense SOS response or strong growth inhibition. Although ethanol at 10 μℓ led to growth inhibition of ~40 %, little occurred at the solvent volume typically used in the *umu* test (4 μℓ). Since only a slight growth inhibition was observed with this amount, ethanol could also be used as a solvent for the *umu* test. Of the 15 types of solvent we tested, we found acetonitrile, methanol, acetone, and cyclohexane to be most suitable for use in both luminescent and conventional *umu* tests.

Luminescent umu test with methanol used as solvent

Of the 5 effective solvents, high-purity methanol can be obtained at low cost. We therefore used methanol as the solvent in luminescent *umu* tests in which we evaluated 2 typical mutagens, sodium azide and dichloromethane extract from vehicle exhaust particles

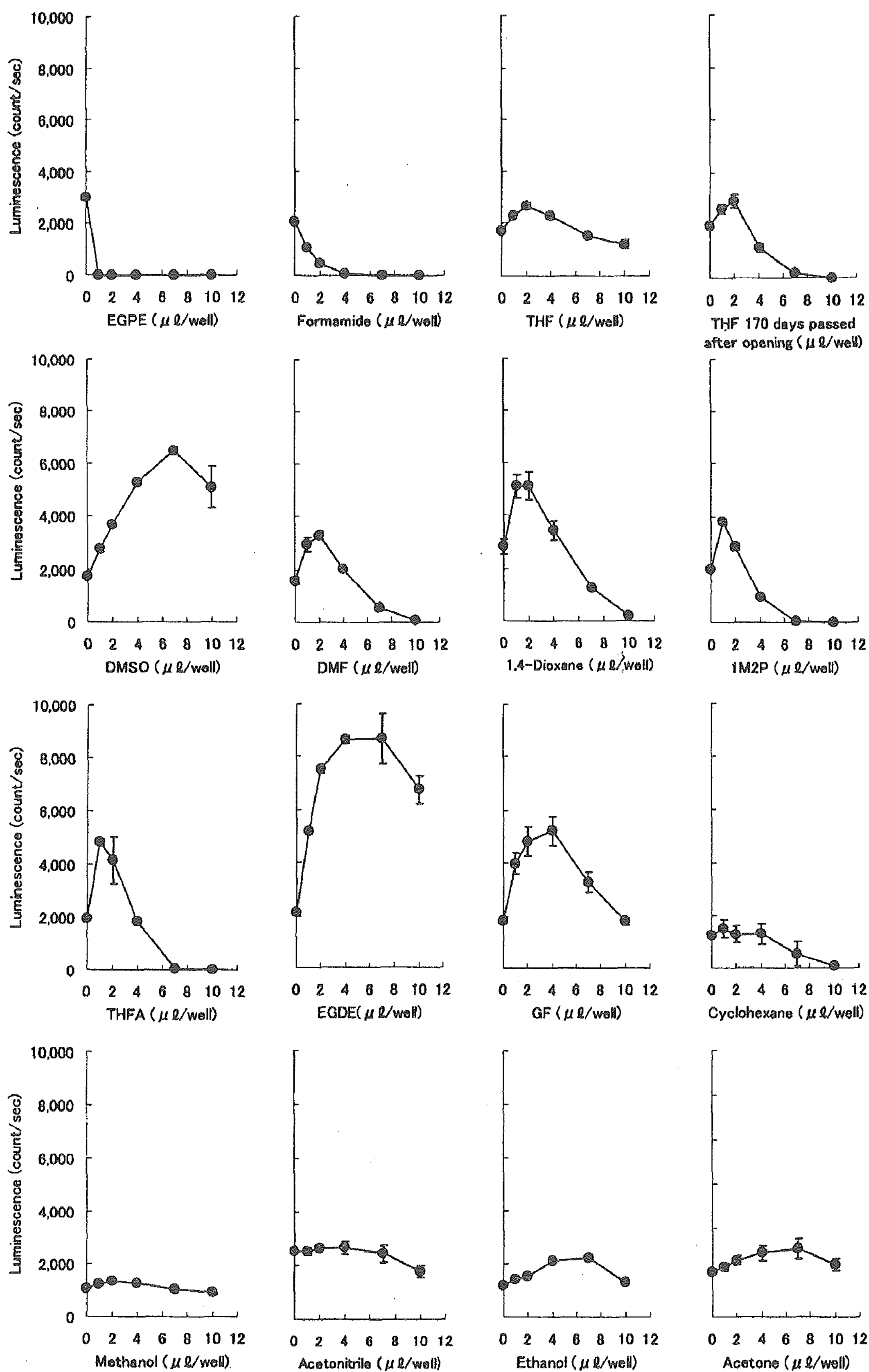

Fig. 1 Results of luminescent *umu* tests using various organic solvents

(NIES No. 8). For comparison purposes, we also tested samples diluted in DMSO, a solvent used in the Ames method. Both mutagens displayed a pronounced dose-response effect, and the amount of luminescence of the methanol-containing samples was twice as large as that of DMSO within the concentration range of the test, a positive result (Fig. 3).

The dose-response relation of the 2 mutagens was lower when methanol was used instead of DMSO, and the amount of luminescence for both decreased similarly. This result indicates that DMSO causes a relatively high background and therefore is unsuitable for

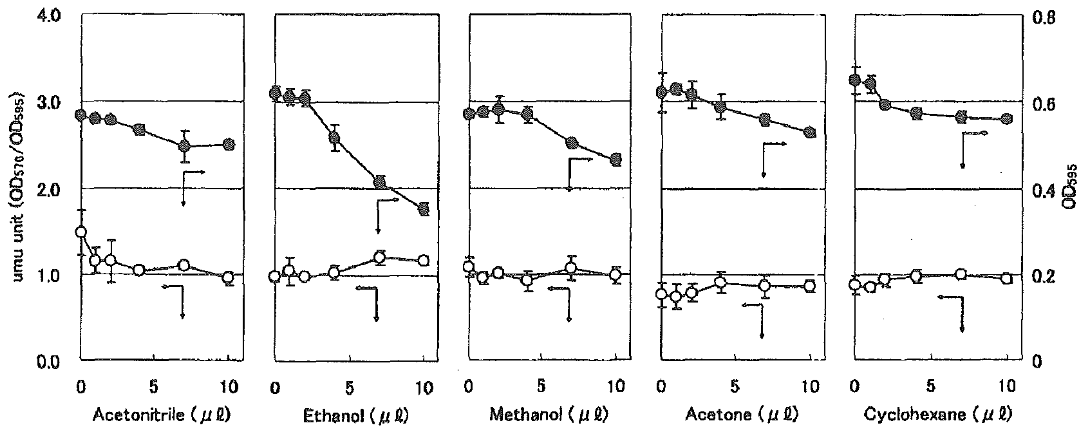

Fig. 2 Results of conventional *umu* tests using various organic solvents
○, *umu* unit (OD_{570}/OD_{595}); ●, OD_{595}

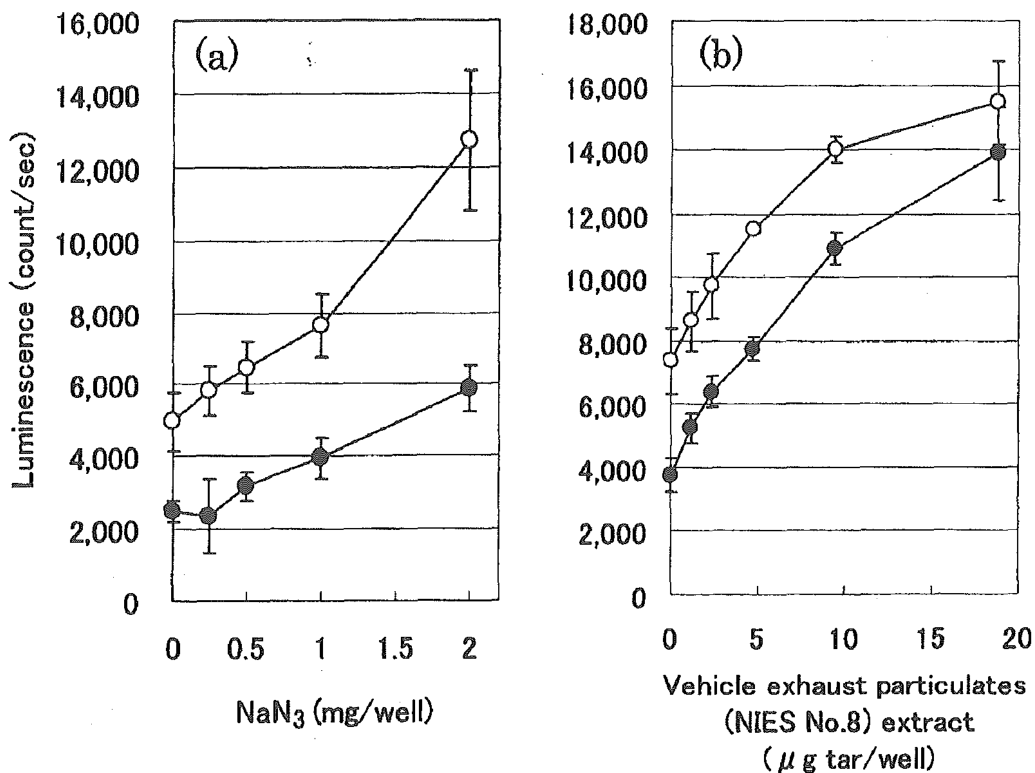

Fig. 3 Results of luminescent *umu* tests of (a) sodium azide and (b) vehicle exhaust particles (NIES No. 8) in which DMSO and methanol were used as solvents
○, DMSO; ●, methanol

the detection of substances of low mutagenicity (e.g., in situations of positive versus negative judgment). In contrast, methanol, which causes less background, is suitable for the detection of mutagenicity of minute substances. In addition, compared with DMSO, methanol also showed slightly higher sensitivity, on condition that the variation of test results is the same.

Together, the results of our current study indicate that cyclohexane, methanol, acetonitrile, ethanol, and acetone can be used for the luminescent *umu* test and that methanol is a good choice when detecting the mutagenicity of environmental samples. Given the fact that the luminescence response of DMSO, which has thus far been widely used for the test, was relatively high, we feel that the remaining 4 solvents will be more effective than DMSO for testing samples with low SOS response.

ACKNOWLEDGEMENT

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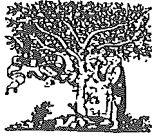
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Rejection properties of aromatic pesticides with a hollow-fiber NF membrane

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Abstract

NF membranes have been drawing much attention in the field of drinking water treatment because they can remove hazardous organic micro-pollutants such as pesticides and THM precursors, as well as hardness. The rejection properties for 10 kinds of aromatic pesticides (0.045–0.194 $mg \cdot L^{-1}$) were investigated with a bench-scale flow cell equipped with a hollow-fiber NF membrane (HNF-1, the nominal desalting degree 35% at 0.3 MPa). Both the permeate and retentate were returned to the feed tank, and separation experiments were conducted for 10 days. Although the feed and retentate concentrations decreased in the first half of the experimental period, stable rejections were obtained in the latter half of the period. The rejections were calculated based on the average concentrations of the retentate and feed, and the removals were on the initial feed concentration. The removals were in the range of 45.0–93.8%, but the rejections based on the feed concentrations were relatively low (41.0–88.4%). The logarithm of the solute permeability (log B) correlated linearly with the molecular weight of the pesticides. In addition, the batch-type adsorption experiments indicated the following results: all pesticides were adsorbed on the membrane and the adsorption properties were controlled not only by hydrophobicity (log P) of the pesticides but also by molecular planarity.

Keywords: Hollow-fiber NF membrane; Aromatic pesticide; Molecular weight; Log P; Adsorption

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1. Introduction

The increased use of pesticides has led to many benefits such as advanced productivity, lower maintenance costs in agriculture, and the improvement of public health. On the other hand, their adverse effects have also grown [1]: an increase of the risks to the ecosystem and human health.

One of the sources for the risk of human health is drinking water contaminated by hazardous micro-organic pollutants including pesticides and endocrine disrupting chemicals. Activated carbon adsorption processes have been commonly applied to remove trace organic pollutants such as pesticides [2].

Removal performance in the activated carbon adsorption processes strongly depends on the physicochemical properties of pollutants [3], and therefore membrane separation technology is focused on as an alternative water treatment process. NF membranes are useful for drinking water treatment because they can operate at relatively lower pressure and can effectively remove both hardness and THM precursors [4–6].

Many researchers have reported that NF membranes can also effectively remove hazardous organic micro-pollutants [7–14]. The factors controlling the rejection properties of organic solutes have been discussed based on a variety of physicochemical properties of solute; however, only limiting information is available.

In our previous works where the rejection properties of pesticides and alkyl phthalates were examined with flat-sheet-type NF membranes [8–10], the following results were obtained: (1) higher desalting NF membranes rejected almost all solutes at more than 95%, (2) some compounds were rejected effectively even by lower desalting membranes, (3) the rejection properties were influenced not only by steric hindrance but also by an affinity to the membrane.

The rejection properties of a hollow-fiber membrane (HNF-1) for non-phenilic pesticides were also investigated in our previous work [11] where the rejection properties were discussed on the basis of short-term (5 h) of membrane separation experiments. The fact that the pesticides were adsorbed on the membrane suggested that it is necessary to conduct the experiments for longer periods in order to evaluate the effects of the adsorption. In addition, it was found that aromatic pesticides were adsorbed more than non-aromatic pesticides.

Some researchers indicated that the adsorption of hazardous organic micro-pollutants affected their rejection [15–17]. In our previous works [8,10], it was found that other NF membranes also adsorbed almost all pesticides. In this work the membrane separation experiments were conducted with a hollow-fiber membrane (HNF-1) for 10 days where 10 kinds of aromatic pesticides with a wide range of hydrophobicity were used as solutes. The concentration variations of the feed solution and the permeates were examined and the factors controlling the rejection properties were discussed. In addition, the adsorption properties of the pesticides on the membrane were also examined.

2. Experimental

2.1. Membrane and solutes

Mini-modules of the HNF-1 membrane used in this work were prepared for a bench-scale cell: 400-mm-long filament, total area of 228.5 cm^2, and average pure water flux of 324 $L{\cdot}m^{-2}{\cdot}d^{-1}$ (RSTD = 4.48%) at 0.3 MPa. A prototype module of HNF-1 was prepared as CM-10 (membrane area 9 m^2; length 440 mm; diameter 100 mm) [18]. The characteristics of the HNF-1 are summarized in Table 1.

The pesticides used in this work are listed in Table 2, where all solutes are presently included in the WHO regulation for drinking water quality or endocrine disruptors, and they have a wide variety of log P values. The pesticides were used

Table 1
Nominal properties of HNF-1 membrane

Structure and materials	Hollow fiber (composite membrane) Skin layer:polyamide; Support membrane:polysulfone
Dimension	o.d. 350 μm; i.d. 200 μm
Membrane performance and operation condition	Solution flux 250 $L \cdot m^2 \cdot d^{-1}$; Salt rejection 35% Salt; NaCl 500 $mg \cdot L^{-1}$; Pressure 0.3 MPa; pH 6.0
Other property	$MgSO_4$ rejection 93% (at 0.5 MPa)

Table 2
Properties and rejections of the pesticides

No.	Pesticide	Initial conc. (mg/L)	Molecular weight	Molecular width (nm)	log *P*	Dipole moment (D)	Rejection (%)	Removal (%)
1	Propiconazole	0.120	342.2	0.481	4.58[a]	3.81	82.3	86.3
2	Carbaryl (NAC)	0.111	201.2	0.377	2.36	3.24	70.9	86.9
3	Chlorothalonil	0.045	265.9	0.320	2.90	2.10	69.5	80.1
4	Propyzamide	0.114	256.1	0.437	3.17[a]	2.61	64.9	72.2
5	Chloroneb	0.102	207.1	0.365	3.09	3.04	88.4	93.8
6	Methyl dymron	0.151	268.4	0.406	2.56[a]	3.54	64.4	77.9
7	Fenobucarb	0.137	207.3	0.411	2.78	4.84	64.1	68.1
8	Tricyclazole	0.194	189.2	0.331	1.70	6.09	58.1	74.9
9	Esprocarb	0.134	265.4	0.446	4.52[a]	1.97	55.4	77.5
10	Mefenacet	0.173	298.4	0.354	3.23	6.09	41.0	45.0

[a]Measured in this work; the others from Hansh et al. [19].

without further purification. The pesticide stock solutions were prepared by the method described in our previous work [11], and the feed solutions were prepared by the dilution of the stock solutions. The molecular width was also calculated by the method described in our previous work [10]. The logarithm of *n*-octanol/water partition coefficient (log *P*) for pesticides was used as a parameter of hydrophobicity in this work; some values were measured based on the method in our previous work [10], and the others were obtained from Hansh et al. [19].

2.2. Membrane separation experiments

A bench-scale flow cell used in this work was the same one as shown in a previous work [11]: the volume of a 10 L feed tank and a cell (23.0 mm i.d.×1,150 mm long) equipped with the HNF-1 membrane.

The membrane separation experiments were conducted under the following conditions: the temperature was at 25°C, the applied pressure was 0.3 MPa, the flow rate in the cell was ca. 16.4 $cm \cdot s^{-1}$, and the experimental period was 10 days. The initial concentrations (0.045–0.194 $mg \cdot L^{-1}$) of feed solution are listed in Table 2.

In general, the retentate and permeate were returned to the feed tank. The samples for analysis were obtained daily from the feed tank, the permeate stream, and the retentate stream. A new membrane was used for each pesticide to prevent the effects of contamination of the pesticide used

previously. The separation experiment for each pesticide was conducted after the measurement of the pure water flux with milli-Q water. After the separation test for each pesticide, the pipeline of the apparatus was washed with methanol and rinsed with pure water. Subsequently, additional experiments without the membrane were performed as a blank test in the same way as the membrane separation tests.

The rejection (R_j) was calculated based on the average concentration of feed and retentate as expressed by Eq. (1). The removal (R_m) was calculated based on the initial feed concentration as expressed by Eq. (2).

$$R_j = 1 - \frac{C_p}{(C_r + C_f)/2} \tag{1}$$

$$R_m = 1 - \frac{C_p}{C_{f,0}} \tag{2}$$

where C_p, C_r, C_f, and $C_{f,0}$ are the concentrations of the permeate, retentate, feed solutions, and the initial feed solution, respectively.

In this work the effects of the concentration polarization were not considered. The pesticide concentrations were analyzed by the HPLC-direct injection method [20]. Since this method does not require any pretreatments including extraction and enrichment, any losses of target compounds are negligible in the analytical procedure, and the quantification levels were μg/L or sub-μg/L.

2.3. Adsorption properties of the pesticides on the membrane

The adsorption properties of the pesticides on the HNF-1 membrane were examined by batch-type experiments. An appropriate amount of cut membrane filaments was immersed into a vial containing pure water, and the pesticide stock solution was added after degassing by suction. The solution was shaken at 70 rpm for 15 days to obtain equilibrium conditions.

3. Results and discussion

3.1. Rejection properties

In the case of hydrophilic compounds such as saccharides, the concentrations of the feed and the retentate were stable within a few hours as described in our previous work [12]. In the case of the pesticide solutions, however, the concentrations for some pesticides did not reach a stable level within a few hours, as shown in Fig. 1. Therefore, the separation experiments for the pesticides were carried out for 10 days. The rejection profiles of some pesticides are shown in Fig. 2. Although the rejections varied in the first half of the experimental period, relatively stable rejections were obtained in the latter half of it.

The concentration profiles of the feed, retentate, and permeate for propyzamide and chloroneb are shown in Fig. 3. The concentrations of the feed and the retentate decreased obviously during the first half of the experimental period. Similar results were also observed for carbaryl, chlorotalonil, and propiconazole. The results may

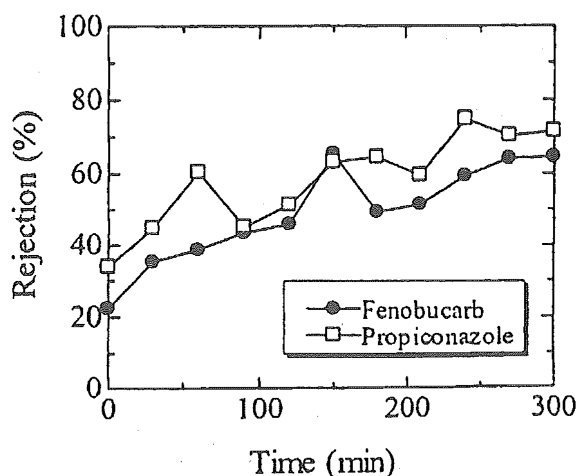

Fig. 1. Rejection profile in the short-term operation (5 h).

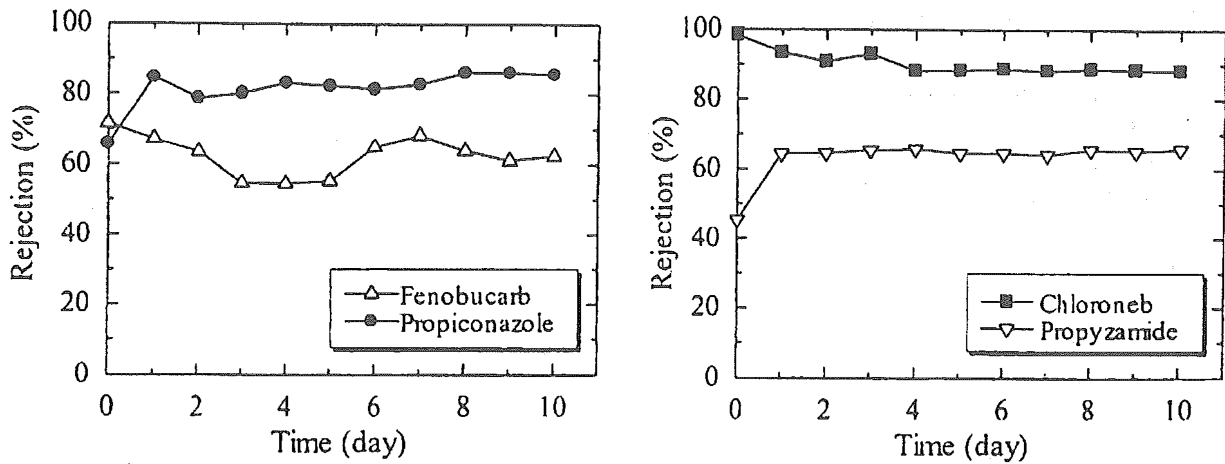

Fig. 2. Rejection profile in the short-term operation (10 days).

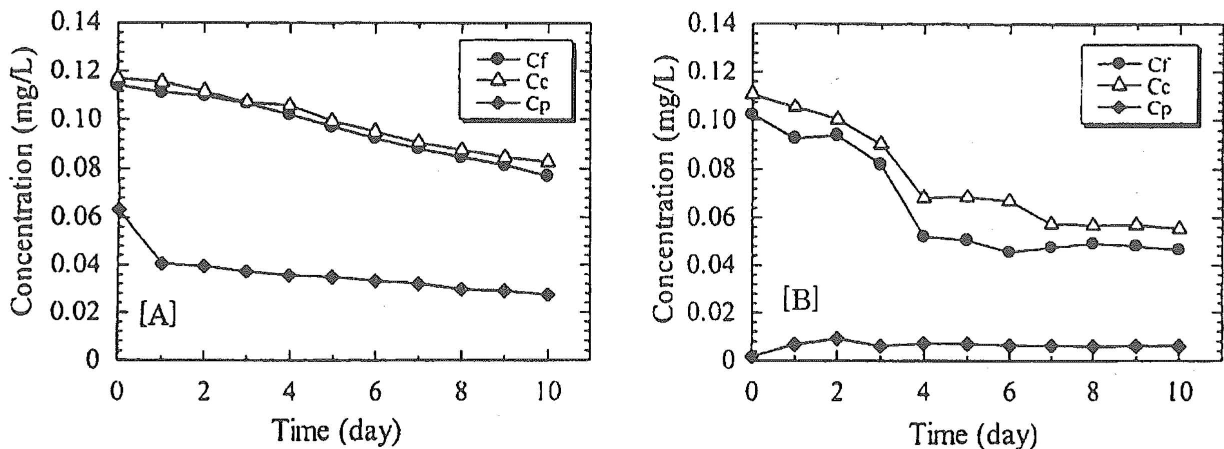

Fig. 3. Concentration profiles of feed, retentate and permeate. [A] propyzamide, [B] chloroneb. C_f, feed; C_r, retentate; C_p, permeate.

be caused by the following factors: vaporization, decomposition, and/or adsorption on the membrane and the pipeline in which polymeric materials were used as an end-cap and O-ring. However, the effects of vaporization may be small because of very low volatile compounds. Similar results were also found in the blank tests conducted without a membrane, and therefore, the effects of the adsorption on the membrane were not evaluated explicitly in the experiments.

It is pointed out that the retentate concentrations were significantly higher than the feed concentration, and the results obviously indicate that the pesticides were rejected by the membrane. Moreover, it was found that the solution fluxes were almost equal to the pure water fluxes. Therefore, even if the pesticides were adsorbed on the membrane, the adsorption did not influence the solution flux.

The rejections (R_j) were also varied due to the decrease of the feed concentrations; however, they indicated relatively stable values in the latter half of the experimental period as shown in Fig. 2. The average values of the rejections for

7–10 days were used as the rejections in later discussions. The rejections were in the range of 41.0 to 88.4% as shown in Table 2. The rejections of the pesticides are significantly lower than those of glucose and sucrose, although the molecular weights of the pesticides (189.2–342.2 Da) are similar to those of saccharides. Considering that the rejection is mainly controlled by the molecular sieving effect [11], the results for pesticides suggest some influence by other factors such as interaction with the membrane.

The removals (R_m) based on the initial feed concentrations are also shown in Table 2: the removal includes the effects of the disappearance of the pesticides as mentioned above. The values of R_m were 45.0–93.8%; however, the values are also lower than the rejections of saccharides.

3.2. Adsorption properties

Although the adsorption amounts of the pesticides were not evaluated in the blank tests, significant adsorption was observed by the batch adsorption experiments where a state of equilibrium was obtained over 10 days. Our previous works [8–11] showed that almost all pesticides used for the examination were adsorbed on several types of NF membranes, and the adsorption property was correlated with hydrophobicity of the solute. Therefore, the logarithm of the partition coefficients (log K) were plotted against log P as shown in Fig. 4, where the partition coefficient is described as the following equation:

$$K = \frac{q}{C} \tag{3}$$

where K is the partition coefficient $[(\mu g \cdot cm^{-2})(mg \cdot L^{-1})^{-1}]$, q the adsorption amount $[\mu g \cdot cm^{-2}]$, and C the concentration of bulk solution $[mg \cdot L^{-1}]$.

Since log K and log P are the functions of free energy for partition and adsorption, respectively, a correlation between these parameters may be expected. However, the correlation coefficient for all solutes was very low. When the solutes were classified into two groups (A and B), log K for both groups A and B was correlated with log P, respectively. The groups were classified based on molecular planarity, where the molecular planarity was evaluated with the P-MWd decided as follows: when a phenyl group is fixed on the X–Y plane, the length of a molecule in the Z-axis direction was calculated and one-half of this length was taken as P-MWd [10]. Group A, shown in Fig. 4, includes planar compounds (P-MWd <0.3 nm) and group B non-planar compounds (P-MWd >0.3 nm). The planar compounds were adsorbed more effectively than the non-planar compounds. The result is similar to the case of the NTR membrane [10] where bulky substituents to the phenyl group hinder adsorption. However, the following must be pointed out: the solutes were adsorbed not only on the skin layer (polyamide) but also on the support layer (polysulfone); therefore, the adsorption amounts are the sum of those in both phases. In the membrane permeation process, since a part of the solute permeated through the skin layer may be entrapped on the support layer, the effects of the adsorption may appear more clearly after a much longer experimental period.

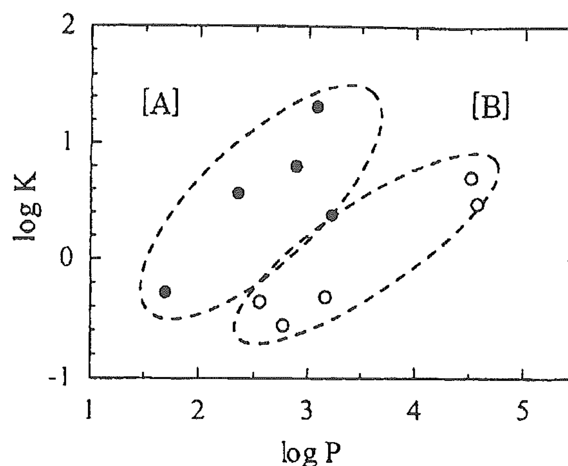

Fig. 4. Relationship between adsorption property (log K) and log P.

The logarithm of the partition coefficient, log K, was also plotted against the dipole moment of solutes as shown in Fig. 5. When the datum of tricyclazole was neglected, both parameters were well correlated. Considering the fact that the dipole moment was not correlated with log *P*, polar effects may also play a significant role for adsorption.

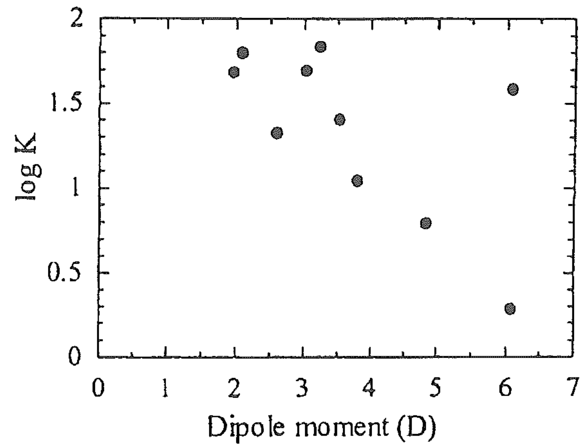

Fig. 5. Relationship between adsorption property (log K) and dipole moment.

3.3. Factors controlling solute separation

Rejections of organic compounds have been discussed with a number of physicochemical properties such as molecular size, solubility, dissociation, polarity, hydrophobicity, etc. [5,

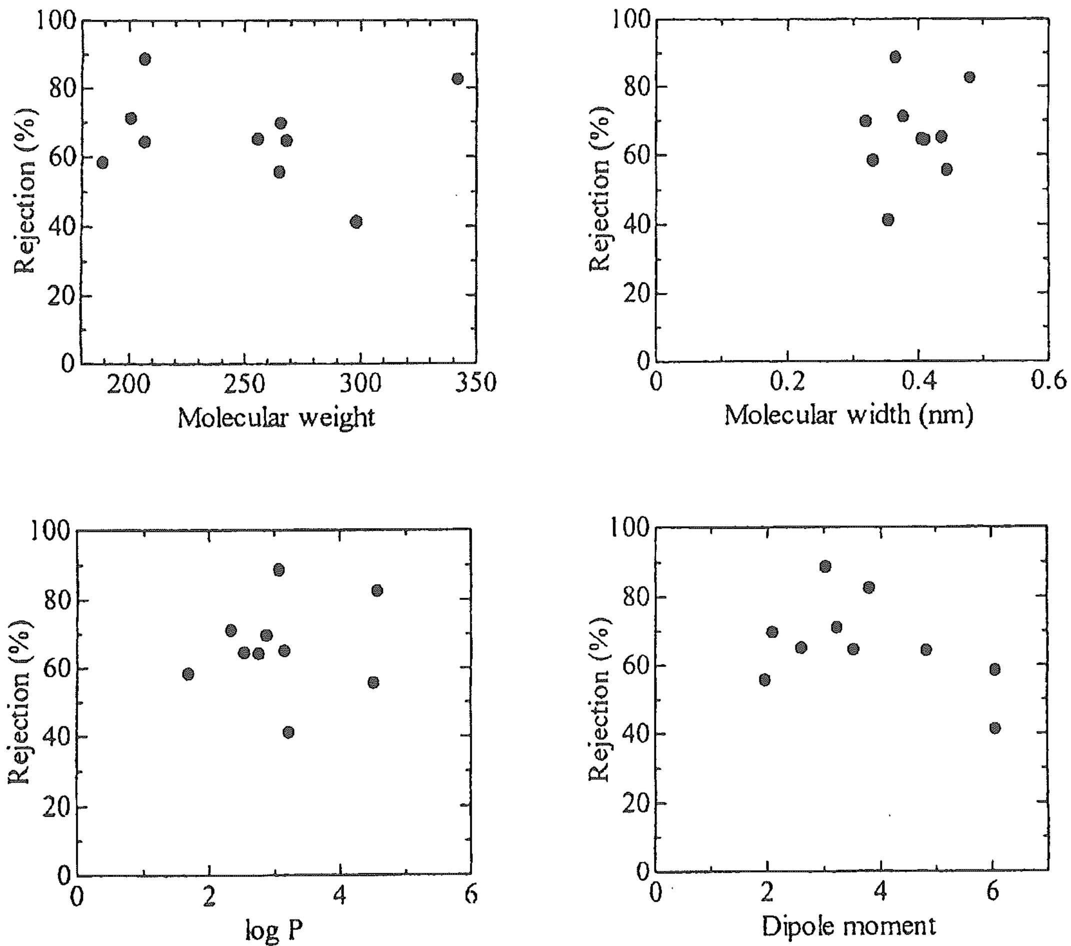

Fig. 6. Relationship between rejection and properties of the solute

8–17,21]. As a first step, the influencing factors for the rejection were examined with the properties related to a whole molecule. Fig. 6 shows the plots of the rejection against molecular weight, molecular width, dipole moment, or log P. The former two parameters are size parameters and the latter parameters are ones in relation to interaction between membrane and solute. Van der Bruggen et. al. [21] suggested that the dipole moment is related to the orientation to the membrane pore. However, no parameters were significantly correlated with the rejection.

The logarithm of solute permeability (log B), which is defined by Eq. (4), was correlated with molecular weight as shown in Fig. 7.

$$J_S = B\left(C_f - C_p\right) \quad (4)$$

where J_s is the solute flux, and C_f and C_p are concentrations of the feed and permeate solutions, respectively.

In nanofiltration processes, a part of a solute may permeate by convective transport as well as diffusive transport. Since the solute permeability is based on a solution–diffusion model, it does not include the effect of convective transport. The solute permeability used in this work is only an apparent parameter. In the case of the HNF-1 membrane, a similar relationship was obtained for non-phenylic pesticides appeared in our previous work [11], although for hydrophilic compounds log B was correlated linearly with molecular width. On the other hand, in the systems of hydrophobic compounds and NTR membranes, log B was not correlated linearly with molecular width but with log P (for alkyl phthalates) [9] or linear combination of log P and log K (for pesticides) [10]. Based on these results, it can be seen as a feature of the HNF-1 membrane that log B and molecular weight are useful descriptors for estimation of the rejection.

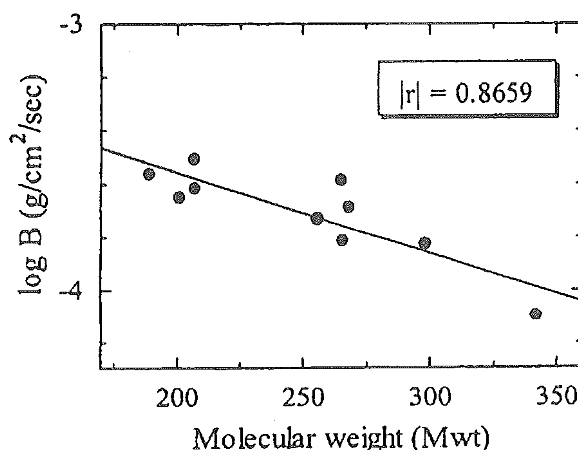

Fig. 7. log B vs. molecular weight.

4. Conclusions

The rejection properties of 10 kinds of aromatic pesticides were examined with a hollow-fiber NF membrane (HNF-1) and the results are summarized as follows:

1. Concentrations of the feed and retentate solutions decreased in the experimental period of 10 days, and stable rejections were obtained in the latter half of the experimental period.
2. Rejections of the pesticides were in the range of 41.0 to 88.3%, and the removals based on the initial feed concentration were in the range of 45.0 to 93.8%.
3. It was found that all of the pesticides were adsorbed on the membrane and that the adsorption property was influenced not only by hydrophobicity but also by molecular planarity of the solute.
4. The apparent solute permeability is correlated linearly with the molecular weight of the pesticide.

The relationship between the pesticide permeability and the molecular weight was also observed for non-phenilic pesticides, and therefore, the results may characterize a feature of the HNF-1 membrane, although the factors controlling the solute permeability were not identified. It is pointed out that the effects of adsorption on the

solute permeation will be one of the important subjects in discussing the membrane separation mechanism.

Acknowledgement

This work was financially supported by a Grant-in-Aid for Scientific Research (15360285) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the 21st Century COE Program "Ecological Engineering for Homeostatic Human Activities" from the Ministry of Education, Culture, Sports, Science and Technology.

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環境水中のエンドトキシン存在形態に関する研究

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Detection of Total- and Free-Endotoxin in Environmetal Water, by Yumiko OHKOUCHI, Kyosuke TAKAHASHI, Keisuke KODERA, Sadahiko ITOH (Dept. of Urban Management, Kyoto Univ.)

1．はじめに

水環境中には多種多様な微生物が存在しており，凝集-沈殿-ろ過の一連の浄水処理を通してある程度除去されるものの，一部は不活化された状態で水道水中に残存している。本研究では，微生物（グラム陰性細菌・シアノバクテリア）の細胞外膜構成物質に起因する生物活性（エンドトキシン）に着目する。エンドトキシンはヒトの細胞においても Toll-like レセプターを介して強い免疫反応を惹起する化学物質として知られており[1]，その活性は由来する微生物の種や量・細胞膜の状態により変化する特性があることから，水温／水質の変動に連動して活性量が大きく変動する可能性もある。そこで，本研究では環境水ならびに水道水中のエンドトキシン活性とその存在状態ならびに微生物量を調べた。

2．実験方法

琵琶湖・淀川水系から採取した水試料ならびに水道水を対象として，微生物量ならびに総エンドトキシン・遊離型エンドトキシンをそれぞれ測定した。微生物量は標準寒天培地および R2A 寒天培地を用いた平板培養法，ならびに DAPI 蛍光染色法により定量した。エンドトキシン活性は，カブトガニ血球抽出成分 LAL (エンドスペシー ES-50M，生化学工業)を使用したエンドポイント比色法(トキシカラー DIA-MP，生化学工業)により測定し，培養液を 14,000 (rpm)で 10 分間遠心分離した上清を遊離型エンドトキシン測定に供した。また，各試料とも TOC，E_{260}，濁度（660nm における吸光度），pH，懸濁物質の各項目について測定を行った。

3．結果と考察

琵琶湖・淀川水系において採取した水試料（No.1, 4-13）ならびに水道水（No.14, 15）のエンドトキシン活性測定結果を表 1 に示す。河川水・湖沼水ともに 100－2500(EU/mL)の範囲にあった。本調査は冬季に実施したが，一部の採水地点については夏季調査も実施しているものの，エンドトキシン活性の季節変動は確認できなかった。なお，下水処理放流水は 1.08×10^4（EU/mL）と自然水と比較して高い値を示した。総エンドトキシンに対する遊離型エンドトキシンの比率を表 1 に併せて示した。この比率は試料間で大きく異なり，水道水中では約 80%が遊離型で存在することがわかった。さらに，遊離型エンドトキシンが総エンドトキシンを上回る結果も複数得られており，無処理の環境水中に混入した懸濁物質または微生物細胞のミセル形成によりエンドトキシンの活性部位が阻害されている可能性も考えられる。

標準寒天または R2A 寒天培地を用いた平板培養による微生物量と総エンドトキシン量の関係を図 1 に示す。平板培養計数結果と総エンドトキシン活性の間には明確な相関は認められなかった。この理由として，1) 環境水中には VBNC(Viable but nonculturable) 微生物を始めとした種々の微生物が共存しており微生物種ごとにエンドトキシン活性を左右する Lipid A の組成が異なる[2]こと，あるいは 2) 微生物細胞外膜の状態は多様であり遊離型エンドトキシンの存在が大きく影響していることが挙げられる。

表 1　環境水中のエンドトキシン活性とその形態

No.	河川/湖沼	採水地点	総Et (EU/mL)	遊離型Et (EU/mL)	比
1	桂川	下水処理場放流水	1.08×10^4	1.26×10^3	0.117
4	琵琶湖南湖	大津港	4.28×10^2	2.20×10^2	0.514
5	琵琶湖南湖	なぎさ公園	3.31×10^2	2.30×10^2	0.695
6	琵琶湖南湖	矢橋	2.43×10^3	3.30×10^2	0.136
7	木津川	三川合流地点	6.21×10^2	1.77×10^3	>1
8	宇治川	三川合流地点	2.03×10^3	8.26×10^2	0.407
9	淀川	枚方大橋	1.51×10^3	2.87×10^2	0.190
10	琵琶湖疏水	北大路通	1.16×10^2	2.95×10^2	>1
11	琵琶湖疏水	若王子橋	1.46×10^2	1.77×10^2	>1
12	琵琶湖疏水	蹴上	1.55×10^2	1.82×10^2	>1
13	琵琶湖疏水	冷泉橋	2.57×10^2	1.94×10^2	0.755
14	水道水	京都市	1.99×10^0	1.53×10^0	0.769
15	水道水	京大	1.12×10^1	9.70×10^0	0.866

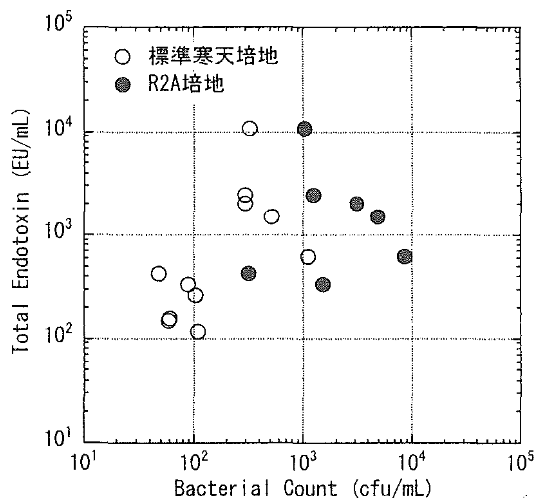

図 1　微生物量とエンドトキシン活性の比較

4．まとめ

本研究では，湖沼水・河川水・水道水中のエンドトキシン活性とその存在状態ならびに微生物量を調べた。その結果，遊離型エンドトキシンの比率は試料毎に大きく異なった。また，平板培養による微生物量はエンドトキシンの指標としては有効性が低いことから，エンドトキシンの代替指標探索が望まれる。

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B－14 不活化した大腸菌の損傷に関する研究

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1．はじめに

環境中には，悪条件や人工的な抗生物質にさらされることで損傷した細菌が存在する。これらの損傷した細菌は通常用いられる培地および培養条件では検出できない可能性があることが報告されている。さらに，自然系内の微生物には各々が環境中のストレスに応答するため，“無傷”，“様々な程度の損傷”“不可逆的(回復可能性の無い)損傷”の3つの状態が存在すると指摘されている[1]。

現在，塩素消毒だけでなく様々な消毒方法が検討されている。もし，病原細菌に対する消毒による損傷が，回復しうるような可逆的な損傷であった場合，危険が残る可能性がある。また新たな消毒方法の導入あるいは既存の消毒方法を考える際に，それらの消毒方法の効果，原理，特徴の違いによって，細菌への損傷程度の測定法への反映のされ方も異なると考えられる。本研究では2種の消毒処理により不活化された大腸菌の損傷程度と測定方法毎による差異と調べることによって，損傷程度の特徴を捉えることを目的とした。

2．実験方法

2．1 不活化処理

モデル微生物には*E.Coli* K 12(NBRC3301)を使用し，平面培地で37 ℃で24 時間培養して形成された大腸菌コロニーを釣菌し，リン酸緩衝液に溶解させて使用した。

（1）紫外線照射処理

初期条件は，大腸菌濃度が約 10^6 CFU/ml とした。濁質有り(落合水再生センターの一次処理水を使用)と無しの条件を設定した。濁質有りの場合は，濁度は約 240 NTU～260 NTU，吸光度(波長 254 nm)1.5 cm^{-1}～1.9 cm^{-1}であり，濁質無しでそれぞれ約 1 NTU～3 NTU，0.04 cm^{-1}～0.2 cm^{-1} であった。

光源として低圧水銀ランプ(20W　東芝殺菌ランプ)を使用した。試料をシャーレ(直径 5.7 cm)に入れて，スターラーで攪拌しながら紫外線を照射した。

試料の採取により液体体積が減少し，照射時の水深は 1.03 cm から 0.73 cm となるが，平均値 0.93 cm を平均線量率の計算に使用した。水面における紫外線線量率をヨウ酸カリウムとヨウ化カリウムを利用した化学線量計[2]にて測定し，平均値 0.37 mJ/s/cm^2 を平均線量率の計算に使用した。

（2）塩素処理

初期条件は大腸菌濃度が約 10^6 CFU/ml，濁度が 1.0 NTU～1.3 NTU とした。

塩素剤として次亜塩素酸ナトリウム溶液(キシダ化学(株))を使用し，初期塩素濃度を 0.07 mg/l～0.4 mg/l となるように投入し，各々の濃度において投入の 1 分後に試料を採取した。試料 10ml に対する脱塩素剤として，3％チオ硫酸ナトリウム(キシダ化学(株))を 0.1 ml 使用した。

2．2 大腸菌の測定方法

（1）コロニー形成法

細菌の増殖能力を測定するため，コロニー形成法を用いた。測定には，大腸菌群の選択培地であるデスオキシコーレイト寒天培地(栄研器材(株))と，非選択培地である Tryptic Soy Agar(Difco 和光(株))を使用し，共に重層寒天法により大腸菌濃度を測定した。

（2）ATP 法

細菌の代謝活性を測定するために，ATP 量を測定した。測定には，ATP 発光キット(東洋ビーネット(株))，ATP 抽出キット(東洋ビーネット(株))，Luminescence　JNR-ⅡAB2300(ATTO(株))にて ATP 量を求めた。

（3） 回復量測定法

細菌の損傷程度を測定するために不活化後の回復量を測定した。不活化後の試料を標準液体培地に投入し37℃で培養し，回復させた。

3． 実験結果と考察

Fig.1～Fig.3 に，紫外線処理および塩素処理後のコロニー形成法による濃度測定結果を示した。また Fig. 4～Fig. 6 に，それぞれの場合における 2 つのコロニー形成法測定結果の相関を示した。

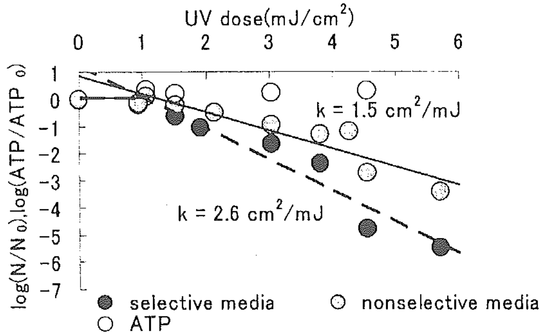

Fig. 1 inactivation by UV irradiation

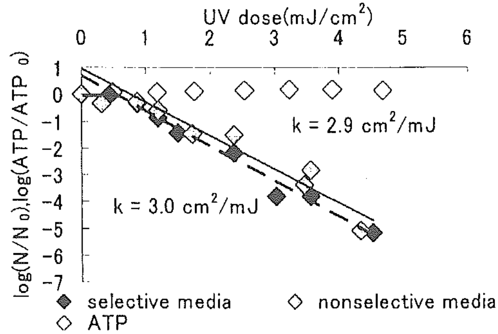

Fig. 2 inactivation by UV irradiation (with sludge)

紫外線による微生物数の減少は式(a)に従う。

$$\ln(N/N_0) = -kIt \qquad \text{(a)}$$

N：照射後微生物数，N_0：照射前微生物数

k：不活化速度定数，I：紫外線線量率，t：照射時間

Fig. 3 inactivation by Chlorine treatment

Fig. 1, Fig. 2 より濁質有りのほうが不活化速度定数が小さいことから，紫外線に対する感受性が大きいことがわかった。よって，濁質中には紫外線による DNA 損傷を補助する因子か，まったく別の損傷を与える因子が存在すると考えられる。選択培地と非選択培地によるコロニー形成能の差は濁質有りの方が小さかった。このことから，濁質中での紫外線不活化では濁質無しの場合と比べて深い損傷を与えられている可能性がある。ATP 量はほとんど変化せず，不活化後の大腸菌も ATP を保持していると考えられる。

塩素処理による大腸菌の減少は式(b)に従う。

$$\ln(N/N_0) = -k\int C\cdot dt \qquad \text{(b)}$$

N：塩素処理後微生物数，N_0：塩素処理前微生物数，k：不活化速度定数，C：塩素濃度，t：塩素処理時間

Fig. 3 より選択培地により培養できる大腸菌の減少は式 (b) に従ったが，非選択培地においては不活化が遅れた。このことから塩素濃度の違いにより不活化過程が異なることが考えられる。ATP 量の変化は小さく，紫外線処理と同様に，代謝機能の阻害は明確にはならなかった。

Fig. 4～Fig. 6 において，選択培地で培養できる状態を “active condition”，非選択培地で培養できて選択培地で培養できない状態を “slight damage”，両培地で培養できない状態を “severe damage or dead” と定義した。

紫外線処理(濁質無)と塩素処理を比較すると，選択培地による生存率($\log(N/N_0)$)が 0 から約-3 の範囲では同様の状態を示し，-3 以下では塩素処理の方が深い損傷状態に移行する細菌数が多かった。このことから，ある程度以上の不活化量では,紫外線処理(濁質無) よりも，塩素処理のほうが確実に損傷させることができることがわかる。紫外線処理(濁質有)と塩素処理を比較すると，紫外線処理(濁質有)では不活化後の大腸菌のほとんどが深い損傷状態になっているため，紫外線処理(濁質有)のほうが確実に損傷させると考えられる。

Fig. 7～Fig. 8 に回復後の結果を示す。回復時の ATP 生成量の増減より代謝機能の状態について考えた。選択培地により培養できる大腸菌の濃度が初期濃度に対して 10^{-2}，10^{-4}，10^{-6} となるように不活化し，各々を回

復させた。

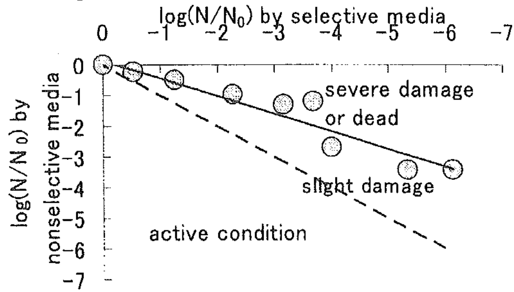

Fig. 4 damage of *E.Coli* K 12 after UV irradiation

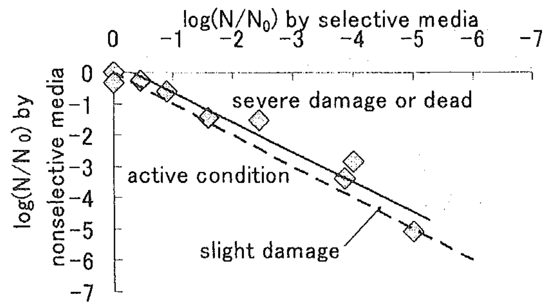

Fig.5 damage of *E.Coli* K 12 after UV irradiation(with sludge)

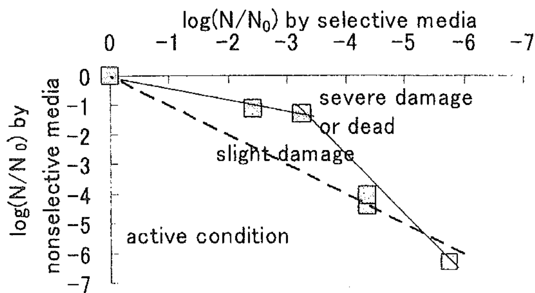

Fig. 6 damage of *E.Coli* K12 after Chlorine treatment

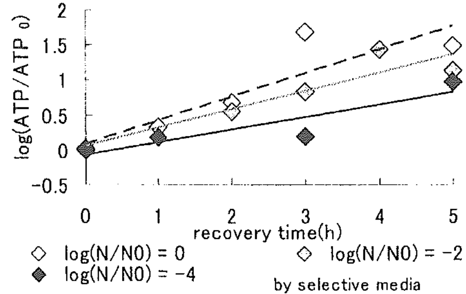

Fig. 7 recovery form UV damage

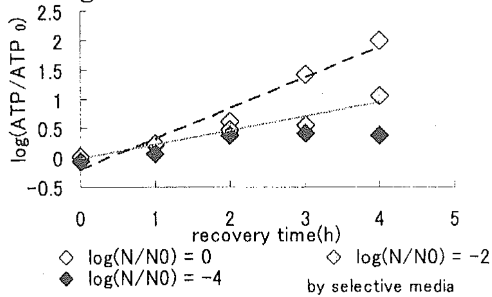

Fig. 8 recovery from UV damage (with sludge)

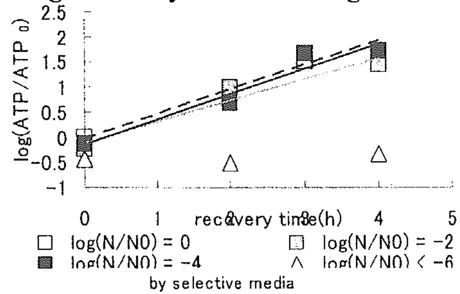

Fig. 9 recovery from Chlorine damage

Fig. 7，Fig. 8 より，紫外線処理では不活化により ATP 回復量が減少した。濁質無しでは代謝が活性化しているが 濁質有りでは，10^{-4} まで不活化した大腸菌の代謝活性化は停止した。よって紫外線処理の濁質共存下であればDNA 損傷の他に，代謝機能を阻害する因子が働くと考えられる。Fig. 9 より低濃度の塩素処理後の大腸菌は代謝が活発化した。低塩素濃度で処理した場合は可逆的な代謝阻害をし，高塩素濃度で処理した場合は不可逆的な代謝阻害をしたと考えられる。

4．まとめ

紫外線処理における濁質の有無による違いについては，濁質共存下のほうが不活化されやすく，選択培地で測定した不活化量が同じでも，深い損傷を与えている割合が多いことが示唆された。

塩素処理については，塩素濃度によって不活化過程が異なるが，紫外線処理とは異なる傾向を示すことがわかった。低塩素濃度では可逆的な代謝機能の阻害をするが，高塩素濃度では不可逆的な代謝機能の阻害をすることが考えられた。

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