すると，管路施設ばかりでなく浄水施設についても，どこかで給水停止をせざるを得ない事態を招くと憂慮せざるを得ない。現に，クリプトスポリジウムによる感染症の発生や塩素注入管理が不適切のため感染症が発生して給水停止を実施せざるを得ない事態が発生している。

1965年の年間給水量は約70億m^3/年であり，河川の自流水が約55億m^3，その他の水源が30億m^3であった。水道普及率の向上とともに年間給水量が増加するにつれて，水道水源の構成が大きく異なるようになっている。すなわち，地盤沈下対策のために地下水取水量の増加は少なく，河川自流水および伏流水の占める割合が低下し，それを補うようにダム・湖沼等新規に開発された水資源施設からの取水量が増加している。しかし，湖沼等停滞水域の環境基準の達成率は40％台に留まっており，植物プランクトン等の影響を受けた水源となっている。しかも，ダムから直接取水して浄水施設まで導水している水道事業体が少ないため，流域から排出される排水や農業排水等非点源負荷の影響を強く受けていることになる。

さらに，化学物質のリスク管理が強化されることに従って水質基準が強化されることに伴い，浄水施設の高度化を図らなければならない。

1. 浄水施設の高度化とは

水質基準項目のうち，発がん性の観点から基準が定められている項目について，水道原水および水道水についてそれぞれの発がんリスクを求め，それらの総和をもって水道原水および水道水の発がんリスクとし，それらの値を首都圏の地方自治体別に示すと**図1**のようになる。さらに，水道水の発がん物質の構成を示すと**図2**のようになる。

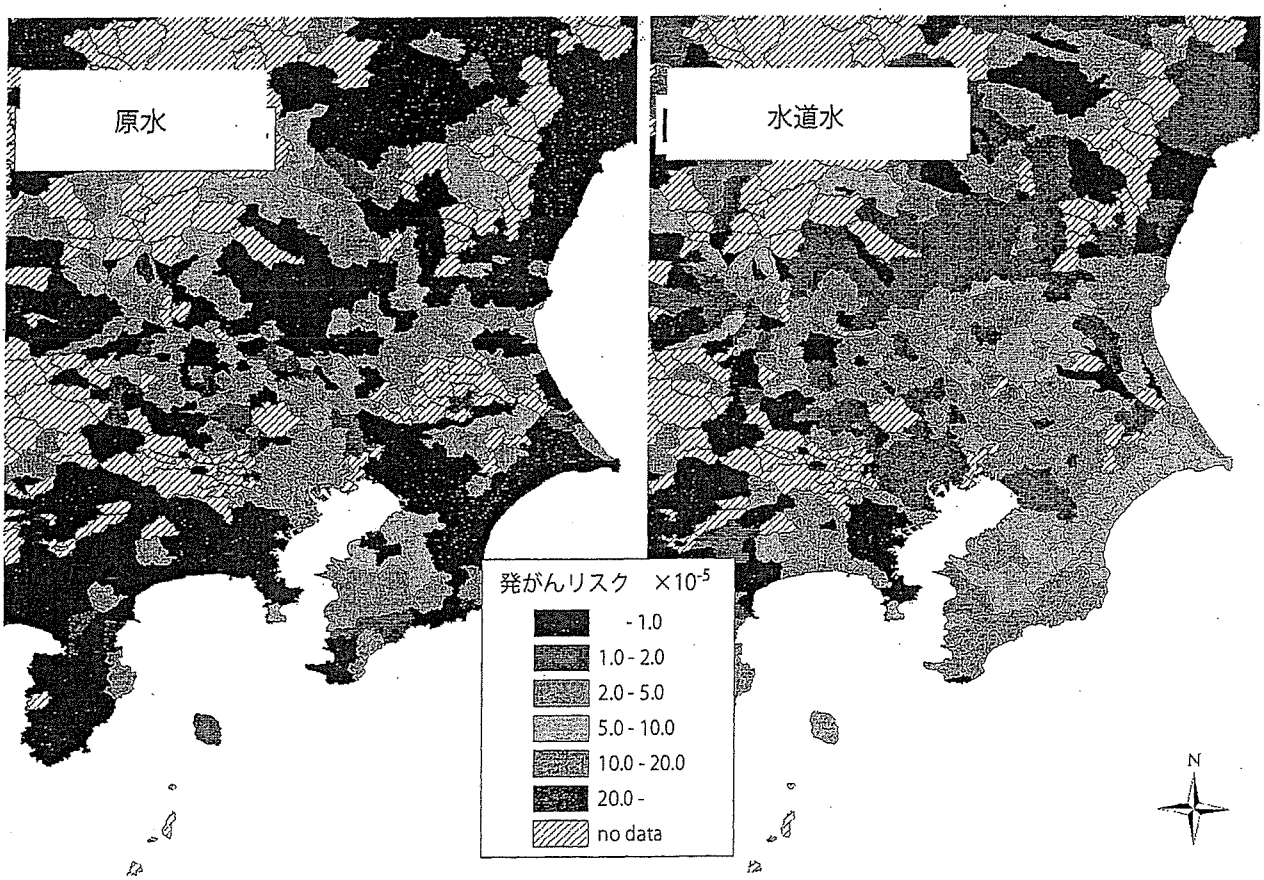

図1　首都圏の水道原水および水道水の発がんリスク分布

水道水の発がんリスクは，個々の発がん物質のリスクレベルを10^{-5}として水質基準が定められているが，10^{-4}オーダーの水道原水が存在している。さらに，その発がんリスクは消毒副生成物による寄与が高い。これは，塩素消毒によって発がん性を有するハロゲン化消毒副生成物が生成するためである。浄水処理によって，消毒副生成物の前駆物質の一部は除去されているので，浄水処理によって発がんリスクは低下していることは確かである[1)]。

水道原水としての公共用水域や地下水の状況，既存の水道施設の水質制御能力を考えたとき，良質で，違和感がなく，利便性障害の少ない水道水を利用できるようにするためには，浄水処理施設をレダンダントで，まさにマルチバリアーとしての機能を持たせたものとしなければならない。たとえば，今後の浄水処理のフローを選択用件との関係から示すと図3のようになろう。

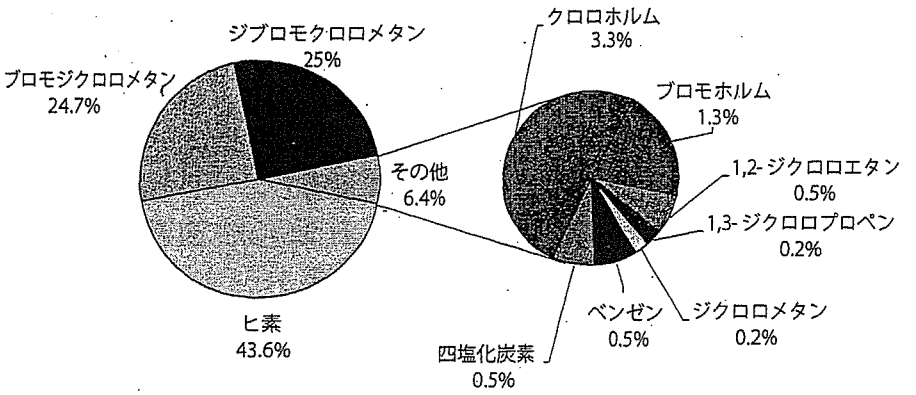

図2　水道水の発がんリスクの構成

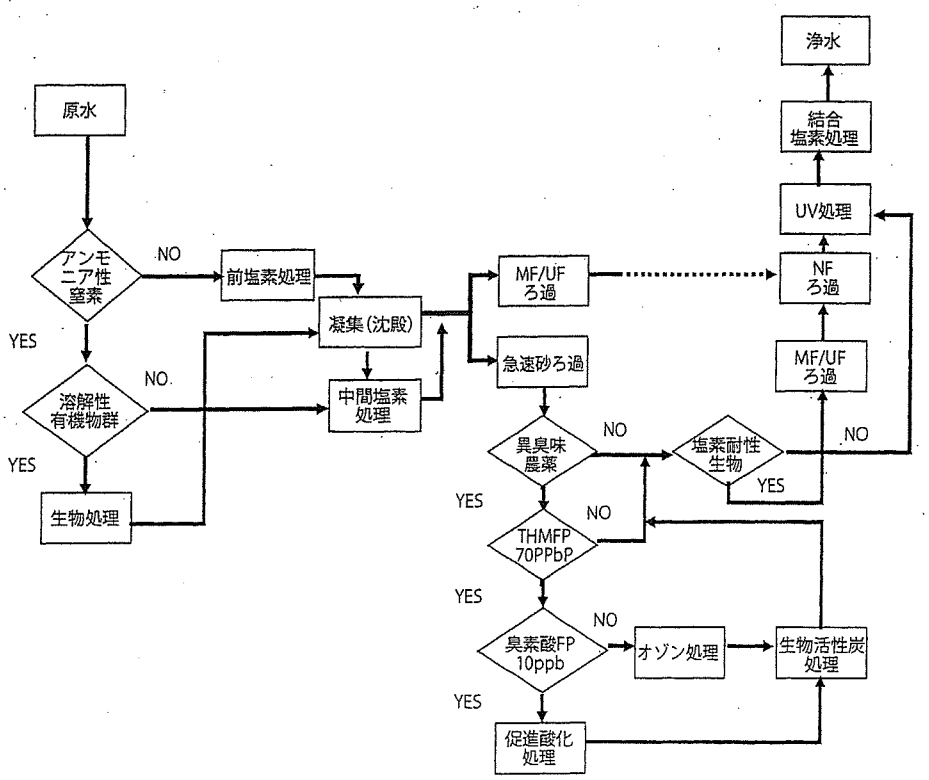

図3　浄水処理システムの構成

WHO飲料水水質ガイドラインが示しているように，水道は感染症対策としての意義がもっとも高い。また，ダム等停滞水域では貧酸素状態が生じると鉄やマンガンのような還元性無機物質が存在するようになり，それらの対策として浄水システム内は酸化的な条件下にしなければならない。塩素等の酸化剤が用いられているが，塩素消費量のもっとも高い還元物質はアンモニア性窒素であり，その濃度変動が大きい場合には酸化反応条件の制御が困難になる。

また，アンモニア性窒素が高い原水では溶解性有機物質も多く，これらの溶解性有機物質は凝集剤として汎用されているアルミ系凝集剤と錯体を形成するなどして凝集反応を阻害する。この錯体となったアルミは浄水に残留することから利便上の障害を生むことがある。このような汚濁した原水では生物処理により，アンモニアや溶解性有機物を可能な限り低減化することが必要となる。なお，アルミ系凝集剤に比べて鉄系凝集剤は溶解性有機物の除去性が高いこと等から，鉄系凝集剤に転換する利点は大きいと考える。

濁質成分を除去する固液分離プロセスとしての凝集沈殿砂ろ過プロセスにおいて，ヒ素のような有害無機物質を除去するために，酸化剤の存在が必要であるため，塩素のような酸化剤を用いた酸化処理が必要になる。凝集沈殿砂ろ過プロセスに代わるMF／UFろ過は，クリプトスポリジウム等耐塩素性生物やピコプランクトンを完璧に分離できる技術であり，汎用浄水技術として扱われるべきものと考える。

砂ろ過やMF／UFろ過は固液分離処理プロセスであり，溶解性有機物質についてはその処理効果を実質的に期待することができない。植物プランクトン等が生産するジオスミンや２MIBのような異臭味物質は活性汚泥法等の生物処理でも生産されることから，水資源を繰り返し利用している水域での障害が発生する可能性が高い。農薬類は季節的に使用されているため，農薬使用時の水道原水中での濃度は高く，慢性影響の観点から健康影響リスク管理目標が設定されているものの，毎年使用されるものであるから季節的な変動を考慮して制御する必要がある。

水道水の発がんリスクは消毒副生成物の寄与が高いことから，消毒副生成物の生成能が高い水道原水では，その前駆物質であるフミン等溶解性有機物をオゾン・活性炭吸着処理によって除去しなければならない。水道原水中に臭化物イオンが存在するとオゾンにより発がん性の強い臭素酸が生成するため，このような場合には臭素酸が生成しないような促進酸化処理が適用されることになる。

異臭味，農薬，消毒副生成物，臭素酸等溶解性の有機物質や浄水処理過程で生成する副生成物を制御するためには，オゾン・活性炭吸着といった高度浄水処理システムを導入しなければならない。しかし，それらの代替システムとして，ナノろ過(NF)膜をベースにした水処理システムが導入される可能性が非常に高い。とくに欧州各国では，蒸発残留物などに起因する水処理上の課題が山積している国際河川ラインを水源とする国々を中心に，オゾン・活性炭からナノろ過膜処理へと転換しつつある。

NF膜ろ過は海水の淡水化で用いられている逆浸透膜の特性とUF膜ろ過の特性を兼ね備えている膜を用いた処理技術であり，一つのプロセスで溶解性有機物も無機イオンも除去できる。運転管理もMF／UF膜ろ過と同様に簡易で自動制御が可能であることから，人的資源の限界がある小規模な浄水場から導入されていくものと考えられるが，大規模な浄水場でもオゾン・活性炭吸着を補完する高度浄水処理施設として導入されるものと考える。NF膜ろ過はペットボトル水の生産工程にも利用されており，さらには中国の一部で整備されている給水拠点でNF膜ろ過処理し

た直飲水道水と常用水道水を供給する二元水道でも利用され，水道のような公共サービスでも適用可能なコストに耐えられるレベルに達している技術である。このようなことから，さらに技術革新が期待できる技術である。

感染性生物対策で今後とも有効なのは塩素処理であることは確かである。しかしながら，塩素剤は反応性の高い酸化剤であるため，水道原水中の有機物を低分子化して，生物資化性の高い有機物(AOC)を生成し給配水系での細菌の再増殖や，トリハロメタンやハロ酢酸等の健康影響リスクを有する有機物を生成する。また，塩素特有の臭気は水道水の不快さの原因にもなる。

ダムで貯留され植物プランクトンによる有機物質を含んだ水道原水の占める割合が高くなり，さらに河川上中流部の公共下水道等生活排水処理施設整備の遅れから，生活雑排水の影響を受けた水道原水ということになると，必然的に塩素消費物質が多くなり，塩素注入制御の困難さが高まる。結果的には，残留塩素濃度が高くなり水道水の快適性が損なわれ，同時に塩素消毒副生成物の濃度も高くなる。

このようなことから，浄水システム内で感染性微生物に対して十分なバリアーを構築した上で，給配水系でのリスク管理と塩素臭に対する違和感がない消毒技術として結合塩素処理が導入されることになる。遊離塩素処理を原則としている米国においてすら，ヨーロッパ諸国と同じように結合塩素処理に転換したり，転換することを決定した大都市水道も存在するようになってきている。

おわりに

水道の運営基盤の強化を図るために給水にかかるコストを低減化するとしても限界があり，公平で適正な費用負担を求めつつサービス水準の向上をめざさなければならない。水道施設の更新を含めて水道事業を再活性化するためには，より効率的な事業運営を展開することが求められる。ISO／WD24512の附属文書として扱われることとなっている日本水道協会規格である「水道事業ガイドライン」は，水道事業サービスの実態をできるだけ定量的に評価できる137の指標を規格として示している。指標についての規格であり，規格に基づいてたとえば水質基準適合率という値が求められたとしても，いくらの値であれば適正，的確に水道事業が展開されていると評価できるものではない。

しかし，その指標を用いることで，水道水の発がんリスクのみを他の水道事業体と比較するのではなく，そのリスクの背景となっている水源，浄水コスト，人的資源，水道料金等の各種指標と合わせて比較することが可能となったのである。すなわち，自己評価するためのツールが規格となったのである。

つまり水道水の水質は，水質基準というナショナルミニマムは満たしているが，詳細に見ていくと地域的には大きな差異があり，その原因が何であるかを明らかにすることが可能となったのである。このようなツールを活用することによって，レダンダントな浄水技術を，これまでと同じように浄水施設で適用するか，あるいはブロックや給水拠点で適用することによって，上質な水道水と水質基準を満たす水道水を供給する多元水道の意義が明らかになるものと考える。

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Estrogenic activities of chemicals related to food contact plastics and rubbers tested by the yeast two-hybrid assay

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Abstract
Food contact plastics and rubbers possibly contain many kinds of chemicals such as monomers, oligomers, additives, degradation products of polymers and additives, and impurities. Among them, bisphenol A, nonylphenol, benzylbutyl phthalate, styrene oligomers and hydroxylated benzophenones have been reported to possess estrogenic activities. In this study, other chemicals related to food contact plastics and rubbers, and their metabolites induced by the S9-mixture were tested for their estrogenic activities using the yeast two-hybrid assay. Among the 150 chemicals, 10 chemicals such as bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol, 4-phenylphenol, 4,4′-isopropylidenediphenol alkylphosphite, two type of styrenated phenol (including mono type), tris(nonylphenyl) phosphite, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-methoxybenzophenone and 2,4-diphenyl-4-methyl-1-pentene, their metabolites and the metabolites of 6 other chemicals, such as 2-(phenylmethyl) phenol, styrenated phenol (di and tri type), 1-(*N*-phenylamino)naphthalene, 4-*tert*-butylphenylsalicylate, nonylphenol ethoxylates and 2-methyl-6-*tert*-butylphenol, displayed estrogenic activities. All of them contained a phenol group in their chemical structures or formed one easily by hydrolysis or metabolism. However, most of the chemicals related to food contact plastics and rubbers, and their metabolites did not show any estrogenicity.

Keywords: *Estrogenic activity, food contact plastics, rubbers, yeast two-hybrid assay, 2-(phenylmethyl) phenol, 4,4′-isopropylidenediphenol alkyl(C_{12}-C_{15}) phosphite, 1-(N-phenylamino) naphthalene, styrenated phenol, tris(nonylphenyl) phosphite, 4-*tert*-butylphenylsalicylate, 2,4-diphenyl-4-methyl-1-pentene, 2-methyl-6-*tert*-butylphenol*

Introduction

Food contact plastics and rubbers may contain many kinds of chemicals such as free monomers, oligomers, additives, degradation products of polymers and additives, and impurities. Among these substances, several are recognized as endocrine disruptors that have estrogenic activities. Bisphenol A, a monomer for polycarbonate resin and epoxy resin, has been reported to have an estrogenic activity in ovariectomized rats (Dodds and Lawson 1938), in MCF-7 human breast-cancer cells (Krishnan et al. 1993) and in the rat uterine-cytoplasma fraction (Olea et al. 1996), and another report has shown that the prostatic weight of the male offspring was increased 6 months after birth (Nagel et al. 1997). Nonylphenol is well-known for its estrogenic activity in MCF-7 cells and in ovariectomized rats after release from a polystyrene test tube (Soto et al. 1991), and it was also determined in several kinds of food contact plastics which originated from the degradation of tris(nonylphenyl) phosphite or tris(mono and/or dinonylphenyl) phosphite used as an antioxidant (Kawamura et al. 1999, 2000). Benzylbutyl phthalate, a plasticizer used for polyvinyl chloride, has been reported to have a weak binding property against the estrogen receptors in MCF-7 (Jobling et al. 1995). Styrene dimers and trimers have been found in polystyrene products (Kawamura et al. 1998a) and also in instant noodles where they had migrated from the polystyrene cup (Kawamura et al. 1998b). Their estrogenic activity in a yeast two-hybrid assay, MCF-7 assay and competitive binding assay have been reported (Nishihara et al. 2000; Ohyama et al. 2001). UV stabilizers, 2-hydroxy-4-methoxybenzophenone have also been reported

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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 cerevisiae* 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 molel^{-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} moll^{-1} (final

Table I. Estrogenic activities of monomers and known estrogens.

Compounds (Other name or abbreviated name) [main product]	CAS No.	REC_{10} (moll^{-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 4 h. 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

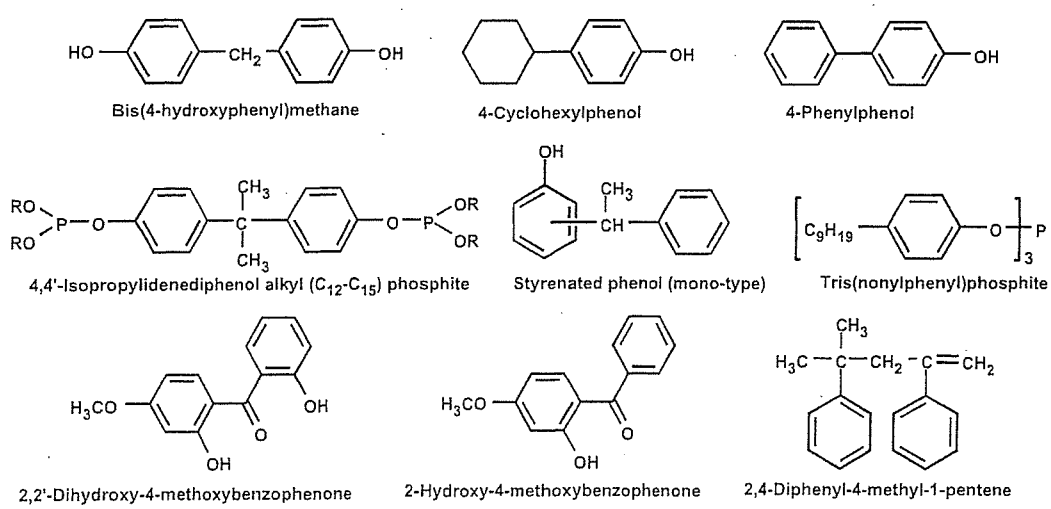

Figure 1. Structures of 9 estrogenic chemicals.

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

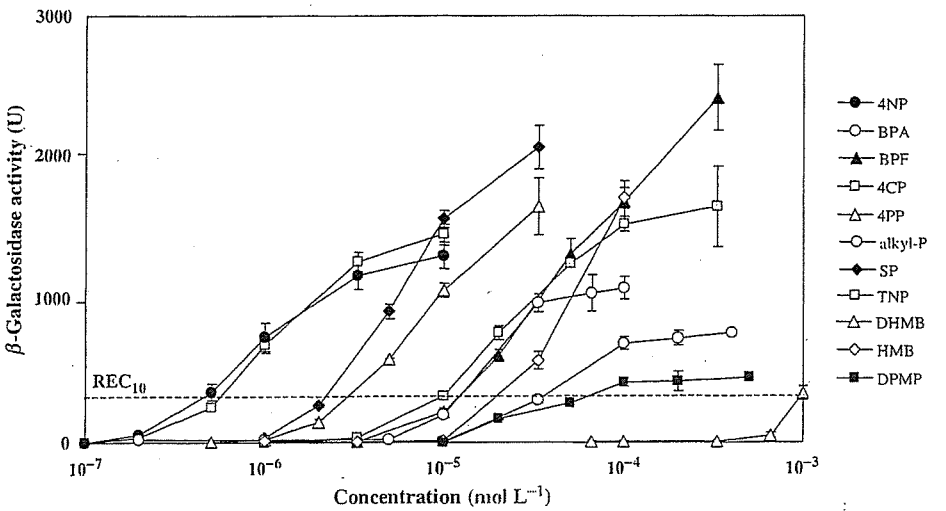

Figure 2. Dose response curves of estrogenic activity of nonylphenol (4NP), bisphenol A (BPA) bis (4-hydroxyphenyl) methane (BPF), 4-cyclohexylphenol (4CP), 4-phenylphenol (4PP), 4,4′-isopropylidenediphenol alkylphosphite (alkyl-p), styrenated phenol (mono 74%, SP), tris (nonylphenyl) phosphite (TNP),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

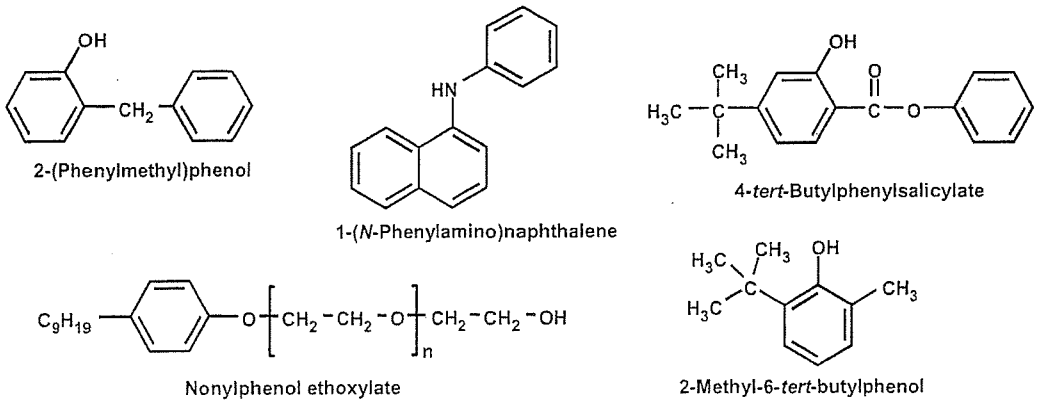

Figure 3. Structures of 5 chemicals which metabolites were estrogenic.

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.

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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

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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-

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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.

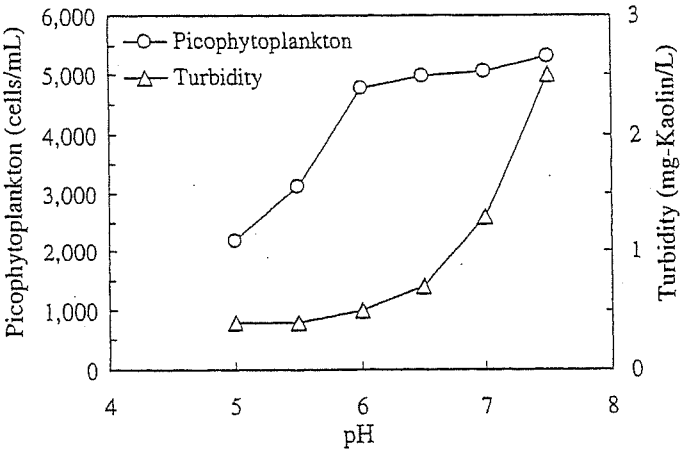

Figure 2 Residual picophytoplankton at each coagulation pH (initial picophytoplankton: 12,983 cells/mL; initial turbidity: 6.3 mg-Kaolin/L; coagulant dosage: 4 mg-Al/L; sedimentation: 15 min)

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%.

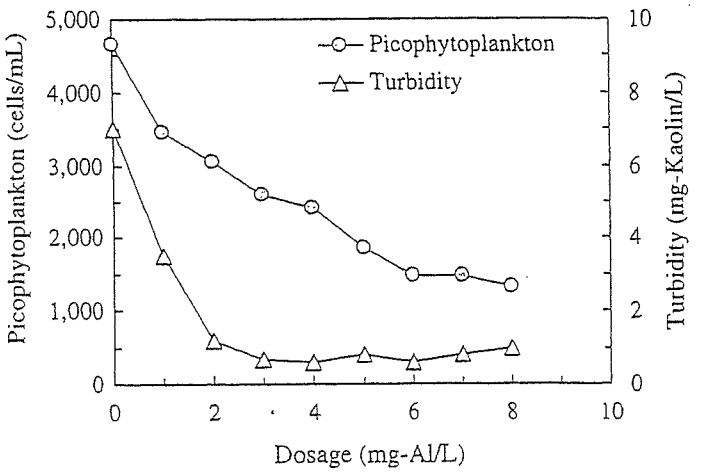

Figure 3 Residual picophytoplankton at each coagulant dosage (initial picophytoplankton: 4,658 cells/mL, initial turbidity: 7.0 mg-Kaolin/L; coagulation pH: 6.0; sedimentation: 15 min)

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.

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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

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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

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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

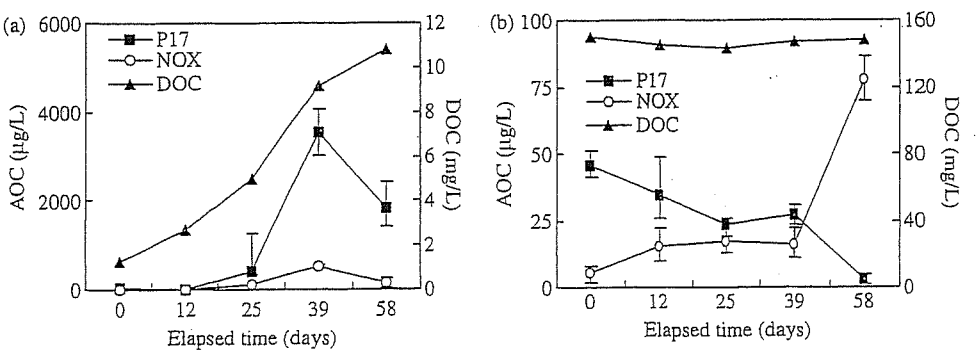

Figure 1 The variation of DOC and AOC (P17, NOX) during *P. tenue* culture: (a) M-11 culture medium; and (b) CT culture medium