to be positive in the MCF-7 assay and the Uterotropic assay (Schlumpf et al. 2001).

Some chemicals related to food contact plastics and rubbers have similar structures to that of the estrogenic chemicals mentioned above and were suspected to possess estrogenic activities. Therefore, we tried to test these many chemicals for estrogenic activity.

For this purpose, there are many useful *in vitro* screening tests such as the MCF-7 assay, receptor binding assays, reporter gene expression assays and so on. We chose the yeast two-hybrid assay which is a kind of reporter gene expression assays, because it was very simple and highly repeatable (Nishikawa et al. 1998, 1999). The yeast two-hybrid assay is based on the ligand-dependent interaction of the estrogen receptor (ER) α and the coactivator TIF2, and the estrogenic activity was detected as the β-galactosidase activity. Two expression plasmids, pGBT9-ERLBD and pGAD424-TIF2, were introduced into yeast cells (*Saccharomyces cervisiae* Y190), which carry a β-galactosidase reporter gene and require tryptophan and leucine for growth. By this method, more than 500 chemicals have already been tested for estrogenic activity, and the structure-activity relationships have been proposed (Nishihara et al. 2000). We also tested UV stabilizers and benzophenone derivatives by this method (Kawamura et al. 2003), and these results had a good relation with the results tested by a human estrogen receptor mediated mammalian reporter gene assay (Kawamura et al. 2005).

In this study, 150 chemicals were tested which comprised monomers, antioxidants, plasticizers, lubricants, vulcanizing agents, vulcanization accelerators and others, including previously reported UV stabilizers (Kawamura et al. 2003). We also tested all of their metabolites which were prepared with the S9-mixture according to the method by Takatori et al. (2003).

Materials and methods

Reagents

The test chemicals listed in Tables I–VIII were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma-Aldrich Japan Co. (Tokyo, Japan), or obtained from the manufacturers. Zymolyase 20T was purchased from Seikagaku Co. (Tokyo, Japan). *o*-Nitrophenyl-β-D-galactoside (ONPG) was purchased from Sigma-Aldrich Japan Co. and dissolved in 0.1 mole l^{-1} phosphate buffer (pH 7.0). The S9-extracts (rat liver 9,000 × g supernatant fraction induced by phenobarbital and 5,6-benzoflavone) and cofactor were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Cofactor was dissolved in 49 ml of milli-Q water. The S9-mix was prepared with 1 ml of S9-extract and 49 ml of cofactor solution, which contained 20 µl ml^{-1} S9, 0.8 µmol NADPH, 0.8 µmol NADH, 1.0 µmol glucode-6-phosphate (G6P), 0.4 U G6P dehydrogenase, 20 µmol Na_2HPO_4, 20 µmol NaH_2PO_4, 6.6 µmol KCl and 1.6 µmol $MgCl_2$.

Preparation of test chemicals

The test chemicals were dissolved in dimethylsulfoxide (DMSO) at 10^{-1} to 10^{-5} mol l^{-1} (final

Table I. Estrogenic activities of monomers and known estrogens.

Compounds (Other name or abbreviated name) [main product]	CAS No.	REC_{10} (mol l^{-1})	
		Parent comp.	Metabolite
‡17β-Estradiol (E_2)	87-18-3	3.4×10^{-10}	–
‡Bisphenol A	50-28-2	1.1×10^{-5}	–
‡4-Nonylphenol	80-05-7	4.6×10^{-7}	–
†*trans*-Stylbene	103-30-0	$>1.0 \times 10^{-3}$	1.1×10^{-5}
Acrylonitrile [polyacrylonitrile, AS resin, ABS resin, AB rubber]	107-13-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Adipic acid [polyamide, polyurethane]	124-04-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡Bis(4-glycidyloxyphenyl)methane [epoxy resin]	2095-03-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2-Bis(4-glycidyloxyphenyl)propane [epoxy resin]	1675-54-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Bis(4-hydroxyphenyl)methane (Bisphenol F) [epoxy resin]	620-92-8	1.3×10^{-5}	2.1×10^{-5}
Cyclohexanone-iso-oxine (ε-Caprolactam) [polyamide]	105-60-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡4-Cyclohexylphenol [phenolic resin]	1131-60-8	6.2×10^{-7}	2.1×10^{-5}
Diphenylmethane-4,4′-diisocyanate [urethane elastomer]	101-68-8	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Methylmethacrylate [polymethylmethacrylate]	80-62-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
α-Methylstyrene [polystyrene, ABS resin]	98-83-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†2-(Phenylmethyl)phenol (*o*-Benzylphenol) [phenolic resin]	28994-41-4	$>1.0 \times 10^{-3}$	2.5×10^{-5}
‡4-Phenylphenol [phenolic resin]	92-69-3	4.7×10^{-6}	1.3×10^{-5}
Styrene [polystyrene, AS resin, ABS resin]	100-42-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Toluene-2,4-diisocyanate [polyurethane]	584-84-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

‡Parent compound was positive, †Only metabolite was positive

Table II. Estrogenic activities of antivities of antioxidants.

Compounds (Other name or composition)	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
Bis(3,5-di-*tert*-butyl-4-hydroxybenzylphosphoric acid ethyl)calcium	65140-91-2	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Bis(2,4-di-*tert*-butylphenyl)pentaerythritol diphosphite	26741-53-7	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
2,4-Bis-(*n*-octylthio)-6-(4-hydroxy-3,5-di-*tert*-butylanilino)-1,3,5-triazine	991-84-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4,4'-Butylidenebis(3-methyl-6-*tert*-butylphenol)	85-60-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4,4'-Butylidenebis(3-methyl-6-*tert*-butylphenyl ditridecyl)phosphite	13003-12-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,5-Di-*tert*-amylhydroquinone	79-74-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,6-Di-*tert*-butyl-4-ethylphenol	4130-42-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,5-Di-*tert*-butylhydroquinone	88-58-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
3-(3',5'-Di-*tert*-butyl-4'-hydroxyphenyl)propionic acid stearyl ester	2082-79-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,6-Di-*tert*-butyl-4-methylphenol	128-37-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dimethylsuccinate polymer with tetramethyl hydroxy-1-hydroxyethyl piperidine	65447-77-0	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
N,N'-Di-2-naphtyl-4-phenylenediamine	93-46-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Distearyl pentaerythrityl diphosphite	3806-34-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2'-Ethylidenebis(4,6-di-*tert*-butylphenol)	35958-30-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4-Hydroxy-3-*tert*-butylanisole	25013-16-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4-Hydroxymethyl-2,6-di-*tert*-butylphenol	88-26-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡4,4'-Isopropylidenediphenol alkyl(C_{12}-C_{15})phosphite	3315-29-5	4.4×10^{-5}	2.1×10^{-5}
2,2'-Methylenebis (6-*tert*-butyl-4-methylphenol)	119-47-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4,4'-Methylenebis (2,6-di-*tert*-butylphenol)	118-82-1	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
2,2'-Methylenebis(4-ethyl-6-*tert*-butylphenol)	88-24-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2'-Methylenebis(4-methyl-6-methylcyclohexlphenol)	77-62-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Octadecyl-3,5-di-*tert*-butyl-4-hydroxyhydrocinnamate	2082-79-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2'-Oxamidobis[ethyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate]	70331-94-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†1-(*N*-phenylamino)naphthalene	90-30-2	$>1.0 \times 10^{-3}$	1.2×10^{-5}
Poly{[6-[(1,1,3,3-tetramethylbutyl)aminol-*s*-triazine-2,4-diyl][2,2,6,6-tetramethyl-4-piperidyl)imino]hexamethylene[(2,2,6,6-tetramethyl-4-piperidyl)imino]}	71878-19-8	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
‡Styrenated phenol (mono or di or tri)	61788-44-1	3.4×10^{-5}	4.7×10^{-5}
‡Styrenated phenol (mono 74%, di 24%)	61788-44-1	2.9×10^{-6}	1.3×10^{-5}
†Styrenated phenol (di 93%, tri 7%)	61788-44-1	$>1.0 \times 10^{-3}$	7.5×10^{-4}
Tetrakis[methylene-3-(3',5'-di-*tert*-butyl-4'-hydroxyphenyl)-propionate]methane	6683-19-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4,4'-Thiobis(3-methyl-6-*tert*-butylphenol)	96-69-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
3,3'-Thiodipropionic acid di-*n*-dodecyl ester (Dilauryl thiodipropioate)	123-28-4	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
3,3'-Thiodipropionic acid di-*n*-octadecyl ester (Distearyl thiodipropioate)	693-36-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
3,3'-Thiodipropionic acid di-*n*-tetradecyl ester (Dimyristyl thiodipropioate)	16545-54-3	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Triethyleneglycolbis[3-(3-*tert*-butyl-5-methyl-4-hydroxyphenyl) propionate	36443-68-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,3,5-Trimethyl-2,4,6-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl) benzene	1709-70-2	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Tris(2,4-di-*tert*-butylphenyl)phosphite	31570-04-4	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
1,3,5-Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6-(*1H,3H,5H*) trione	27676-62-6	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
1,3,5-Tris(4-*tert*-butyl-3-hydroxy-2,6-dimethylbenzyl)-1,3,5-triazine-2,4,6-(*1H,3H,5H*) trione	40601-76-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,1,3-Tris(2-methyl-4-hydroxy-5-*tert*-butylphenyl)butane	1843-03-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡Tris(nonylphenyl)phosphite	26523-78-4	1.0×10^{-5}	4.0×10^{-4}

‡Parent compound was positive, †Only metabolite was positive

concentrations: 10^{-3} to $10^{-7}\,mol\,l^{-1}$). When the chemical could not be dissolved at $10^{-1}\,mol\,l^{-1}$, the concentration was changed to 10^{-2} to $10^{-5}\,mol\,l^{-1}$ (final concentrations: 10^{-4} to $10^{-7}\,mol\,l^{-1}$). The concentration of DMSO was 1% in the assay, which did not inhibit the yeast growth. Each experiment was accompanied by 17β-estradiol (E_2) as the positive control and DMSO as the negative control.

Measurement of estrogenic activity by yeast two-hybrid assay

The yeast two-hybrid cells were preincubated overnight at 30°C with vigorous shaking in a SD medium which was free from tryptophan and leucine. The culture was diluted with 4 volumes of the fresh SD medium and 250 µl of this solution put into a small test tube. The test chemical solution (2.5 µl) was added and incubated for 4 h at 30°C.

After incubation, 150 µl of the culture solution was placed into each of the 96 wells of a microplate and the absorbancy measured at 595 nm. The rest of the culture was centrifuged at 10,000 rpm for 7 min, after which the supernatant was removed. The cells were enzymatically digested by incubation with $1\,mg\,ml^{-1}$ Zymolyase 20T (200 µl) at 30°C for 15 min. The cell lysate was mixed with $4\,mg\,ml^{-1}$ ONPG (40 µl) and incubated at 30°C for exactly 30 min. The reaction was stopped by the addition of $1\,mol\,l^{-1}$

Table III. Estrogenic activities of plasticizers.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
Bis(2-ethylhexyl)azelate	103-24-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
n-butyl benzyl phthalate	85-68-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di(ethylene glycol)dibenzoate	120-55-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di(propylene glycol)dibenzoate	27138-31-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-2-ethylhexyl adipate	103-23-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-2-ethylhexyl phthalate	117-81-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diacetyllauroyl glycerol	30899-62-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dibenzyl adipate	2451-84-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dibutyl sebacate	109-43-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dicyclohexyl phthalate	84-61-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diheptyl phthalate	41451-28-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisobutyl adipate	141-04-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisobutyl phthalate	84-69-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisodecyl adipate	27178-16-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisodecyl phthalate	26761-40-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisononyl adipate	33703-08-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisonyl phthalate	28553-12-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisooctyl phthalate	27554-26-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisopropyl adipate	6938-94-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dimethyl phthalate	131-11-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-alkyl adipate (C = 6, 8, 10)	–	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-butyl adipate	105-99-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dibutyl maleate	105-76-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-butyl phthalate	84-74-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dinonyl phthalate	84-76-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-octyl adipate	123-79-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-octyl phthalate	117-84-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-propyl adipate	106-19-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dioctyl sebacate	122-62-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diphenylcresyl phosphate	26444-49-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Ditridecyl phthalate	75359-31-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Epoxidized soybean oil	8013-07-8	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Heptylnonyl adipate	68515-75-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Phosphoric acid diphenyl 2-ethylhexyl ester	1241-94-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tributyl 2-acetylcitrate	77-90-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tri-*n*-butyl phosphate	126-73-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Trimellic acid tris(2-ethylhexyl)ester	3319-31-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	6846-50-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

Table IV. Estrogenic activities of lubricants.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
trans-trans-2,4-Decadienal	25152-84-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Docosanoic acid amide	3061-75-4	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
cis-13-Docosenic acid amide	112-84-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Hexadecanoic acid amide	629-54-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Octadecanoic acid amide	124-26-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
9-Octadecenoic acid amide	301-02-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Oleic acid	112-80-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

Na_2CO_3 (100 µl). After centrifugation at 10,000 rpm for 5 min, the supernatant (150 µl) was placed into each well of a microplate. The absorbances at 420 and 570 nm were read using a microplate reader. The β-galactosidase activity was calculated using the following equation:

$$U = 1000 \times ([OD_{420}] - [1.75 \times OD_{570}])/ ([t] \times [v] \times [OD_{595}])$$

where t = time of reaction (min), v = volume of culture used in the assay (ml), OD_{595} = cell density at the start of the assay, OD_{420} = absorbance by *o*-nitrophenol at the end of the reaction, and OD_{570} = light scattering at the end of the reaction.

The β-galactosidase activity was expressed as the mean and standard deviation of the results from three separate test tubes.

Preparation of metabolites and their measurement of estrogenic activity

To a tube containing 990 µl of the S9-mix, 10 µl of the test chemical solution (mainly 10^{-1} to

Table V. Estrogenic activities of UV-stabilizers.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
2,5-Bis(5′-*tert*-butyl-2′-benzoxazolyl)thiophene	7128-64-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
†4-*tert*-Butylphenylsalicylate	87-18-3	$>1.0 \times 10^{-3}$	2.7×10^{-5}
2,4-di-*tert*-Butylphenyl-3,5-di-*tert*-butyl-4-hydroxybenzoate	4221-80-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡2,2′-Dihydroxy-4-methoxybenzophenone	131-53-3	1.0×10^{-3}	1.3×10^{-4}
2-[2′-Hydroxy-3′,5′-bis(α,α-dimethylbenzyl)phenyl]-2H-benzotriazole	70321-86-7	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
2-(2′-Hydroxy-3′-*tert*-butyl-5′-methylphenyl)-5-chlorobenzotriazole	3896-11-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
2-(2′-Hydroxy-3′,5′-di-*tert*-amylphenyl) benzotriazole	25973-55-1	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
‡2-Hydroxy-4-methoxybenzophenone	131-57-7	6.6×10^{-4}	2.0×10^{-5}
2-(2′-Hydroxy-5′-methylphenyl) benzotriazole	2440-22-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-Hydroxy-4-*n*-octyloxybenzophenone	1843-05-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

‡Parent compound was positive, †Only metabolite was positive

Table VI. Estrogenic activities of vulcanizing agents and vulcanization accelerators.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
1,4-Benzoquinone dioxime	105-11-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Benzoyl-peroxide	94-36-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
n-Cyclohexyl-2-benzothiozolyl sulfenamide	95-33-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2′-Dibenzothiazolyl disulfide	120-78-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
4,4′-Dibenzoylquinone dioxime	120-52-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
1,3-Diphenylguanidine	102-06-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4,4′-Dithiodimorpholine	103-34-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Hexamethylenetetramine	100-97-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-Mercaptobenzothiazole	149-30-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-(4-Morpholinodithio)benzothiazole	95-32-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-(Morphilinothio)benzothiazole	102-77-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Piperidinium pentamethylenedithiocarbamate	98-77-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Sodium dimethyldithiocarbamate dihydrate	72140-17-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tetraethylthiuram disulfide	97-77-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tetra-*n*-butylthiuram disulfide	1634-02-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1-(2-Tolyl)biguanide	93-69-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

Table VII. Estrogenic activities of miscellaneous additives.

Compounds (Other name)	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
2,5-Bis(*tert*-butylperoxy)-2,5-dimethylhexane	78-63-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*tert*-butyl-peroxide	110-05-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡2,4-Diphenyl-4-methyl-1-pentene	6362-80-7	6.6×10^{-5}	6.6×10^{-5}
Glycerol trilaurate (Trilaurin)	538-24-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Isopropyl benzene (Cumene)	98-82-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†Nonylphenol ethoxylate	26027-38-3	$>1.0 \times 10^{-3}$	4.5×10^{-5}
Oleyl alcohol	143-28-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

‡Parent compound was positive, †Only metabolite was positive

$10^{-5}\,mol\,l^{-1}$ which corresponds to 5×10^{-4} to $5 \times 10^{-8}\,mol\,l^{-1}$ of final concentration) was added, incubated at 37°C for 4 h and then stored at −80°C until the yeast two-hybrid test was run as metabolite solution. Each experiment was accompanied by *trans*-stylbene to confirm the metabolic activity.

The yeast two-hybrid cells were pre-incubated overnight at 30°C with vigorous shaking in a SD medium free from tryptophan and leucine, then diluted with 1.5 volumes of fresh 2 × SD medium. In a small test tube, 125 µl of the cell solution and 125 µl of the metabolite solution were mixed and then

Table VIII. Estrogenic activities of other chemicals.

Compounds (Other name)	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
Bisphenol A bis(2,3-dihydroxypropyl)ether	5581-32-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Bisphenol A bis(3-chloro-2-hydroxyphenol)ether	4809-35-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-*tert*-Butyl-4-methylphenol	2409-55-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,3-Diphenylpropane	1081-75-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,4-Di-*tert*-butylphenol	96-76-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,3-Dimethylbenzene (*m*-Xylene)	108-38-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,4-Dimethylbenzene (*p*-Xylene)	106-42-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
3,5-Dimethylphenol (3-xylenol)	108-68-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dodecamethylcyclohexanesiloxane	540-97-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,2-Epoxyethylbenzene (Styrene oxide)	96-09-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†2-Methyl-6-*tert*-butylphenol	2219-82-1	$>1.0 \times 10^{-3}$	3.5×10^{-4}
Phosphoric acid tris(3-methylphenyl)	563-04-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Phosphoric acid tris(4-methylphenyl)	78-32-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Poly(bisphenol A-co-epichlorohydrin)	25036-25-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Poly(bisphenol A-co-epichlorohydrin)glycidyl end capped	25036-25-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
β-Sitosterol	83-46-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Stigmasterol	83-48-7	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
1,3,5-Tri-*tert*-butylbenzene	1460-02-2	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$

†Only metabolite was positive

incubated at 30°C for 4h. Thereafter, the same procedure as the *Measurement of estrogenic activity by yeast two-hybrid assay* was carried out.

Data analysis

The results were evaluated on the basis of the relative activity, expressed as 10% relative effective concentration (REC_{10}), which is the concentration of the test chemical showing 10% of the agonist activity of $10^{-6}\,mol\,l^{-1}$ E_2, the highest activity level of E_2. When the activity of the test chemical was higher than the REC_{10} within the concentration range tested, the chemical was judged to be positive. When it was judged to be negative, more than the highest dose tested was indicated.

Results and discussion

Estrogenic activity of monomers

Fourteen kinds of monomers used for food contact plastics and/or rubbers were tested for their estrogenic activity and the results are shown in Table I. This table also shows the activity of E_2, bisphenol A and nonylphenol for comparison which are already known to possess the estrogenic activity as mentioned above. Three chemicals, bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol and 4-phenylphenol, displayed estrogenic activities and their activities were comparable to nonylphenol and bisphenol A. These three chemicals have already been reported to be estrogenic, the bis(4-hydroxyphenyl) methane based on the MCF-7 assay (Pérez et al. 1998), 4-cyclohexylphenol based on the uterotrophic assay (Yamasaki et al. 2003), and 4-phenylphenol based on the recombinant yeast screen assay (Routledge et al. 1997).

Their metabolites also possessed this activity, though they were weaker than the parent compound. The metabolite of 2-(phenylmethyl) phenol also showed a potency. However, the other 10 monomers and their metabolites did not have any estrogenicity.

Estrogenic activity of antioxidants

Forty kinds of antioxidants used for food contact plastics and/or rubbers and their metabolites were tested, and these results are shown in Table II. Four chemicals, 4,4′-isopropylidenediphenol alkyl(C_{12}–C_{15}) phosphite, two type of styrenated phenol, and tris(nonylphenyl)phosphite, displayed estrogenic activities. Their activities were comparable to nonylphenol and bisphenol A. Their metabolites also possessed such an activity and the metabolite of 1-(*N*-phenylamino)naphthalene and one type of styrenated phenol also showed a potency. However, the other 34 antioxidants and their metabolites did not have any estrogenicity.

Regarding three kinds of styrenated phenols, that containing mono-type 74% and di-type 24% showed the strongest activity, and that containing mono, di and/or tri-type (percent compositions were unknown) showed next activity. While, that containing di-type 93% and tri-type 7% did not show the activity, though its metabolite possess a weak activity. It is presumed that mono-type of styrenated phenol plays predominant role in the estrogenic activity.

Estrogenic activity of plasticizers

Thirty-eight kinds of plasticizers used for food contact plastics and rubbers and/or their metabolites were tested, and these results are shown in Table III. All the plasticizers and their metabolites did not display any estrogenicity.

Estrogenic activity of lubricants

Seven kinds of lubricants used for food contact plastics and/or rubbers and their metabolites were tested, and these results are shown in Table IV. All the lubricants and their metabolites did not display any estrogenicity.

Estrogenic activity of UV-stabilizers

Ten kinds of UV-stabilizers used for food contact plastics and/or rubbers and their metabolites were tested, and these results are shown in Table V. Two chemicals, 2,2′-dihydroxy-4-methoxybenzophenone and 2-hydroxy-4-methoxybenzophenone, displayed estrogenic activities based on the same assay (Kawamura et al. 2003) and the latter has been reported based on the MCF-7 cell assay and the Uterotropic assay (Schlumpf et al. 2001). Their metabolites showed such an activity here and already reported on the same assay (Takatori et al. 2003). The metabolite of 4-*tert*-butylphenylsalicylate also showed a potency. However, the other 7 UV-stabilizers and their metabolites did not show any estrogenicity.

Estrogenic activity of vulcanizing agents and vulcanization accelerators

Sixteen kinds of vulcanizing agents and vulcanization accelerators used for food contact rubbers and their metabolites were tested and these results are shown in Table VI. All the vulcanizing agents and vulcanization accelerators and their metabolites did not display any estrogenicity.

Estrogenic activity of miscellaneous additives

Seven kinds of miscellaneous additives used for food contact plastics and/or rubbers and their metabolites were tested, and the results are shown in Table VII. The chain transfer agent for the acrylonitrile butadiene styrene (ABS) plastic and rubber, 2,4-diphenyl-4-methyl-1-pentene, and its metabolites displayed estrogenic activities. The metabolite of an emulsifier, nonylphenol ethoxylate showed an estrogenicity. Nonylphenol ethoxylate is known to be decomposed to nonylphenol, therefore, the estrogenicity of its metabolite was presumed to originate in nonylphenol. However, the other 5 additives and their metabolites did not show any estrogenicity.

Estrogenic activity of other chemicals

Eighteen kinds of oligomers, degradation products and other impurities of plastics or rubbers and their metabolites were tested, and these results are shown in Table VIII. The metabolite of 2-methyl-6-*tert*-butylphenol, which is a material of several antioxidants and also their decomposition products, displayed an estrogenic activity. However, the other 17 compounds and their metabolites did not show any estrogenicity.

Estrogenic chemicals detected in this test

As a result of this test, 10 out of 150 chemicals, bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol, 4-phenylphenol, 4,4′-isopropylidenediphenol alkylphosphite, two type of styrenated

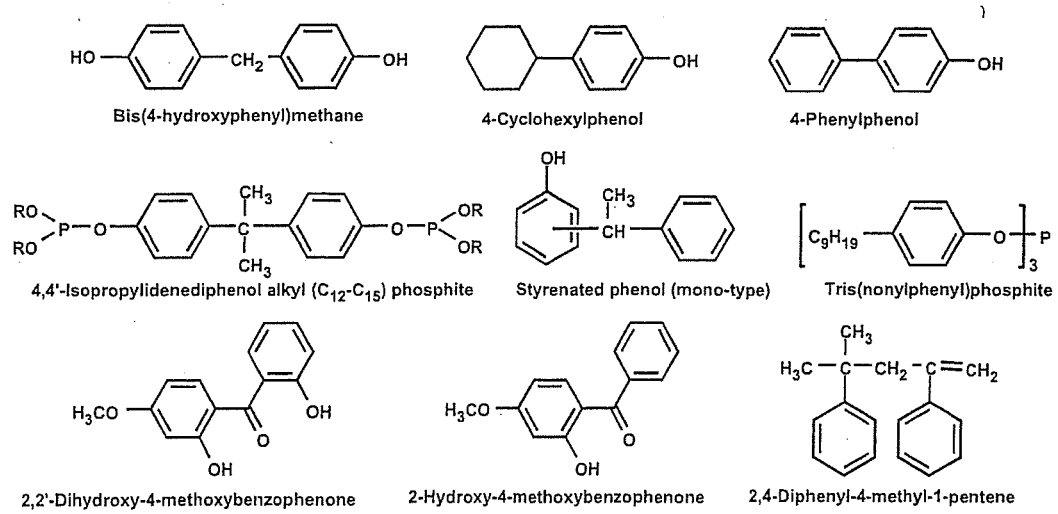

Figure 1. Structures of 9 estrogenic chemicals.

phenol, tris(nonylphenyl) phosphite, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-methoxybenzophenone and 2,4-diphenyl-4-methyl-1-pentene, displayed estrogenic activities. Their chemical structures are shown in Figure 1. They contained a phenol group or formed one easily by hydrolysis. The dose response curves of their estrogenic activity are shown in Figure 2, together with those of well-known estrogenic chemicals, nonylphenol and bisphenol A. Their activities were almost between nonylphenol and bisphenol A.

Moreover, the metabolites of these 10 chemicals and other 6 chemicals, 2-(phenylmethyl) phenol, 1-(*N*-phenylamino) naphthalene, one type of styrenated phenol, 4-*tert*-butylphenylsalicylate, nonylphenol ethoxylate and 2-methyl-6-*tert*-butylphenol, also displayed the estrogenic activities. The structures of these 5 chemicals except styrenated phenol are shown in Figure 3. They also contained a phenol group or formed one by metabolism. The REC_{10} of the metabolites were between 1.2×10^{-5} mol l^{-1} and 3.5×10^{-4} mol l^{-1}.

Among them five chemicals, bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol, 4-phenylphenol, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone, have already been reported to have estrogenic activities as mentioned above. The other chemicals were newly found to possess an estrogenic activity in the present study.

Conclusions

Our study showed that most of the chemicals related to food contact plastics and rubbers, and their

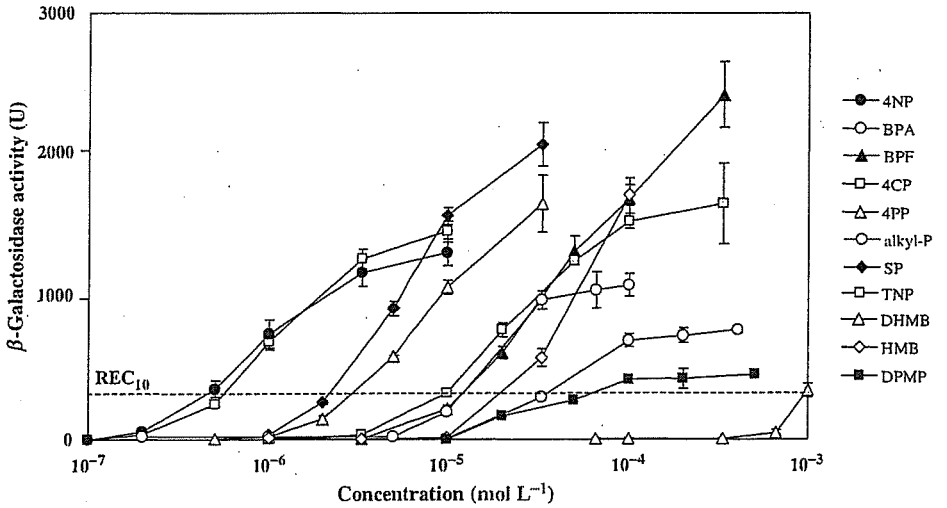

Figure 2. Dose response curves of estrogenic activity of nonylphenol (4NP), bisphenol A (BPA) bis (4-hydroxyphenyl) methane (BPF), 4-cyclohexylphenol (4CP), 4-phenylphenol (4PP), 4,4′-isopropylidenediphenol alkylphosphite (alkyl-p), styrenated phenol (mono 74%, SP), tris (nonylphenyl) phosphite (TNP), 2,2′-dihydroxy-4-methoxybenzophenone (DHMB), 2-hydroxy-4-methoxybenzophenone (HMB) and 2,4-Diphenyl-4-methyl-1-pentene (DPMP).

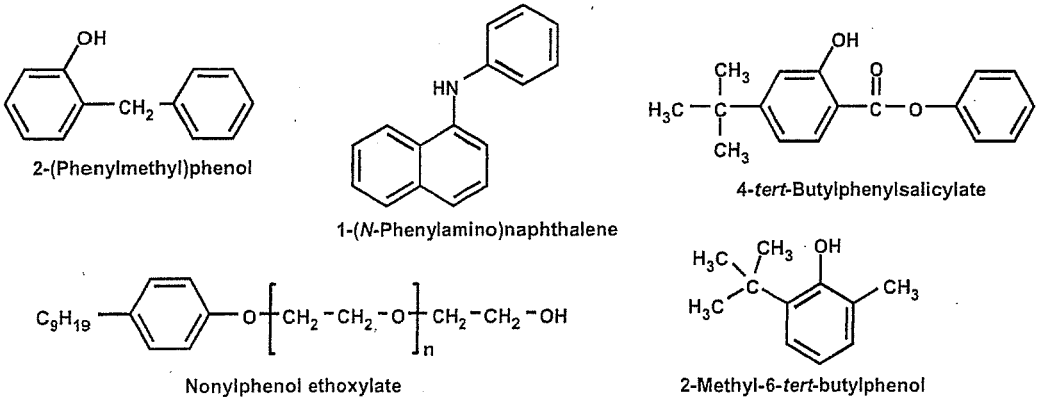

Figure 3. Structures of 5 chemicals which metabolites were estrogenic.

metabolites did not possess any estrogenicity. However, 10 chemicals and 6 other metabolites revealed estrogenic activities based on the yeast two-hybrid assay. These chemicals will need further investigations regarding their toxicity.

Acknowledgements

The authors thank Professor T. Nishihara and J. Nishikawa in Osaka University for providing the yeast two-hybrid cells and useful advice. This research was partially supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports and Technology, Japan.

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Assimilable organic carbon (AOC) originating from picophytoplankton in drinking water

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Abstract The removal efficiency of conventional drinking water for picophytoplankton and the contribution of picophytoplankton to AOC were investigated in this research. The removal ratio during coagulation–sedimentation step was determined by jar test using PAC (poly-aluminium chloride). Lower coagulation pH showed better picophytoplankton removal in coagulation–sedimentation. The optimum coagulant dosage for picophytoplankton was twice or more than that for turbidity. The removal efficiency of picophytoplankton was 44–60% at lowest pH in water quality standard (5.8) and at an optimum coagulant dosage for turbidity. The removal ratio of picophytoplankton in rapid sand filtration was determined by pilot scale column experiments with sand and anthracite. The average removal percentage was 16.3% without PAC addition and chlorination before sand filtration; on the other hand it was 51.5% with PAC and chlorination. AOC increased by the chlorination of picoplankton including 6,800 cells/L of picophytoplankton was 21 μg-acetateC/L at 0.1 mg/L of residual free chlorine. The AOC was increased by the increase of residual chlorine concentration, and leveled off at 0.3 mg-Cl/L. From the result, the AOC originating from picoplankton (maximum AOC from picophytoplankton) could increase up to 155 μg-acetateC/L in this reservoir. It indicates that the removal of picoplankton (picophytoplankton) in drinking water treatment process is important from the viewpoint of AOC control.

Keywords Assimilable organic carbon; coagulant; coagulation conditions; drinking water; picoplankton

Water Science & Technology: Water Supply **Vol 6 No 2 pp 169–176** © IWA Publishing 2006

Introduction

Picoplankton is a small plankton with size range 0.2–2.0 μm, and can be classified roughly into picophytoplankton and heterotrophic picoplankton. Picophytoplankton (autotrophic picoplankton, photoautotrophic picoplankton) is a phytoplankton with the size range. Intensive research on picophytoplankton was initiated in marine ecosystems. The significance of picophytoplankton in freshwater systems was first reported in 1980s, when it was found that picophytoplankton is ubiquitous in all aquatic systems. It is also investigated that picophytoplankton contributes to the total phytoplankton biomass in lakes and the primary production decreases as the trophic level of the lake increases. In oligotrophic and/or mesotrophic regions of oceans and lakes, the picoplankton largely contributes to energy flow and material recycling (Stockner, 1991; Barkmann, 2000; Okunishi *et al.*, 2003).

Recently, turbidity of treated water is required to be less than 0.1 mg-Kaolin/L in Japanese drinking water treatment, because of the countermeasure for *Cryptosporidium* oocyst removal. Some researchers showed that picoplankton, especially picophytoplankton, contributed to the turbidity of treated water (Nakamura *et al.*, 1997; Hoson *et al.*, 2002). In addition, toxic compounds produced by picoplankton, especially by picoplankton cyanobacterium, were also reported (Kaya *et al.*, 1993). Therefore, picophytoplankton has recently become one of the most important targets in drinking water treatment. How-

doi: 10.2166/ws.2006.066

ever, the research about the treatment of picophytoplankton and picoplankton in drinking water treatment is very limited in comparison with upper basic studies.

Some researchers reported the difficulty of removing picophytoplankton in conventional drinking water treatment processes, i.e. coagulation–sedimentation and rapid filtration. The residual picophytoplankton in treated water is killed and its cell wall is damaged by conventional disinfection processes using chlorine. Therefore, the extracellular organic matter coming from picophytoplankton would contribute to Assimilable Organic Carbon (AOC) in treated drinking water. AOC is one of the regrowth potentials of bacteria (microorganisms) in distribution systems, AOC promotes bacterial growth and may be related to the occurrence of coliform bacteria in distribution systems (Volk and Lechevallier, 2002). It is known that there was no breeding of *Bacillus coli* at less than 50 μg-acetateC/L of AOC, and no breeding of heterotrophic bacteria at less than 10 μg-acetateC/L of AOC (*Standard Methods for Examination of Water*, 2001). However, the contribution of picophytoplankton to AOC has not yet been studied, neither has a systematic study about the effect of each coagulation parameter on coagulation efficiency of picophytoplankton removal. The removal efficiency of traditional drinking water for picophytoplankton and the contribution of picophytoplankton to AOC were investigated in this research.

Materials and methods

Raw water

Picophytoplanktons were collected from a eutrophic reservoir in Japan. The volume of the reservoir is approximately 2,600,000 m^3, and sample was taken from the surface of the reservoir. Water temperature ranged from 5 to 30 °C. Turbidity ranged 10–30 mg-kaolin/L; the highest turbidity was observed in summer and the lowest turbidity was observed in winter. DOC ranged from 2–5 mg-C/L, and alkalinity ranged from 30–45 mg-$CaCO_3$/L. The pH was almost stable at 7–8 throughout the year.

Jar test

About 500 mL of raw water was coagulated in a 1 L beaker. Polyaluminium chloride (PAC) was used as coagulant, the dosage was 1–6 mg-Al/L. The solution was mixed at 150 rpm for 3 minutes by jar tester with one paddle, then the mixing speed was reduced to 60 rpm for 15 minutes flocculation. The samples for analysis were taken from 3 cm below the surface after 30 minutes sedimentation. The coagulation pH was adjusted 5.0–7.5 by hydrochloric acid and sodium hydroxide.

Rapid sand filtration

A sand layer with anthracites (particle size: 1.2 mm) layer of 20 cm depth on quartz sands (particle size: 0.6 mm) layer of 60 cm depth was established in a 2 m acrylic column of 10 cm diameter. The flux was adjusted around 150 m/day, which is the conventional value in real drinking water treatment plants in Japan. Picophytoplankton suspension was prepared by the following method. Picoplankton was separated from other bigger particles using filtration with 2.0 μm filter, then the incubation of picophytoplankton was conducted in CT culture media (Hoson *et al.*, 2002). The picoplankton suspension with about 4,000,000 cells/mL was obtained after incubation. It was diluted 50 times with tap water and supplied for experiments. Sampling was conducted every hour in a 10 hr experiment.

Generally, there are picophytoplankton cells adsorbing PAC on their surface and cells not adsorbing PAC after coagulation–sedimentation. It is therefore necessary to study the difference in removal efficiency both with and without PAC adsorption. In addition, coagulant addition before sand filtration is known to enhance the efficiency of sand

filtration (Ebie *et al.*, 2001; Yazawa, 2002). Therefore, to investigate the effect of coagulant on picophytoplankton removal, the experiment was conducted with and without PAC addition (0.1 mg-Al/L). The solution was mixed 30 seconds after addition of PAC to homogenize coagulant and the mixing was conducted slowly to prevent coagulation and flocculation of picophytoplankton. Therefore, PAC would be adsorbing on to the picophytoplankton surface or coexisting in the surrounding solution. Chlorination before sand filtration is generally applied in real drinking water treatment, and so the effect of chlorination is also studied using sodium hypochlorite at 0.5 mg-Cl/L dosage.

Picophytoplankton analysis

Previous work in this area has been by Ichiyanagi *et al.* (1997) and Barkmann (2000). The separation of picoplankton from other plankton was conducted by the filtration with 2.0 μm Millipore filter. Samples were preserved with cold formalin (2% final concentration) and filtered by 0.2 μm Millipore polycarbonate filter. Picophytoplankton on the filter was enumerated by epifluorescence microscopy (Nikon, Optiphot-2) with a 100W super high pressure mercury lamp (HB-1010 AF). Previously, it was shown that there were very few picoplankton cells in this reservoir under blue excitation. Therefore, a green excitation filter set (BA590/DM580/EX510-560) was used and the dominant picoplankton fluoresced red under the green excitation. Generally red fluorescence shows that they were rich in the photosynthetic pigment, phycocyanin (light red, Cyanobacteria) and chlorophyll (weak red, Eukaryotes). Ten fields and at least 200 cells (normally around 500) were counted. For the coagulated samples and filtrated sample (sand filtration), the separation of picoplankton using 2.0 μm membrane was not conducted to avoid the trap of picoplankton by the membrane, because a 20–40% decrease of picophytoplankton by filtration with 2.0 μm membrane was observed in an earlier experiment in the coagulated solution. The counting of picoplankton could easily be conducted on the basis of size even in the case of the sample without the 2.0 μm membrane filtration.

Chlorination of picoplankton and AOC analysis

Heterotrophic plankton (animal plankton) of size 0.2–2.0 μm is not separated from picophytoplankton suspension by filtration method, so the contribution of total picoplankton for AOC production was investigated in this research. Raw reservoir water was filtered with 2.0 μm membrane to remove bigger plankton and particles than picoplankton. The picoplankton suspension was concentrated by centrifugation, one tenth volume of solution was collected from the bottom. To decrease background DOC before chlorination, nine times by volume of organic free physiological saline was added into the concentrated suspension and picoplankton was concentrated by centrifugation again. Sodium hypochlorite was used as chlorination agent. In Japan, the residual free chlorine in tap water of user is regulated to be more than 0.1 mg-Cl/L by the water quality standard. Therefore, chlorination of picoplankton was conducted with 0.1–0.5 mg-Cl/L of free chlorine concentration after 24 hours.

AOC concentration was determined using the pour plate method (*Standard Methods for the Examination of Water and Wastewater*, 1998, 2001). Water samples were collected in carbon-free 100 mL glass bottles with screw cap. After neutralization of disinfectant residuals by sodium thiosulfate, samples were inoculated with approximately 5,000 colony-forming units (CFU)/mL of either *Pseudomonas fluorescens* P17 or *Spirillum* NOX. After incubation in 7–11 days at 15 °C, CFU (either P17 or NOX) were measured by pour plate and converted into AOC units on the basis of the relationship between the amount of sodium acetate and growth cell number of either P17 or NOX.

The AOC is expressed as micrograms per litre of equivalent acetate-carbon (acetateC), and total AOC concentration is the sum of AOC-P17 and AOC-NOX.

Other analytical methods

Dissolved organic carbon (DOC) was determined by total organic carbon analyzers (TOC-5000, Shimadzu). pH was determined by a pH meter (F-8, Horiba). Turbidity was determined using a turbidimeter (ANA-148, Tokyo Photoelectric).

Results and discussion

Number of picophytoplankton in raw water

Figure 1 shows the seasonal change of picophytoplankton density in raw water. The picophytoplankton density ranges from 3,000–132,000 in 2003 and 2004, and it was found that maximum density was observed in summer season (from July to September). This value agrees with other researches in Japan (Ichiyanagi *et al.*, 1997; Nakamura *et al.*, 1997; Yazawa, 2002) and other countries (Stockner, 1991). Some of these researches reported that the maximum picoplankton density increased up to 1,000,000 cells/mL.

Removal of picophytoplankton in conventional drinking water treatment

The effect of coagulation and sedimentation conditions (coagulant dosage, pH and sedimentation time) on picophytoplankton removal was investigated to determine the range of picophytoplankton removal during drinking water treatment and to compare with turbidity removal. Figure 2 shows the effect of coagulation pH on picophytoplankton and turbidity removal. Both picophytoplankton and turbidity decreased at lower pH. The residual picophytoplankton decreased slowly from pH 7.5 to 6.0 and sharply decreased at lower than 6.0. The residual turbidity decreased sharply when pH decreased from 7.5 to 6.5, and slowly decreased at pH lower than 6.5. The difference of pH effect between turbidity and picophytoplankton removal indicates the more negative charge of picophytoplankton surface than that of turbid materials. Decreasing the pH to lower than 6.0 is effective to improve picophytoplankton removal. However, the drinking water pH should be adjusted from 5.8 to 8.6 on the basis of water quality standard in Japan, so drinking water treatment is generally conducted in this range. Therefore, the percentage range of picophytoplankton removal in actual and effective coagulation pH 6.0–7.0, for both turbidity and picophytoplankton, was 61–63%.

Figure 3 shows the effect of coagulant dosage on picophytoplankton removal and turbidity removal. The residual picophytoplankton decreased continuously up to a dosage

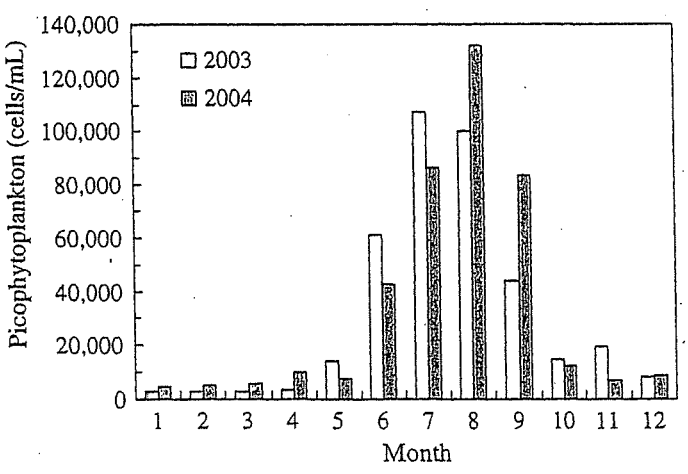

Figure 1 Seasonal change of picophytoplankton density

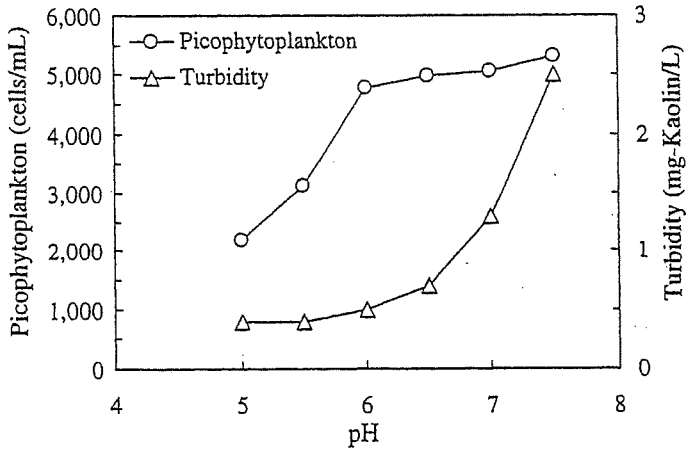

Figure 2 Residual picophytoplankton at each coagulation pH (initial picophytoplankton: 12,983 cells/mL; initial turbidity: 6.3 mg-Kaolin/L; coagulant dosage: 4 mg-Al/L; sedimentation: 15 min)

of 6 mg-Al/L; while the residual turbidity had already leveled off at a dosage of around 3 mg-Al/L. The coagulation–sedimentation is generally conducted at the optimum dosage for turbidity (smallest dosage in the range of level off). This was 3–5 mg-Al/L on the basis of Figure 2. Therefore, it is found that the percentage of picophytoplankton removal ranges from 44–60%. In addition, the result indicates that there was a factor of two difference of optimum dosage between turbidity removal and picophytoplankton removal.

Figure 4 shows the residual picophytoplankton and turbidity as a function of sedimentation time. Both quickly decreased in initial 5 minutes and then slowly decreased after 5 minutes. There was only small difference after 30 minutes in both residual picophytoplankton and turbidity. It was found that the optimum sedimentation time for picophytoplankton removal was the same as that for turbidity removal.

Figure 5 shows the residual picophytoplankton in rapid sand filtration. The average removal percentage was 16.3% without PAC addition, while it was 45.1% with PAC addition. This indicates that the adsorption and/or coexisting of PAC helps to trap picophytoplankton during rapid sand filtration, and can increase removal percentage by a factor of three. The chlorination could also increase the removal efficiency, because it increased the removal percentage up to 51.5% with the addition of PAC and chlorination. These indicate that the variation of removal rate in sand filtration process ranges from 16 to 52%.

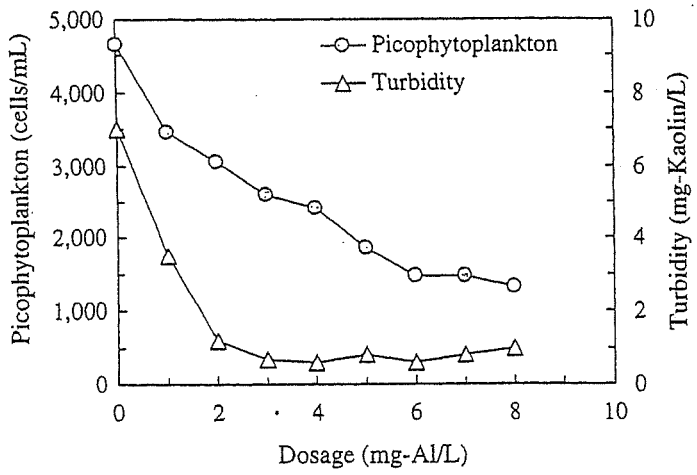

Figure 3 Residual picophytoplankton at each coagulant dosage (initial picophytoplankton: 4,658 cells/mL, initial turbidity: 7.0 mg-Kaolin/L; coagulation pH: 6.0; sedimentation: 15 min)

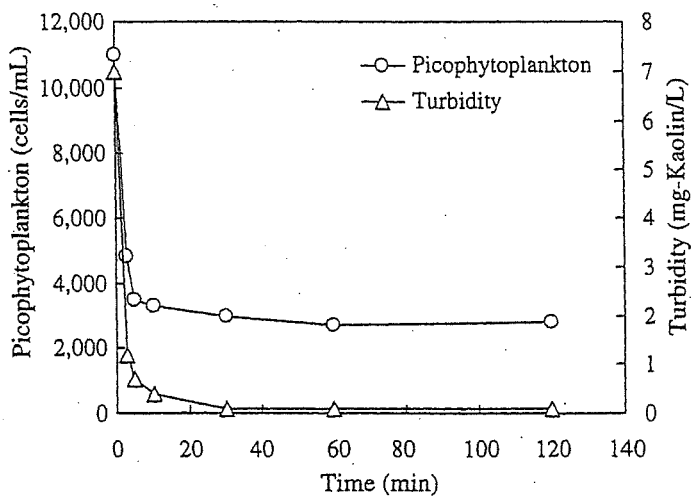

Figure 4 Residual picophytoplankton with sedimentation time (initial picophytoplankton: 11,012 cells/mL; initial turbidity: 7.1 mg-Kaolin/L; coagulant dosage: 4 mg-Al/L; coagulation pH: 6.0)

AOC from picoplankton

Table 1 shows the AOC from the chlorination of picoplankton at three residual chlorine concentrations. The AOC originating from picoplankton (AOC-pico) includes both AOC from picophytoplankton and from other heterotrophic picoplankton (including bacteria), because picophytoplankton are not isolated in this research. Background AOC without picoplankton was 0.38 μg-acetateC/L and AOC-pico is shown in this table. This result indicates that picoplankton itself is not the origin of AOC from this result. AOC was increased by the increase of residual chlorine and was leveled off at around 90 μg-acetateC/L of AOC-pico with 0.3 mg-Cl/L.

If heterotrophic picoplankton and other organic particles with 0.2–2.0 μm could be negligible, the AOC-pico equal to the AOC caused by picophytoplankton (AOC-phyto), meaning the AOC-pico indicates the maximum AOC-phyto. Maximum AOC-phyto per cell can be calculated using the maximum AOC-phyto and shown in Table 1. This result shows that maximum AOC-phyto was about 2.5 pg-acetateC/cell at 0.1 of residual chlorine. The picophytoplankton density range in raw water was 3,000–132,000 cells/mL in this reservoir. On the basis of this value and lowest removal percentages of picophytoplankton in coagulation–sedimentation and sand filtration (44% and 16%, respectively),

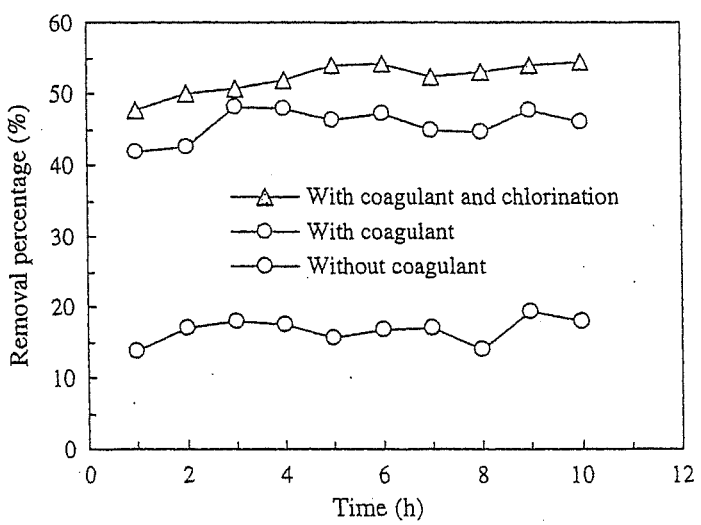

Figure 5 Residual picophytoplankton at each sedimentation time (initial picophytoplankton: about 80,000 cells/mL without turbid materials; coagulant dosage: 0.1 mg-Al/L; chloride dosage: 1 mg/L)

Table 1 AOC increased by chlorination of picoplankton (AOC-pico, maximum AOC-phyto)

Chlorine dosage (mg-Cl/L)	Residual chlorine concentration (mg-Cl/L)	AOC caused by picoplankton (AOC-pico, maximum AOC-phyto) (μg-acetateC/L)	Maximum AOC caused by one picophytoplankton cell (pg-acetateC/cell)
0.0	0.0	0*	almost 0
0.4	0.1	21**	3
0.6	0.3	80**	13
0.8	0.5	89**	13

*Picophytoplankton density: 120,000 cells/mL
**Picophytoplankton density: 6,800 cells/mL

maximum AOC-phyto can be increased up to 155 μg-acetateC/L at 0.1 mg-Cl/L of residual chlorine concentration. Because the breeding of bacteria in distribution systems is significant at over 50 μg-acetateC/L of AOC, this result indicates that the removal of picoplankton (picophytoplankton) in drinking water treatment processes is important from the viewpoint of AOC control.

Conclusions

The purpose of this study was to investigate the picophytoplankton removal efficiency of conventional drinking water treatment and the contribution of picophytoplankton (picoplankton) to AOC. The specific conclusions derived from this study are as follows:

- Lower coagulation pH showed better removal of picophytoplankton in coagulation–sedimentation treatment.
- The optimum coagulant dosage for picophytoplankton was twice or more higher than that for turbidity.
- The removal percentage of picophytoplankton in coagulation–sedimentation treatment was 44–60% at lowest pH in the range of water quality standard (5.8) and optimum coagulant dosage for turbidity.
- The average removal percentage in sand filtration was 16.3% without PAC and chlorination, while it was 51.5% with PAC and chlorination.
- The removal of picoplankton (picophytoplankton) in drinking water treatment process seemed to be important from the viewpoint of AOC control.

Acknowledgements

The authors appreciate the strong cooperation of Kure City waterworks bureau. The authors would also like to thank Katsunori Kihara, Tomoaki Nakasaka and Yuuki Uehara for their help.

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Assimilable organic carbon formation from algogenic organic matter and its variation by chlorination

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Abstract The objective of this study is to assess the importance of algae and algogenic organic substances concerned with the formation of assimilable organic carbon (AOC) during algal growth and chlorination process. Laboratory tests were carried out using cultures of algal species (*Phormidium tenue*) with different culture medium, M-11 and CT. Extracellular organic matter (EOM) produced during the stationary phase and death phase of *P. tenue* (10^6 cells/mL) with M-11 culture medium led to significant increase of the AOC concentration, up to 100 times as high as its initial concentration. In case of CT culture medium containing a high DOC component, the AOC concentration did not increase significantly during *P. tenue* culture. The formation and removal of AOC derived from EOM and intercellular organic matter (IOM) by chlorination were also examined. The AOC concentration after chlorination of EOM and IOM became maximum with 2 mg/L of chlorine dosage while it decreased with increase of chlorine dosage when the dosage was higher than 2 mg/L, suggesting that necessary chlorine dosage to not form AOC is higher than 2 mg/L.

Keywords Algae; AOC; chlorination; EOM; formation; IOM

Water Science & Technology: Water Supply **Vol 6 No 2 pp 239–244** © IWA Publishing 2006

Introduction

Bacterial regrowth in the drinking water distribution system has become a topical issue in recent years due to many operational problems such as deterioration of water quality, corrosion of pipes for water distribution, bad tastes and odors. The heterotrophic bacteria adhered on the surface of pipes repeat the proliferation and the detachment during the formation of biofilms on the surface. Some bacteria in biofilms would not be eliminated by high chlorine residual for disinfection (LeChevallier and Au, 2004; Tsai *et al.*, 2004). Bacteria surviving in the biofilms would exfoliate from the surface of pipes after regrowth. The exfoliated bacteria would deteriorate quality of water over and over during water distribution system. Bacterial regrowth should be controlled to maintain the safety of drinking water. Generally, the bacterial regrowth is associated with assimilable organic carbon (AOC) (Escobar and Randall, 2001; Liu *et al.*, 2002). Van der Kooij (1990) suggested that the AOC should be less than 10 μg/L to limit the growth of heterotrophic plate counts bacteria in unchlorinated systems. However, it seems difficult to control the concentration of the AOC with conventional treatment processes for the drinking water (Huck *et al.*, 1991; Lehtonen *et al.*, 2002). During the treatment of raw water including humic substances derived from algogenic organic matter (AOM), the fraction of DOC would be not easily degraded chemically and biologically (Charnock and

doi: 10.2166/ws.2006.075

Kjønnø, 2000; Hem and Efraimsen, 2001). Occasional algal blooms consisting of blue–green algae and diatoms could also make the control of DOC (dissolved organic carbon) difficult because of the release of AOM into water extracellularly and, upon cell lysis, intracellularly. Algae and their extracellular organic matter (EOM) could be precursors for disinfection by-product formation related to chlorination treatment and could cause the increase of AOC concentration (Graham *et al.*, 1998; Plummer and Edzwald, 1998; Schmidt *et al.*, 1998).

In the present study, we carried out a series of experiments to assess the variation of AOC concentration by algal growth and chlorination. Firstly, we investigated the difference in the formation of algogenic organic matter and AOC with two kinds of algal culture media: M-11 and CT medium. The M-11 culture medium has properties as follows: (1) the concentration of DOC is low; and (2) the mineral composition is similar to that of tap water. On the other hand, the CT culture medium is characterized to contain high concentration of DOC. We also performed the chlorination of EOM and IOM (intracellular organic matter) to assess the fate of AOC derived from algae and the relationship between chlorination dosage and the formation and removal of AOC derived from EOM and IOM by chlorination was discussed.

Materials and methods

AOC determination

The determination is based on the measurement of the maximum extent of growth of a selected pure bacterial culture in representative water samples in which the indigenous bacteria have been killed or inactivated by heat treatment. Colony counts are used for determining bacterial densities. The analytical method developed by van der Kooij (1995) was adopted in the present study. The method employs *Pseudomonas fluorescens* strain P17 and *Spirillum species* strain NOX. These two microorganisms prefer different groups of compounds. P17 has a great nutritional versatility, and may grow on a variety of carbohydrates, aromatic acids and amino acids. It can also grow on carboxylic acids, with the exception of formic, glyoxylic and oxalic acids as typical by-products of ozonation. Therefore, the growth of strain P17 was used for the determination of the concentration of aromatic and amino acids etc. In case of NOX, it cannot utilize carbohydrates, alcohols, or aromatic acids but a wide range of carboxylic acids. Strain NOX can also utilize a few amino acids; however, this organism cannot assimilate amino acids when growing on mixtures of compounds. Therefore, the growth of strain NOX was used for the determination of the concentration of carboxylic acids in water (Orlandini *et al.*, 1997). All glassware was thoroughly cleaned and rendered organic carbon-free by combustion at 550 °C for 1 hour. The samples were incubated at 15 ± 0.5 °C and the number of colony forming units (CFU) was measured in nine petri-dishes for each sample water after 7, 9 and 11 days. The colonies were measured with the plate count technique after 72 ± 3 hours incubation at 28 ± 1 °C on R2A agar. The total AOC in micrograms per litre of equivalent acetate-carbon is the sum of AOC–P17 and AOC–NOX. In the present study, the yield coefficients of P17 and NOX were measured as 4.1×10^6 cfu/μg acetate-carbon and 1.2×10^7 cfu/μg acetate-carbon, respectively.

Algal culture

The blue green alga *Phormidium tenue* (*P. tenue*, ATCC49642) was selected as test alga to form AOM. It was cultivated using the M-11 culture medium and CT culture medium under the illumination condition of 3000lx with 12 h/12 h of light/dark cycle in the incubator. The temperature was maintained at 22 °C. Table 1 shows the components of each culture medium.

Table 1 The components of culture media

M-11 culture medium		CT culture medium	
$NaNO_3$	10 mg	$Ca(NO_3)_2{\cdot}4H_2O$	15 mg
K_2HPO_4	1 mg	KNO_3	10 mg
$MgSO_4{\cdot}7H_2O$	7.5 mg	β-Na_2 glycerophosphate·$5H_2O$	5 mg
$CaCl_2{\cdot}2H_2O$	4 mg	$MgSO_4{\cdot}7H_2O$	4 mg
Na_2CO_3	3 mg	Vitamin B_{12}	0.01 μg
$FeSO_4{\cdot}7H_2O$	0.1 mg	Biotin	0.01 μg
$Na_2EDTA{\cdot}2H_2O$	0.1 mg	Thiamine HCl	1 μg
Distilled water	100 ml	PIV metals	0.3 ml
pH	8.0	TAPS	40 mg
		Distilled water	99.7 ml
		pH	8.2

Chlorination

Sodium hypochlorite (NaOCl) solution was standardized by titration and then diluted with distilled water. The chlorine dosages used were 2, 5, 10, 20, 30 mg/L. Sealed 3 L amber glass bottles were used for all chlorination experiments. The amber glass bottle was also covered with aluminium foil to prevent photodegradation of the solutions. The contents of amber glass bottles were stirred continuously with a magnetic stirrer. After chlorination, the sample was withdrawn from each bottle, and then filtered with GF/C filter and terminated by addition of sodium thiosulfate immediately after collection. Chlorination was terminated by addition of sodium thiosulfate immediately after collection.

Results and discussion

Firstly, we examined the variation of the concentrations of DOC and AOC (P17, NOX) produced extracellularly during *P. tenue* cultivation. Figure 1 shows their variation according to the elapsed time for each culture medium (M-11 and CT). In case of M-11 culture medium in Figure 1a, the initial concentration of DOC before inoculating the *P. tenue* (10^6 cells/mL) was about 1.5 mg/L. The DOC concentration had a tendency to increase with elapsed time and reached 9.2 mg/L at the stationary phase (39 days). The DOC increased more during the death phase (after 39 days). The variation of DOC concentration was considered due to the increasing EOM. The increasing EOM also led to the significant increase of the AOC concentration. As shown in Figure 1a, the AOC concentration increased to about 4,000 μg/L at the stationary phase which was 100 times as high as the initial AOC concentration. The result showed that the AOC-P17 occupied more than 90% of AOC during the cultivation. In other words, the AOC concentration in culture solution depended on the AOC-P17 component such as carbohydrates, aromatic acids and amino acids. While the DOC concentration for CT culture medium (Figure 1b) was much higher

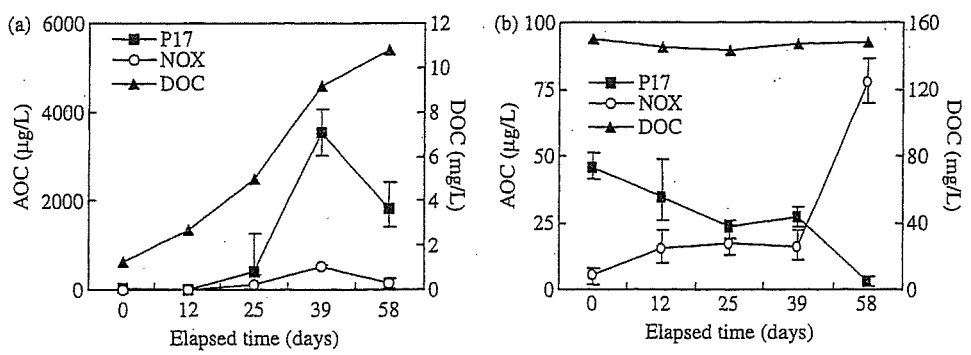

Figure 1 The variation of DOC and AOC (P17, NOX) during *P. tenue* culture: (a) M-11 culture medium; and (b) CT culture medium

than that for M-11 culture medium. The initial concentration of DOC was about 150 mg/L in CT culture medium and the concentration of DOC was constant during *P. tenue* culture. Unlike M-11 culture medium, the CT culture medium would not lead to a significant variation of AOC concentration although some variation was observed in AOC-P17 and AOC-NOX at the death phase (after 39 days). From the results, it was considered that the generation and the ingredients of EOM derived from algae could be changed by the condition of growth medium. Moreover, the AOC formation seemed to increase enormously in culture medium including organic substances with low concentration such as M-11 culture medium. The variation of AOC component derived from EOM produced in M-11 culture medium after 39 days culture was classified by molecular weight fractionation (Figure 2). The EOM was fractionated by ultra filtration membrane into four classes of molecular weight (lower than 1,000Da, 1,000–5,000Da, 5,000–10,000Da and higher than 10,000Da). The AOC produced extracellularly by *P. tenue* was mainly detected at the fraction of lower than 1,000Da of molecular weight. The ratio of AOC-P17 and AOC-NOX at the fraction of lower than 1,000Da was about 80% and 20%, respectively. The ratio of AOC-NOX decreased with increase of molecular weight and it almost disappeared in the fraction of higher than 10,000Da after all. The EOM with low molecular weight is likely to be the main organic substances contributing to the formation of AOC.

We also investigated the relationship between the chlorine dosage and residual chlorine. Figure 3 shows that the residual chlorine increases according to chlorine dosage. The chlorine was not consumed and remained in case of the fresh culture medium excluding *P. tenue*. When the chlorine dose was 30 mg/L, the residual chlorine for EOM and IOM was about 10 and 20 mg/L, respectively. Namely, the chlorine demand for the oxidization of IOM was nearly double that for the oxidization of EOM. Figure 4 shows the variation of AOC components after the chlorination. In Figure 4a, the AOC concentration shows the maximum concentration of 751 μg/L at the low chlorine dosage of 2 mg/L, revealing that AOC increased 18% compared with that before chlorination. The AOC concentration then decreased with the increase of chlorine dosage. The 5, 10 and 30 mg/L of chlorine dosage led AOC to decrease to 336 μg/L, 219 μg/L and 98 μg/L, respectively. Before chlorination, the concentration of AOC–P17 was nearly equivalent to that of AOC-NOX. The ratio of AOC–P17 to AOC, however, had rapidly decreased with chlorination. When the 30 mg/L chlorine was dosed, the AOC–NOX ingredients could account for 90% of the AOC concentration. Considering that the 2 mg/L of chlorine dosage is generally adopted for drinking water treatment, the present system may not sufficiently treat the EOM contaminated into raw water and the AOC may remain even after chlorination. Higher chlorine dosage than usual is needed for treatment of EOM not to form AOC. The AOC variation according to the chlorination of IOM is presented in Figure 4b. IOM

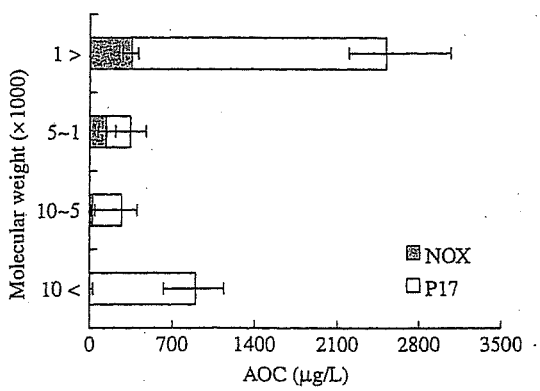

Figure 2 The molecular weight profile of AOC derived from EOM produced by *P. tenue* culture