Use of *Paramecium* Species in Bioassays for Environmental Risk Management: Determination of IC_{50} Values for Water Pollutants

Norikazu Miyoshi,[a] Tomonori Kawano,[b,1] Miho Tanaka,[b,2] Takashi Kadono,[b,1] Toshikazu Kosaka,[b] Manabu Kunimoto,[c,3] Tadao Takahashi,[a] and Hiroshi Hosoya*[,b]

[a]Biological Laboratory, Nishikyushu University, 4490–9 Ozaki, Kanzaki, Saga 842–8585, Japan, [b]Department of Biological Science, Graduate School of Science, Hiroshima University, 1–3–1 Kagamiyama, Higashi-Hiroshima 739–8526, Japan, and [c]Environmental Health Science Division, The National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–8506, Japan

(Received March 10, 2003; Accepted August 22, 2003)

Effect of chemical water pollutants on the growth of two *Paramecium* species (*Paramecium caudatum* and *Paramecium trichium*) were examined. The chemicals used as model chemical pollutants include organic solvents, potential carcinogens, mutagens, metabolic modulators, herbicides, insecticides, fungicides, antimicrobials, heavy metals, and heavy metal-containing chemicals. In this study, the IC_{50} values indicating the concentration of substances inhibiting the proliferation of *Paramecium* cells by 50% were used instead of the LD_{50} value, which indicates the dose of substances killing half the population of organisms, since the former is a more sensitive parameter for assessing the toxicity of substances at lower concentrations. Among 25 chemicals examined, di-(2-ethylhexyl)phthalate, potassium dichromate, 2,4-dichlorophenoxy acetic acid, and paraquat stimulated the growth of paramecia depending on the concentrations used. Dimethyl sulfoxide and formaldehyde were shown to be inert to paramecia in the range of concentrations (up to 1%, v/v) used here. Other chemicals were shown to inhibit the growth of the paramecia and thus the IC_{50} values for those chemicals were determined. Our data presented here may be a useful reference for assessing the impact of water pollutants on aqueous microecosystems consisting of various microorganisms including protozoa.

Key words —— IC_{50}, model chemical pollutants, *Paramecium* species, protozoa, water pollution

INTRODUCTION

Protozoan cells are often used as bioindicators of chemical pollution, especially in aqueous environments.[1)] Among protozoa, *Tetrahymena pyriformis* (*T. pyriformis*) is the ciliated model most commonly used for laboratory research.[2)] Cells of *T. pyriformis* are reportedly useful in the ciliate mobility test carried out in the presence or absence of various components determined to be air pollutants.[3)] In addition to the ciliate mobility test, Sauvant *et al*.[2)] described the methodologic features of recently developed toxicologic and ecotoxicologic bioassays performed with *T. pyriformis*, based on cell growth rate, biochemical markers, and behavioral changes.

The paramecia are also used to study the effect of water pollution by monitoring cell motility and also by studying the frequency of the emergence of abnormal strains.[4)] To study the impacts of model chemical pollutants on paramecia here, two *Paramecium* species, *Paramecium caudatum* (*P. caudatum*) (SJ-4 strain) and *Paramecium trichium* (*P. trichium*) (OH-24b strain, mating type I), were chosen from two major groups of *Paramecium* species, the "aurelia" group and the "bursaria" group, respectively.

In this study, we proposed the use of IC_{50} (the substance concentrations inhibiting the proliferation

[1]Present address: Graduate School of Environmental Engineering, The University of Kitakyushu, 1–1 Hibikino Wakamatsu-ku, Kitakyushu 808–0135, Japan

[2]Present address: INRA, UMR Biologie des Organismes et des Populations Appliquée à la Protection des Plantes [BiO3P], Domaine de la Motte, B.P. 29, 35653 Le Rheu Cedex, France

[3]Present address: Department of Public Health and Molecular Toxicology, School of Pharmaceutical Science, Kitasato University, 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan

*To whom correspondence should be addressed: Department of Biological Science, Graduate School of Science, Hiroshima University, 1–3–1 Kagamiyama, Higashi-Hiroshima 739–8526, Japan. Tel.: +81-824-24-7443; Fax: +81-824-24-0734; E-mail: hhosoya@sci.hiroshima-u.ac.jp

of protozoan cells by 50%) rather than LD_{50} (the substance doses killing half the population of protozoan cells) values as a sensitive parameter for assessing the effects of environmentally toxic substances. Since the chemicals used here showed few lethal effects, it was impossible to determine the LD_{50} value for each chemical. However, most chemicals used here showed inhibitory effects on the proliferation of paramecium cells, and thus IC_{50} values were obtained. Here we present the data as a groundwork that provides useful primary knowledge for assessing the impact of various chemical water pollutants on aqueous microecosystems consisting of various microorganisms including protozoa.

MATERIALS AND METHODS

Chemicals —— Twenty-five substances including potential genotoxic carcinogens, herbicides, insecticides, fungicides, antimicrobials and organic solvents were used as the model chemical pollutants. Dimethyl sulfoxide (DMSO), formaldehyde (HCHO), bis-(dimethylthiocarbamoyl) disulfide kown as thiuram, 2-aminoanthracene (2-AA), 4-nitroquinoline-*N*-oxide (4NQO), *S*-4-chlorobenzyl diethylthiocarbamate (benthiocarb), di-(2-ethylhexyl)phthalate (DEHP), 2,4-dichlorophenoxy acetic acid (2,4-D), methyl viologen dichloride (paraquat), *S*-1,2-bis(ethoxycarbonyl)ethyl-*O*,*O*-dimethylphosphorodithioate (malathion), manganese ethylene-bis-dithiocarbamate known as maneb, $NiCl_2$, $CuSO_4$, $CdCl_2$, potassium dichromate ($K_2Cr_2O_7$), triphenyltin chloride (Ph_3SnCl), tributyltin chloride (Bu_3SnCl), and mercuric chloride ($HgCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). *p*-Nonylphenol (PNPhOH) and bis-phenol-A (bisPhA) were from Tokyo Chemical Industry Co. (Tokyo, Japan). Phenol (PhOH), pentachlorophenol (PCPhOH), and methylmercury chloride (MeHgCl) were from Nacalai Tesque Inc. (Kyoto, Japan). 2,4,4′-Trichloro-2′-hydroxydiphenylether (triclosan) was obtained from Ciba-Geigy Ltd. (Basel, Switzerland). Hexachlorophen (HCP) and gamma-hexachlorocyclohexane (lindane) were purchased from Sigma (St. Louis, MO, U.S.A.).

Water or DMSO (0.1%, v/v) was used as solvent. DMSO, HCHO, paraquat, $NiCl_2$, $CuSO_4$, $CdCl_2$, and $K_2Cr_2O_7$ were dissolved in water. The remaining model chemical pollutants were first dissolved in trace volumes of absolute DMSO and diluted with water. The concentration of solvent DMSO in stock solutions was maintained at 0.1%(v/v). Final concentration of solvent DMSO in the ciliate culture was as low as 0.0001%. In the range of concentrations used here, all chemicals were fully dissolved, and no precipitation of chemicals was observed after the addition of chemicals to the cultures. Various concentrations of the chemicals were added to the paramecium cultures at time zero of the experiments, and the cells were propagated in the presence of the added chemicals throughout the culture periods without washing or exchanging the media.

Food Organisms —— *Enterobacter aerogenes* (*E. aerogenes*) was cultured on agar medium containing meat extracts (2 mg/ml), polypeptone (10 mg/ml), NaCl (5 mg/ml), and glucose (1 mg/ml). Colonies of *E. aerogenes* growing on agar medium were picked up with tip of a platinum loop, dipped into 1000 ml of lettuce infusion, and incubated statically for 3 hr at 23 ± 1°C. This liquid culture of *E. aerogenes* was used as a food organism for ciliates.

Ciliate Cultures —— Two *Paramecium* species, *P. caudatum* (SJ-4 strain) and *P. trichium* (OH-24b strain, mating type I), were used in this study. The paramecia were cultured in lettuce infusion containing a 10% volume of food organism culture (*E. aerogenes*) at 23 ± 1°C. The paramecia were subcultured every 30 days by inoculating the fresh lettuce infusion containing *E. aerogenes* (100 ml) with 5 ml of confluent culture of paramecia in a 200-ml flask.

Determination of Growth Rates and IC_{50} Values —— For determination of growth rates, paramecia were cultured on 12-well microplates. Each well was filled with 1 ml of the lettuce infusion containing *E. aerogenes*, and culture was initiated by the addition of 10 paramecium cells to each well. Then microplates were incubated for 2–5 days at 23 ± 1°C. Cells were sampled 2 or 5 days after initiation of the culture, and the number of cells was counted under a microscope using a hemocytometer. For precise counting of the cells, cell motility was minimized by adding 1–2 drops of Bouin's solution to the cells. Changes in growth (proliferation) rates in the two paramecia were examined in the presence or absence of various model chemical pollutants. The IC_{50} values defined as the concentrations of the substances required for 50% inhibition of proliferation of paramecia were determined as a parameter for toxicity of model chemical pollutants.

Table 1. IC_{50} in *P. trichium* (OH-24b) Determined for Organic Solvents, Carcinogens, Mutagens, and Metabolic Modulators

Chemical	Range of dose examined	IC_{50} (day 2)	IC_{50} (day 5)
DMSO	0.01%(v/v)–1.0%(v/v)	NOEL $\geq$ 1.0%(v/v)	NOEL $\geq$ 0.1%(v/v)
HCHO	12.5 μM–500 μM	NOEL $\geq$ 250 μM	NOEL $\geq$ 500 μM
PhOH	0.5 μM–5 mM	1.28 mM	1.60 mM
Triclosan	0.01 μM–100 μM	2.58 μM	5.4 μM
DEHP	0.25 μM–2.5 mM	Growth stimulated	Growth stimulated
BisPhA	0.1 μM–1.0 mM	37.8 μM	0.85 μM
4NQO	0.5 nM–5 μM	170 nM	90 nM
2-AA	0.02 μM–200 μM	0.78 μM	0.98 μM

IC_{50} values were determined as described in the Materials and Methods. When the growth of cells was stimulated or no inhibition in growth was observed, IC_{50} values were not determined. NOEL, no-observable-effect level. In control cells, day 2 and 5 correspond to the early and late-log phase of proliferation growth.

Table 2. IC_{50} in *P. caudatum* (SJ-4) Determined for Organic Solvents, Carcinogens, Mutagens, and Metabolic Modulators

Chemical	Range of dose examined	IC_{50} (day 2)	IC_{50} (day 5)
DMSO	0.01%(v/v)–1.0%(v/v)	NOEL $\geq$ 1.0%(v/v)	NOEL $\geq$ 0.1%(v/v)
HCHO	12.5 μM–500 μM	115 μM	418 μM[a)]
PhOH	0.5 μM–5 mM	1.48 mM	2.09 Mm[b)]
Triclosan	0.01 μM–100 μM	1.64 μM	1.38 μM
DEHP	0.25 μM–2.5 mM	Growth stimulated	Growth stimulated
BisPhA	0.1 μM–1.0 mM	22.6 μM	10.8 μM
4NQO	0.5 nM–5 μM	0.93 μM[c)]	1.25 μM[c)]
2-AA	0.02 μM–200 μM	0.37 μM	0.15 μM

IC_{50} values were determined. For details, see the text. *a*) HCHO stimulated the proliferation growth of *P. caudatum* in a limited range of concentration between 12.5 μM and 250 μM. *b*) NOEL $\geq$ 0.5 mM. *c*) No effect was observed between 0.5 nM and 0.5 μM.

RESULTS AND DISCUSSION

IC_{50} Values for Organic Solvents, Carcinogens, Mutagens, and Metabolic Modulators

Effects of various model chemical pollutants on the proliferation of two *Paramecium* species were examined 2 and 5 days after initiation of the culture. In the absence of water-polluting substances, day 2 or 5 corresponds to the early or late-log phase of proliferation growth, respectively.

Tables 1 and 2 list IC_{50} values for various organic solvents, carcinogens, mutagens, and metabolic modulators determined in *P. trichium* (OH-24b) and *P. caudatum* (SJ-4), respectively. As listed in the tables, the concentrations of substances used in this study were in the ranges between 0.01% and 1.0%(v/v) for DMSO, 12.5 and 500 μM for HCHO, 0.5 μM and 5 mM for PhOH, 0.1 μM and 1.0 mM for BisPhA, 0.5 nM and 5 μM for 4NQO, 0.01 and 100 μM for triclosan, and 0.02 and 200 μM for 2-AA.

When added alone, DMSO used as a solvent for many other chemicals showed no inhibitory effect on the proliferation of paramecia in the range examined here. Even 1.0%(v/v) DMSO was shown to be at the no-observable-effect level (NOEL). Another widely used organic solvent, HCHO, showed a slight inhibitory effect on the proliferation rates of *P. caudatum* (SJ-4) cells, and IC_{50} values on day 5 were determined to be 418 μM. On the other hand, the IC_{50} value could not be determined in *P. trichium*, but instead NOEL was determined to be greater than 500 μM.

High concentrations of PhOH were required for inhibition of proliferation in both species of *Paramecium*. On day 5, the IC_{50} values for PhOH determined in *P. trichium* and *P. caudatum* were notably high (1.6 mM and 2.09 mM, respectively).

It was shown that *P. trichium* cells are more sensitive to triclosan, since the late-log phase of growth was obviously inhibited by lower concentrations of triclosan in *P. trichium*.

A potential carcinogen DEHP showed no inhibitory effect on the proliferation rates in both paramecia, and thus the IC_{50} value was not determined (Tables 1, 2). Instead, DEHP stimulated the growth

Table 3. IC_{50} in *P. trichium* (OH-24b) Determined for Metallic and Metal-containing Water Pollutants

Chemical	Range of dose examined	IC_{50} (day 2)	IC_{50} (day 5)
$K_2Cr_2O_7$	1 nM–10 μM	NOEL $\geq$ 0.1 μM	Growth stimulated
Maneb	5 nM–50 μM	29.61 μM[a)]	11.3 μM
$NiCl_2$	0.1 μM–1 mM	3.75 μM	2.35 μM
$CuSO_4$	0.25–100 μM	1.62 μM	1.26 μM
$CdCl_2$	0.01–100 μM	12 nM	170 nM
Ph_3SnCl	0.1 nM–1 μM	30 nM	80 nM
Bu_3SnCl	0.2 nM–2 μM	0.30 μM	0.23 μM
$HgCl_2$	0.2 nM–200 μM	0.89 μM[b)]	0.67 μM[b)]
MeHgCl	0.2 nM–20 μM	20 nM	ND

IC_{50} values were determined. For details, see the text. *a*) NOEL $\geq$ 5 μM. *b*) At low concentrations between 0.2 and 20 nM, $HgCl_2$ slightly stimulated proliferation.

Table 4. IC_{50} in *P. caudatum* (SJ-4) Determined for Metallic and Metal-containing Water Pollutants

Chemical	Range of dose examined	IC_{50} (day 2)	IC_{50} (day 5)
$K_2Cr_2O_7$	1 nM–10 μM	NOEL $\geq$ 10 μM	Growth Stimulated
Maneb	5 nM–50 μM	18.37 μM[a)]	17.5 μM[a)]
$NiCl_2$	0.1 μM–1 mM	1.0 μM	8.41 μM
$CuSO_4$	0.25–100 μM	1.68 μM	1.69 μM
$CdCl_2$	0.01–100 μM	0.81 μM	0.84 μM
Ph_3SnCl	0.1 nM–1 μM	6.8 nM	13 nM
Bu_3SnCl	0.2 nM–2 μM	90 nM	57 nM
MeHgCl	0.2 nM–20 μM	0.011 μM	0.035 μM

IC_{50} values were determined. For details, see the text. *a*) NOEL $\geq$ 5 μM

of both *P. trichium* and *P. caudatum* in a dose–dependent manner (data not shown).

BisPhA, 4NQO, and 2-AA were shown to be highly toxic to both species of *Paramecium* and the IC_{50} values for those chemicals were determined to be in ranges between the submicromolar and micromolar levels. 2-AA and 4NQO are genotoxic carcinogens of which the toxic actions are believed to be mediated by the generation of reactive oxygen species.[5,6)] It is possible that the toxic action of 2-AA and 4NQO in paramecia is due to the formation of reactive oxygen species. It has been reported that BisPhA has estrogen-like effects in bird embryos.[7)] However, the mechanism of BisPhA toxicity in paramecia is unknown.

IC_{50} Values for Metallic and Metal-containing Water Pollutants

Tables 3 and 4 list the IC_{50} values for metallic and metal-containing water pollutants determined in *P. trichium* and *P. caudatum*, respectively. The concentrations of water pollutants used in this study were in the ranges between 1 nM and 10 μM for $K_2Cr_2O_7$, 5 nM and 50 μM for Maneb, 0.1 μM and 1 mM for $NiCl_2$, 0.25 and 100 μM for $CuSO_4$, 0.01 and 100 μM for $CdCl_2$, 0.1 nM and 1 μM for Ph_3SnCl, 0.2 nM and 2 μM for Bu_3SnCl, 0.2 nM and 200 μM for $HgCl_2$, and 0.2 nM and 20 μM for MeHgCl.

Ph_3SnCl, Bu_3SnCl, and MeHgCl were shown to be highly toxic to paramecia at nanomolar to submicromolar levels. Organic tin compounds including Ph_3SnCl and Bu_3SnCl have been used as antimicrobials, especially against bacteria, and thus the presence of Ph_3SnCl and Bu_3SnCl in river water drastically changes the bacterial population by eliminating most bacterial species, except for some resistant strains.[8)] Such resistant bacteria include some strains derived from *Pseudomonas diminuta*.[8)] Our knowledge of the effect of such organic tins on protozoan species is limited. The present data suggest that paramecium species are also highly susceptible to organic tin.

In the presence of $K_2Cr_2O_7$, late-log phase proliferation of both *P. trichium* and *P. caudatum* was stimulated for unknown reasons in a dose–dependent manner. The other metal salts $CuSO_4$, $NiCl_2$, and $CdCl_2$ were shown to be inhibitory to both paramecia. Among them, $CdCl_2$ was the most toxic and

Table 5. IC_{50} in *P. trichium* (OH-24b) Determined for Organic Herbicides, Insecticides, Fungicides, and Antimicrobials

Chemical	Range of dose examined	IC_{50} (day 2)	IC_{50} (day 5)
2,4-D	0.2 μM–2 mM	180.1 μM	365.9 μM
Benthiocarb	0.2 μM–2 mM	465.0 μM	1.75 μM
Paraquat	0.1 μM–1 mM	198.0 μM	343.4 μM[a)]
PCPhOH	20 nM–200 μM	5.35 μM	4.55 μM
Lindane	0.2 μM–2 mM	45.5 μM	92.1 μM
Malathion	0.2 μM–2 mM	52.5 μM	50.3 μM
pNPhOH	20 nM–200 μM	6.1 μM	6.4 μM
HCP	2.0 nM–200 μM	0.68 μM	1.5 μM
Thiuram	10 nM–100 μM	0.2 μM	1.1 μM

IC_{50} values were determined. For details, see the text. *a*) In a limited range of concentration between 0.1 and 10 μM, paraquat stimulated the growth of *P. trichium*.

Table 6. IC_{50} in *P. caudatum* (SJ-4) Determined for Organic Herbicides, Insecticides, Fungicides, and Antimicrobials

Chemical	Range of dose examined	IC_{50} (day 2)	IC_{50} (day 5)
2,4-D	0.2 μM–2 mM	0.36 mM	1.30 mM[a)]
Benthiocarb	0.2 μM–2 mM	1.42 μM	1.02 μM
Paraquat	0.1 μM–1 mM	3.92 μM[b)]	2.71 μM[b)]
PCPhOH	20 nM–200 μM	1.98 μM	1.47 μM
Lindane	0.2 μM–2 mM	5.94 μM	Data not available[c)]
Malathion	0.2 μM–2 mM	12.41 μM	43.34 μM
HCP	2.0 nM–200 μM	0.43 μM	0.21 μM
Thiuram	10 nM–100 μM	1.35 μM	2.45 μM

IC_{50} values were determined. For details, see footnotes in Table 1. *a*) Low concentration of 2,4-D (0.1–200 μM) enhanced the proliferation by *ca.* 67–122%. *b*) In the presence of a low concentration (0.1 μM), paraquat stimulated the growth of *P. caudatum*. *c*) IC_{50} (day 5) for lindane was 15–20 μM, but further experiments are required.

thus the IC_{50} value obtained was the lowest in $CdCl_2$-treated paramecia.

Copper is the major inorganic fungicidal component found in the Bordeaux mixture that has been widely used for pest control in orchards for many years, and thus contamination of fruit peels[9)] and orchard soil[10)] with copper has been reported.

The major source of metal contamination in aqueous environments is the disposal of sewage sludge. Recently some countries such as the U.K. have banned the disposal of sewage sludge at sea, which has led to the prediction that land application of sludge will become more widespread.[11)] While agricultural uses of sludge are being encouraged,[12)] the positive aspect of recycling nutrients may be offset by the risk of contamination by heavy metals (such as Ni, Cu, Cd, Zn, and Hg) that are frequently present in sludge at high concentrations.[11,12)] Therefore investigation of the impact of heavy metals on ecosystems and organisms living therein has become important.

Today, the use of mercury and mercury-containing chemicals in both industry and agriculture is restricted in most countries. However, it has been reported that mercury used in the past has accumulated in the biomass of water organisms such as algae.[13)] Thus it is necessary to assess the impact of mercury on aqueous organisms. The IC_{50} values for $HgCl_2$ in *P. trichium* determined here in the early and late-log phases were determined to be 0.89 and 0.67 μM, respectively. However, at lower concnetrations, $HgCl_2$ showed a stimulatory effect on growth at the ranges between 0.2 and 20 nM. After treatment with $HgCl_2$ (200 nM), the rate of proliferation was almost identical to that in control cells. $HgCl_2$ at concentrations higher than 2 μM completely inhibited growth.

IC_{50} Values for Organic Agrochemicals Including Herbicides, Insecticides, Fungicides, and Antimicrobials

Tables 5 and 6 list IC_{50} values for various organic herbicides, insecticides, fungicides, and antimicrobials determined in *P. trichium* and *P.*

caudatum, respectively. The concentrations of chemicals used in this study were in the ranges between 0.2 μM and 2 mM for 2,4-D, 0.2 μM and 2 mM for benthiocarb, 0.1 μM and 1 mM for paraquat, 0.02 and 200 μM for PCPhOH, 0.2 μM and 2 mM for lindane, 0.2 μM and 2 mM for malathion, 0.02 and 200 μM for PNPhOH, 2 nM and 200 μM for HCP, and 0.01 and 100 μM for thiuram.

2,4-D slightly enhanced the proliferation of *P. caudatum* at the range between 100 nM and 200 μM but such effect was not observed in *P. trichium* (data not shown). In the presence of high concentration of 2,4-D (2 mM), growth of both paramecia were completely inhibited. The IC_{50} for 2,4-D was shown to be higher in *P. caudatum*.

2,4-D is one of the oldest chemicals used in agriculture. Herbicide application started in the early 1950s and 1960s with auxin-type compounds, folloewd by inhibitors of cell division and photosynthesis. Auxin-type herbicides such as 2,4-D interfere with plant hormone regulation, but their mode of action is still unclear.[14,15] Since high concentrations of 2,4-D inhibited the growth of paramecia (Tables 5 and 6), this herbicide is not inert to nonplant organisms due to unknown mechanisms.

Paraquat is a widely used photosynthesis-related herbicide. Incubation of *P. trichium* cells with paraquat 1–10 μM resulted in stimulation of growth by 2- to 4-fold. In the presence of paraquat 1–10 μM, the growth rate was *ca*. 2-fold greater than that in control cells. At paraquate 100 μM, the rate of growth weas almost identical to that in control cells. Treatment of *P. trichium* cells with paraquat 1 mM resulted in complete inhibition of growth. On the other hand, concentrations of paraquat higher than 1 μM completely inhibited the growth of *P. caudatum* cells. These data suggest that *P. caudatum* cells are more sensitive to paraquat. In *P. caudatum* cells, stimulation of growth by paraquat was observed only at 0.1 μM, the lowest concentration examined here.

In contrast to 2,4-D and paraquat, the remaining herbicides, insecticides, fungicides, and antimicrobials showed no growth stimulation at any concentration examined and all inhibited the growth of the two paramecia.

In this study, we examined the direct impact of various chemicals on the growth of paramecia. However, in some cases, metabolites of added chemicals are more toxic to organisms. For example, the cases of maneb and structurally related fungicides, which are widely used in the agricultural field to eliminate plant pathogenic fungi from crops and vegetables, are well known. It is considered that the toxicity of maneb is not due to the manganese moiety of the compound, and maneb and related fungicides sprayed on vegetables are reportedly metabolized easily in them. However, it has been reported that maneb and related compounds possess toxic activities attributable to their main metabolite ethylene thiourea.[16] Ethylene thiourea, the toxic catabolite of maneb and related fungicides, is known to remain in the vegetables and 20% of total ethylene thiourea is still detectable in vegetables after 20 days of exposure to fresh air or water.[17] Thus the impact of fungicide catabolites on humans and animals must be examined thoroughly. Maneb and its catabolite remain in vegetables and fruits and are also toxic to the cells of *Saccharomyces cerevisiae*.[16] Therefore we propose that further information on the effects of catabolic products of fungicides, antimicrobials, insecticides, herbicides, and other potential chemical pollutants must be collected.

The IC_{50} values obtained for agriculturally important chemicals such as herbicides, insecticides, and fungicides are not surprisingly low. However, in agricultural fields and surrounding environment, living organisms are often exposed to both organic and inorganic fungicides and herbicides at the same time. In the water environment surrounded by agricultural fields and/or industrial regions, various chemicals are collected by water flow and accumulate there, and thus organisms living therein are exposed to multiple chemical pollutants. Therefore the groundwork to assess the impact of multiagent exposures of living organisms is necessary. Any potential additive actions or any synergism caused by a combination of toxic substances released to the environment should be surveyed. In this study, we examined the impact of a single application of toxic agents to paramecia. We are now planning to carry out further studies to examine the impact of multiple applications of model chemical pollutants on paramecia.

Furthermore, there is no guarantee that the data obtained for aqueous protozoa is applicable to other aqueous organisms such as algae. To survey the toxic compounds targeting both or either animals (protozoa) and plants (algae) in aqueous environments, we are now developing a sensitive biomonitoring system for the detection of toxic compounds using green paramecia as units of plant-animal symbiosis.

REFERENCES

1) Apostol, S. (1973) A bioassay of toxicity using protozoa in the study of aquatic environment pollution and its prevention. *Environ. Res.*, **6**, 365–372.
2) Sauvant, M. P., Pepin, D. and Piccinni, E. (1999) *Tetrahymena pyriformis*: a tool for toxicological studies. *Chemosphere*, **38**, 1631–1669.
3) Graf, W., Graf, H. and Wenz, M. (1999) *Tetrahymena pyriformis* in the ciliate mobility test. Validation and description of a testing procedure for the registration of harmful substances in the air as well as the effects of cigarette smoke on the human respiratory ciliated epithelium. *Zentralbl. Hyg. Umweltmed.*, **201**, 451–472.
4) Komala, Z. (1995) Notes on the use of invertebrates, especially ciliates, in studies on pollution and toxicity. *Folia Biol.* (Krakow), **43**, 25–27.
5) Weisburger, J. H., Hosey, J. R., Larios, E., Pittman, B., Zang, E., Hara, Y. and Kuts-Cheraux, G. (2001) Investigation of commercial mitolife as an antioxidant and antimutagen. *Nutrition*, **17**, 322–325.
6) Botchway, S. W., Chakrabarti, S. and Makrigiorgos, G. M. (1998) Novel visible and ultraviolet light photogeneration of hydroxyl radicals by 2-methyl-4-nitro-quinoline-N-oxide (MNO) and 4,4′-dinitro-(2,2′)bipyridinyl-N,N′-dioxide (DBD). *Photochem. Photobiol.*, **67**, 635–640.
7) Halldin, K., Berg, C., Bergman, A., Brandt, I. and Brunstorm, B. (2001) Distribution of bisphenol A and tetrabromobisphenol A in quail eggs, embryo and laying birds and studies on reproduction variables in adults following *in ovo* exposure. *Arch. Toxicol.*, **75**, 597–603.
8) Harino, H., Fukushima, M., Kurokawa, Y. and Kawai, S. (1997) Susceptibility of bacterial populations to organotin compounds and microbial degradation of organotin compounds in environmental water. *Environ. Pollut.*, **98**, 157–162.
9) Iwane, A. (2000) Studies on mineral components of apples part V. Variation in mineral components of different parts of apple fruit during development. *J. Jpn. Soc. Food Sci. Technol.* (*Nippon Shokuhin Kagaku Kogaku Kaishi*), **47**, 317–326.
10) Narimanidze, E. and Bruckner, H. (1999) Survey on the metal contamination of agricultural soils in Georgia. *Land Degradat. Develop.*, **10**, 467–488.
11) Cole, L. J., McCracken, D. I., Foster, G. N. and Aitken, M. N. (2001) Using Collembola to assess the risks of applying metal-rich sewage sludge to agricultural land in western Scotland. *Agric. Ecosystems Environ.*, **83**, 177–189.
12) Obbard, J. P. and Jones, K. C. (2001) Measurement of symbiotic nitrogen-fixation in leguminous host-plants grown in heavy metal-contaminated soils amended with sewage sludge. *Environ. Pollut.*, **111**, 311–320.
13) Zakova, Z. and Kockova, E. (1999) Biomonitoring and assessment of heavy metal contamination of streams and reservoirs in the Dyje/Thaya River Basin, Czech Republic. *Water Sci. Technol.*, **39**, 225–232.
14) Fedtke, C. (1982) *Biochemistry and Physiology of Herbicide Action*, Springer., Berlin-New York.
15) Loos, M. A. (1975) Phenoxyalkanoic acids. In *Herbicides, Chemistry, Degradation and Mode of Action*, Vol. 1 (Kearney, P. C. and Kaufman, D. D., Eds.), Marcel Dekker., New York-Basel, pp. 1–128.
16) Bertini, S., Del Carratore, R., Giorgi, M., Bronzetti, G. and Della Croce, C. (2000) Genotoxic and monooxygenase system effects of the fungicide maneb. *Arch. Toxicol.*, **74**, 415–420.
17) Knio, K. M., Saad, A. and Dagher, S. (2000) The fate and persistence of zineb, maneb, and ethylenethiourea on fresh and processed tomatoes. *Food Addit. Contam.*, **17**, 393–398.