TOXICITY EVALUATION USING HUMAN CELLS IN WATER TREATMENT PROCESSES

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Abstract : Changes in cytotoxicity and mutagenicity of pesticides (7 pesticides in the quality standard for water), spiked in groundwater, were studied using bioassays by ozonation and activated carbon adsorption. It was shown that the mutagenicity of bentiocarb-added water increased with ozonation time, however, it was not the case with water that contains either dimethyl 2,2-dichlorovinylphosphate (DDVP) or dimethyl 4-nitro-m- tolyl phosphorothioate (MEP) In terms of cytotoxicity, the growth inhibition for human diploid cell (TIG-1) in bentiocarb-added water increased with ozonation time. The cytotoxicity for DDVP-added water remained intact after ozonation, and it was not removed by the additional process of activated carbon adsorption. The changes in pesticides concentration by ozonation were not related to the changes of mutagenicity and cytotoxicity. It was also shown that the mutagenicity and cytotoxicity were varied with the reaction byproducts. Thus, to evaluate toxicity of water on humans, both the cytotoxicity and mutagenicity should be considered, at least in water quality management.

INTRODUCTION

Recently, sources of tap water have been deteriorated with a number of chemicals produced by modern industries and emitted to the environment as solid wastes and/or wastewater. A number of genotoxic health effects, such as cancer, birth defects, and reproductive anomalies, have been reported so far.[1,2] Chemical analysis of particular toxic substances carried out in a raw water quality control, does not take into account the overall toxicity to be appeared by multi-component intakes or the possible changes in toxicity caused by chemical interactions or biological metabolism.

In past decades, the bulk of scientific data have been accumulated regarding the human toxicity of drinking water. Progress in this area is mainly due to the development and widespread use of short-term genetic bioassay, usually as an Ames test developed by Bruce Ames and his colleagues.[3,4] However, the Ames test detects mutagenicity and genotoxicity only. The toxicity to the cells (cytotoxicity) needs to be considered because it is involved with various reactions with individuals, organics and cells. Thus, a toxicity test using cultured cells is the essential unit step for toxic actions occurring on individual level.

Nowadays, various bioassays using cultured cells have been developed instead of an individual test using some kind of animal for drugs or new-chemicals.[5] *In vivo* test by cultured cells was considered to be a useful method for water quality assessment because it detects many kinds of unknown chemicals formatted by design or

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undesign. Besides simplicity of control, rapidity and standardization of testing methods, it can predict the toxicity to the human body by using human cells.[6] Most cell lines from human lose the metabolic function. For this reason, using normal human cells may give more reliable results. Therefore, in order to evaluate toxicity to human bodies, cytotoxicity is also considered to be taken into account in water quality control.[7]

In this study, the cytotoxicity and the mutagenicity of water in drinking water treatment processes were evaluated by the Ames test and growth assay using TIG-1, which is human foetal lung diploid cells (normal human cells). Seven different pesticides were prepared in each groundwater sample. After the addition of pesticides, the groundwater was treated by the ozonation and activated carbon adsorption process.

MATERIALS AND METHODS

Materials

Seven different pesticides, dimethyl 2,2-dichlorovinylphosphate (DDVP), dimethyl 4-nitro-m-tolyl phosphorothioate (MEP) (ISO name is fenitrothion), S-p-chlorobenzyl diethylthiocarbamate(bentiocarb), diisopropyl 1, 3-dithiolane-2-ylidene malonate (isoprothiolane), diethyl 5-phenyl-3-isoxazolyl phosphorothioate (isoxathion), o,o-diisopropyl-s-benzyl-phospholothioate(IBP), p-nitrophenyl phenyl phosphonothionate(EPN) (Wako Pure Chem., Japan) were used as model contaminants, which are listed in the water quality regulations for drinking water in Japan.[8] Activated carbon fiber(ACF, A-15, Osaka Gas Co., Osaka, Japan) was used as a model activated carbon for the sake of experimental convenience due to its ability of fast adsorption and desorption rates.[9]

Water treatment

Pesticides were added to groundwater taken from the IIS (Institute of Industrial Science), University of Tokyo, to prepare the model for raw water. To determine the initial concentration
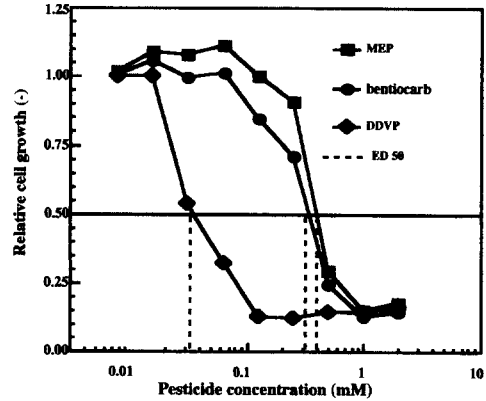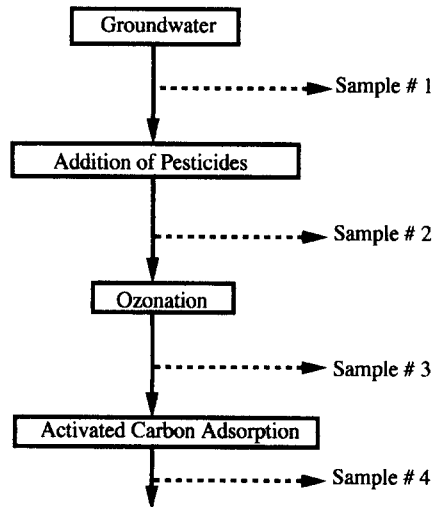

Figure 1. Dose response curve of TIG-1.

Figure 2. The flow of experimental water treatments and preparation of sample waters.

of pesticides, TIG-1 cell growth inhibition to pesticides was observed (Figure 1). ED 50 (Effective Dose), the dose for 50% survival of cells in dose response curves of TIG-1, was selected for easily observing changes of toxicity in the water treatment process. 0.05, 0.34 and 0.37 mM of DDVP, bentiocarb and MEP, were respectively determined as ED 50 for the initial concentration in Figure 1.

Ozonation was carried out for 2, 5, 10, 20 and 30 min by using an ozonizer, POX-10 (Fuji electric, Japan), where ozone concentration was 3 ppm and the flow rate was 0.4 L/hr. After the ozonation, the residual ozone was removed by adding L-ascorbic acid sodium salts. The batch

activated carbon adsorption was carried out after the 5 min of ozonation for 3 hr by using 1 g/L of ACF. The flow of water treatments and the preparation of sample waters are illustrated in Figure 2.

Cell Culture

We have chosen TIG-1 cells (Human foetal lung diploid cells), obtained from the Tokyo Metropolitan Institute of Gerontology, Japan, as a normal human cell for the cell growth assay. This cell line is established from a Japanese human female embryo, and the doubling time does not change until about 60 population doubling levels (PDL).[10] Cells less than 40PDL were used, and these were taken from frozen amples prepared by the first-obtained cell at 24 PDL. The cells were cultured in Dulbecco modified Eagle's medium (Nissui Pharm., Tokyo, Japan) supplemented with 20 mM-HEPES (N-2-hydroxyethylpypelazine-N-2-ethansulfonic acid, Dojindo Lab. Kumamoto, Japan), 5%-fetal bovine serum (FBS), 100 units-penicillin and 100 mg-streptomycin. The cells were inoculated in 96 multi-well plates(Sumilon, Tokyo, Japan) at the initial cell density of 1.0×10^4 cells/cm^2. After 24 hr of culture, the medium was exchanged for the one prepared by the water samples as shown in Figure 2, which consisted of 10 volume-% of a 10-fold concentrated culture medium and 90 volume-% of the water sample. After 6 days, the cell numbers were counted by acid phosphatase (AP) assay as described below in detail.

Measurement of Cell Growth

We employed the acid phosphatase (AP) assay to measure the living cell number because this assay is quick and simple.[11,12] In this method, the living cell numbers are measured by using the light absorbance at a wavelength of 405nm (E405), which in is proportion to it. We confirmed in preliminary experiments that the cell number up to 1×10^6 cells/cm^2, which is over the number expected on 6th day, was well correlated with E405 by a linear equation on our experimental conditions. After the culture medi-

um was removed, the cells were rinsed with 100 µl-PBS/well and soaked in sodium acetate buffer (pH 5.5) containing 0.037 g/L-p-nitrophenylphosphate (Sigma, St. Louis, USA) and Triton X-100 (Wako, Osaka, Japan). After 2 hr of incubation at 37°C, the absorbance developed in each well was measured by a microplate reader (MPR A4i, TOSOH Co., Tokyo, Japan).

Mutagenic Test

The Ames test is widely used for assaying the occurrence of mutagens which are sometimes related to carcinogens. Mutagenicity is one of toxicities of micropollutants in water. It is necessary to introduce other types of biological analysis for toxicity to secure the safety of drinking water.[7]

Salmonella Typhimurium TA100 strain was used for the mutagenesis assay. The assay was carried out according to the manner described by Maron and Ames[4], with a slight modification including a pre-incubation step. First, preincubate for 20 min a solution which is 0.1 mL of an overnight nutrient broth culture of the bacterial strain, 0.1 mL of the water sample, and 0.5 mL of phosphate buffer (pH 7.4). After that, the solution was added to 2 mL of molten top agar supplemented with biotin and a trace of histidine. The positive control mutagens and the solvent control were added to the triplicate tubes. The preparations was mixed and poured on minimal glucose agar plates. The revertant colonies were counted after a 48 hour incubation at 37°C.

RESULTS AND DISCUSSION

Cytotoxicity and Mutagenicity of Water Samples Treated in Typical Conditions

First of all, the changes of cytotoxicity and mutagenicity of the sample waters were observed in typical watertreatment conditions, such as 5 min for ozonation and activated carbon adsorption. They were the main focus to know the effects of ozonation and adsorption treatments in general.

The growth inhibition pattern is shown in Figure 3. In the case of bentiocarb added water, cell growth was inhibited to the same level as that of after the ozone treatment. This fact represents that bentiocarb and its derivatives have similar cytotoxicity. After the carbon adsorption, cell growth was recovered a little, but not to the level of raw water. This is presumed that the derivatives of bentiocarb are not adsorbed to the activated carbon used in the presence of background organics contained in the groundwater. In the case of DDVP, cell growth was more inhibited after the ozone treatment and it was not changed after adsorption. In the case of MEP, its cytotoxicity was completely removed by the ozonation. In these experiments, three typical changes in cytotoxicity by ozonation were observed.

The mutagenicity when each water sample used was shown in Figure 4. The revertants of DDVP-added water were increased a little after ozonation, and the number of revertants was not reduced by carbon adsorption. On the contrary, the number of revertants in MEP-added water was decreased after ozonation, and it was further decreased, below the level of raw water, after carbon adsorption. There was no change in the revertant number for bentiocarb-added water.

As shown in Figure 3 and 4, cytotoxicity and mutagenicity were changed considerably by the ozonation. Therefore, we further investigated the changes of cytotoxicity and mutagenicity with ozonation time.

Changes of Cytotoxicity and Mutageni-City with the Ozonation Time

The pesticides used in this work can be easily degraded by ozonation.[13,14] Time-course changes of relative cell growth with ozonation time were shown in Figure 5, 6 and 7.

In the case of bentiocarb-added water, bentiocarb was easily degraded by ozonation of enough reaction time(Figure 5). After 30 min of ozonation, bentiocarb concentration was reduced to almost zero. The cell growth was once recovered to 75% of that of the raw water
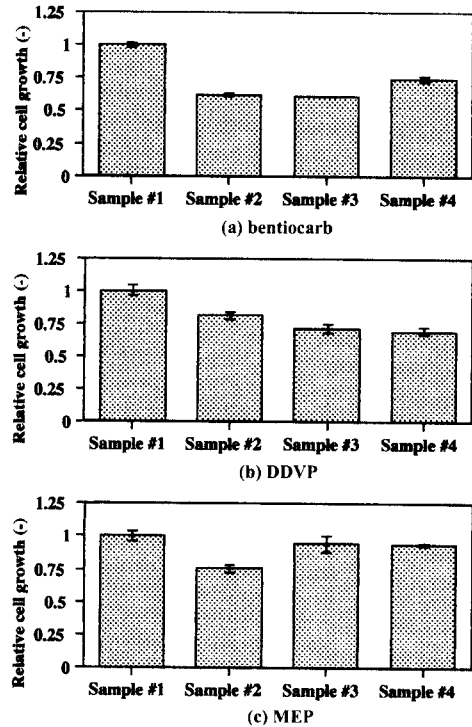

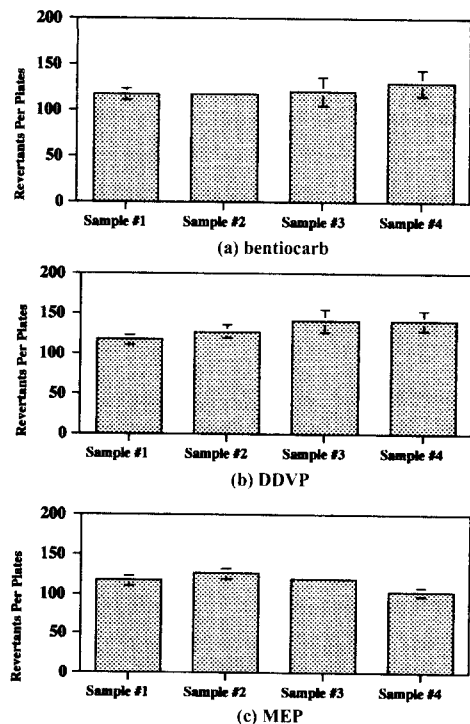Figure 3. Relative cell growth when the water samples used for culture.

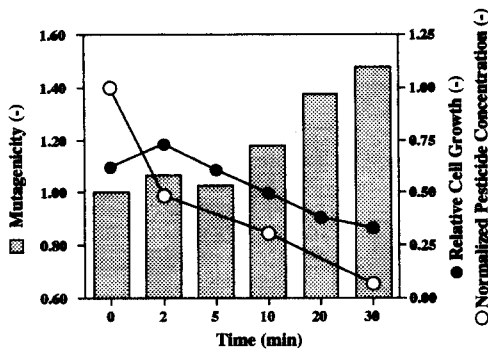Figure 4. Mutagenic activity when the water samples used for culture.

Figure 5. Changes of mutagenecity, cytotoxicity and bentiocarb concentration with ozonation time.
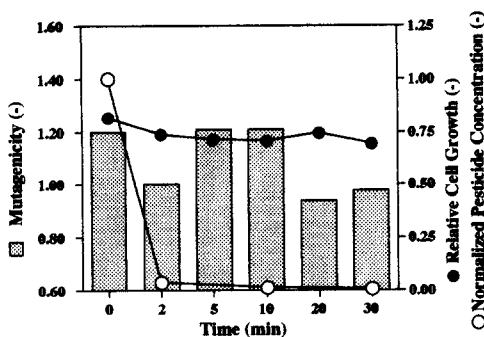

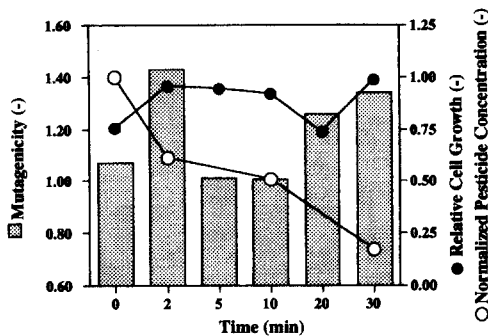Figure 6. Changes of mutagenecity, cytotoxicity and DDVP concentration with ozonation time.

Figure 7. Changes of mutagenecity, cytotoxicity and MEP concentration with ozonation time.

level after 2 min ozonation, but afterward, it gradually decreased with ozonation time. These results represent that the cytotoxicity is enhanced by ozonation as reported in the literature for chlorination.[15] With the elapse of time of ozonation, mutagenic activity was also gradually increased in bentiocarb-added water.

It was found that DDVP very easily decomposed by ozonation, as shown in Figure 6, DDVP degraded 95% and over within 2 min of ozonation. On the contrary, cell growth did not change much. After 10 min ozonation, DDVP concentration was reduced to almost zero, but no significant changes of cell growth were observed during ozonation. On the other hand, the revertants were reduced by ozonation in the case of DDVP-added water.

Figure 7 shows that cell growth in MEP-added water was recovered compared with the condition of raw water after ozonation. Mutagenic activity increased considerably after 2 min of ozonation, and then decreased with ozonation time.

The various appearances of toxicity were found in the pesticide-added groundwater by ozonation. In the case of bentiocarb-added water, it was shown that cytotoxicity and mutagenicity were increased with ozonation time. It was suspected that bentiocarb itself had no mutagenic activity, but mutagenic and cytotoxic derivatives were produced by ozonation. Also, it was found that the DDVP derivatives produced by ozonation have cytotoxicity but do not have mutagenic activity. For that reason, the changes of cytotoxicity and mutagenicity were investigated for other pesticides.

Figure 8 shows the variation of toxicity with ozonation time. The toxicity before ozonation was considered zero. The mutagenicity of bentiocarb and MEP was increased, but it was not over two fold of the number of mutagenic revertants. The cytotoxicity of bentiocarb, IBP and DDVP-added water increased by ozonation, but the cytotoxicity of MEP and isoprothiolane-added water decreased.

In a view of theses results about the 7 pesticides, the decrease of the concentration of pesticides by ozonation had no considerable correlation with cytotoxicity and mutagenicity. And the growth inhibition bioassay using human cells successfully indicated the other toxicities, besides appearing in the Ames test.

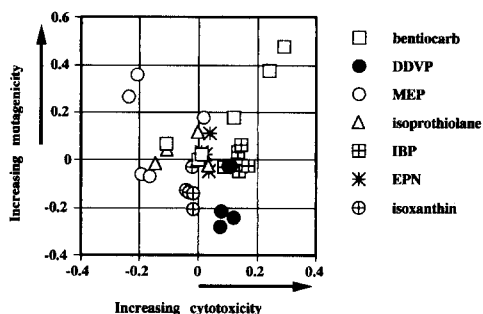Regarding these facts, it is considered that

Figure 8. Comparison of changes of cytotoxicity and mutagenicity of ozonated pesticides solution.

environmental risk management based on concentration of individual hazardous chemicals contains some problems. First of all, we have to continue to set standards for increasing numbers of newly discovered pollutants. In fact, for landfill leachates sampled at various sites in Japan, hazardous pollutants identified by extensive analysis were shown to constitute less than 1% of the total organic carbon concentration (TOC).[16] The second is that current standards do not take into account the situation, where humans or ecosystems are exposed to multiple and unknown hazardous chemicals simultaneously. One promising approach is the use of bioassays in addition to conventional chemical-specific management.

CONCLUSIONS

It was found that ozonation derivatives of pesticides do not always agree with the cytotoxicity and mutagenicity, depending on the pesticides. The evaluation of water treatments by the combination of cytotoxicity and mutagenicity is expected to give much more reliable information regarding the effects on human health.

ACKNOWLEDGEMENT

REFERENCES

1. Grisham, J. W., "Health Aspects of the Disposal of Waste Chemicals," Pergamon Press, New York, USA, pp. 454 (1986).

2. Buffer, P. A., Crane M. and Key M. M., "Possibilities of detecting health effects by studies of populations exposed to chemicals from waste disposal sites," Environ. Health Perspect., 62, 423~456 (1985).

3. Ames, B. N., McCann J. and Yamasaki E., "Methods for detecting carcinogens and mutagenes with the Salmonella/mammalian microsome mutagenicity test," Mutation Res., 31, 347~364 (1975).

4. Maron, D. and Ames. B. N. "Revised methods for the Salmonella mutagenicity test," Mutation Res, 113, 173~212 (1983).

5. Clemedson, C. et al., "MEIC evaluation of acute systemic toxicity, part II. In vitro results from 68 toxicity assays used to test the first 30 reference chemicals and comparative cytotoxicity analysis," ATLA, 24, 273~311 (1996).

6. Ekwall, B., "Correlation between cytotoxicity in vitro and LD50 values," Acta Pharmacol. Toxicol., 52, 80~99 (1983).

7. Utsumi, H., Hamada, A. and Ohno. Y., "Application of cytotoxicity test for toxic micropollutants," J. of Japan Society on Water Environ., 15, 655 (1992).

8. Quality standard for water, Japan, (1993).

9. Sakoda, A., Kawazoe, K. and Suzuki, M., "Adsorption of tri- and tetra-chloroehtylene from aqueous solutions on activated carbon fibers," Water Res., 21, 717 (1987).

10. Ohashi, M., Aizawa, S., Ooka, H., Ohsawa, T., Kaji, K., Kondo, H., Kobayashi, T., Noumura, T., Matsuo, M., Mitsui, Y., Murota, S., Yamamoto, K., Ito, H., Shimada, H. and Utakoji T., "A new human diploid cell strain, TIG-1, for the research on cellular aging," Exp. Geront., 15, 121~133 (1980).

11. Connolly, D. T., Knight, M. B., Harakas, N. K., Wittwer, A. J. and Feder, J. "Determination of the number of endothelial cells in culture using an acid phosphatase assay,"

Anal. Biochem., **152**, 136~140 (1986).

12. Martin, A. and Clynes, M., "Comparison of 5 microplate calorimetric assays for in vitro cytotoxicity testing and cell proliferation assays," *Cytotechnology*, 11, 49~58 (1993).

13. Nakamura, S., Matsubara, M., and Kinoshita, M., "Pesticide degradation in advanced water treatment," *The 44th JWWA*, pp. 216~218 (1994).

14. Tambo, N., "Report on Behavior and assessment of pesticide and byproducts in water utilization system," Ministry of Health and Welfare, Japan (1994).

15. Kawasaki, Yuiji., "The change of toxicity by chlorination of pesticides," *The 31th JWWA*, pp. 511~513 (1980).

16. Yasuhara, A., Shiraishi, H., Nishikawa, M., Yamamoto, T., Nagasugi, O., Kenmotsu, K., Hukui, H., Nagase, M., and Kawagoshi, Y., "Organic components in leachates from hazardous waste disposal sites," *Waste manage Res.*, 17, 186~197 (1999).