

was not greatly affected by pesticide adsorption and degradation rates in paddy field soils.

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Application of “Water Safety Plan” to drinking water quality management in Japan

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Introduction

“Water Safety Plan (WSP)”, a highlight in the third edition of WHO Guidelines for Drinking-water Quality [WHO, 2004], is a very important tool to achieve health-based targets. WSP requires the systematic management of drinking water quality from a source to consumer taps. In this paper, Japan’s approaches to WSP incorporation in drinking water quality management are described.

Japan approaches to WSP incorporation in drinking water quality management

Activities for the introduction and application of WSP to the drinking water quality management in Japan include a research study on WSP application in municipal water supplies and the development of guidelines on WSP application. Key steps in developing a WSP are shown in Figure 1 for reference.

Research study on WSP application in municipal water supplies

A research study, with its objective of exemplifying the way of WSP introduction to municipal water supplies in Japan and funded by a grant of MHLW, started in FY2004. Five water supplies, such as Tokyo, Yokohama, Osaka, Osaka (bulk water supply) and Kobe, are going to reformulate their drinking water quality management programs applying the concept of WSP. The study originally started on a trial basis, but it actually contributes to streamlining and upgrading their current programs of drinking water quality management. Tokyo Metropolitan Water Supply and Osaka Municipal Water Supply are going to obtain or have obtained ISO 9001 certifications regarding to their water treatment and/or distribution systems along with WSP application.

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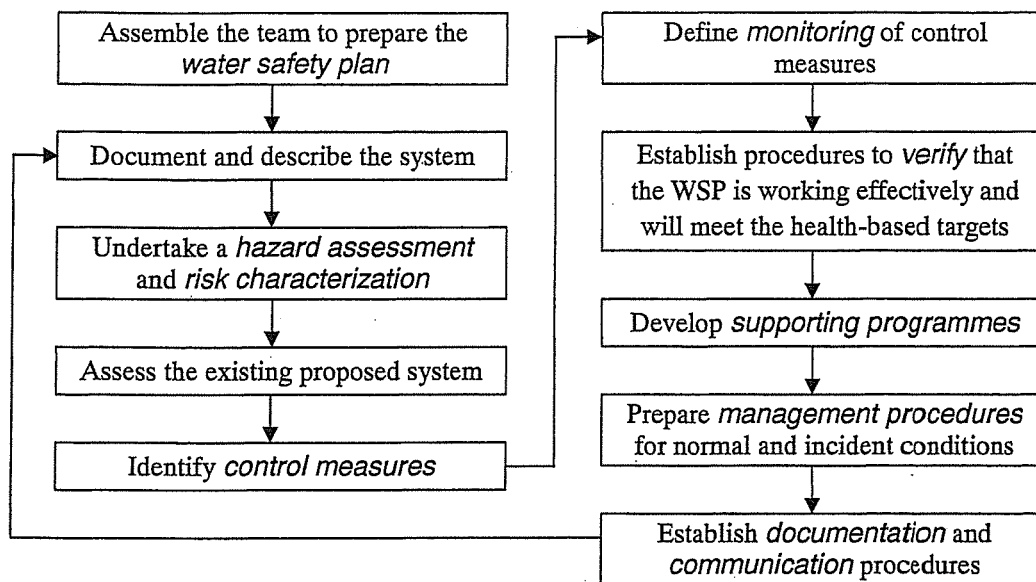


Figure 1 Overview of the key steps in developing a Water Safety Plan (WSP)

Development of guidelines on WSP application

A committee for the development of guidelines on WSP introduction was organized in Japan Water Works Association (JWWA) under the financial support of MHLW in FY2005. A few trials of WSP application to small drinking water supplies are being implemented. There exist more than eight thousand small water supplies, with a population served of 101-5,000 persons, in Japan at present. Their water quality management is rather poor compared with municipal water supplies, and its improvement is of vital importance for preventing waterborne disease outbreaks caused by drinking water contamination. Most of them lack manpower, technology and financial resources. Therefore, small water suppliers will become the main audience of the guidelines on WSP application. Their WSP should be simple, user-friendly and easy to improve. Draft guidelines will be prepared until March 2007.

WSP dissemination to developing countries through international cooperation

NIPH has been serving as the coordinator of Operation and Maintenance Network (OMN) Group since several years ago. OMN is a NGO established in 1990 under WHO for the purpose of improving the operation and maintenance of water supply and sanitation facilities especially in developing countries through the exchange of experiences, knowledge and information. The main activities of OMN include training tool development, convening workshops/seminars and information exchange through the web. Recently, OMN is contributing to the improvement of drinking water quality management through WSP dissemination to developing countries in Asia

collaborating with WHO, JICWELS (Japan International Corporation of Welfare Services) and other institutions as written below.

- JICWELS workshop on WSP and other topics, Hue, Vietnam, June 2005; Collaboration with OMN and WHO/WPRO
- JICWELS seminar on water supply management (WSP and other topics), Tokyo, January 2006; Collaboration with ONM, WHO/WPRO and NZ
- WHO-OMN workshop for PPWSA (Phnom Penh Water Supply Authority) on water supply management (WSP and other topics), Phnom Penh, Cambodia, October 2006

Conclusions

There is no doubt that WSP is essential for ensuring drinking water safety. Hazard identification seems a key component of WSP. Catchment management should be paid more attention to by drinking water suppliers in order to achieve drinking water safety. The way of WSP application may vary according to the situations of individual water supply.

Reference

World Health Organization: Guidelines for Drinking-water Quality, 3rd Edition, Vol.1: Recommendations, 2004

Effect of uncertainties in agricultural working schedules and Monte-Carlo evaluation of the model input in basin-scale runoff model analysis of herbicides

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Abstract In the prediction of time-series concentrations of herbicides in river water with diffuse-pollution hydrological models, farming schedules (the dates of herbicide application and drainage of irrigation water from rice paddies) greatly affect the runoff behavior of the herbicides. For large catchments, obtaining precise data on farming schedules is impractical, and so the model input inevitably includes substantial uncertainty. This paper evaluates the effectiveness of using the Monte-Carlo method to generate sets of estimated farming schedules to use as input to a GIS-based basin-scale runoff model to predict the concentrations of paddy-farming herbicides in river water. The effects of using the Monte-Carlo method to compensate for uncertainty in the evaluated parameters for herbicide decomposition and sorption were also evaluated.

Keywords Farming schedule; herbicide; modeling; pollutograph; runoff prediction

Introduction

Pesticides used in agriculture can enter hydrological catchment systems and contaminate rivers, which are primary sources of drinking water in many regions. Hydrological diffuse-pollution models are designed to simulate the movements of water and pollutants in river basins and thereby aid in assessing water quality. Several models for predicting pesticide concentrations in river water have been proposed and applied. The Hydrologic Simulation Program-FORTRAN (HSPF) (Johanson *et al.*, 1983, 1997) is a comprehensive model of watershed hydrology and water quality that enables integrated simulations of runoff, sediments, and nutrient transport. It can also model pesticide transport (Moore *et al.*, 1988; Laroche *et al.*, 1996; Dabrowski *et al.*, 2002), but this requires accurate agricultural as well as hydrological, meteorological, and geographical data as input. Accurate hydrological, meteorological, and geographical data are collected throughout Japan and are available to researchers. For large target catchment areas, however, acquisition of precise data on farming schedules, including the amounts of pesticides used and the dates of application, is impossible; the data acquired inevitably involves substantial uncertainty. Moreover, many factors affect the processes of sorption and decomposition of pesticides in soil and water. Owing to a lack of information on the reaction environment, however, it is impossible to quantify specific reaction rates. Generally, reported values are subject to various kinds of uncertainties.

Accordingly, the purpose of our work was to study the effects of uncertain input data regarding agricultural work schedules on model-based predictions of herbicide concentrations in river water. This paper evaluates the effectiveness of using the Monte-Carlo method to create estimates of input data, using a river-basin model composed of a large area divided into small compartments. The effects of uncertainty in the input parameters for herbicide decomposition and sorption are also discussed.

Model description

Compartment model

A compartment model was used to describe the movement of herbicides in a river basin and to create herbicide pollutographs. In the model, a river basin was divided into a grid of 1 km × 1 km grid cells. Each grid cell was subdivided into 12 compartments: each compartment was defined as consisting mainly of a river-water compartment (R compartment), a river-bed compartment (S compartment), a paddy-field-water compartment (W compartment), 2 paddy-field-soil compartments (X and Y compartments), or others (Figure 1). Water and herbicides from all compartments except the C compartment move laterally to the R compartment of one of the immediately surrounding 8 grid cells, specifically, to the cell along the steepest downhill slope from the source cell. Lateral movement from the C compartment goes to the R compartment of the next grid cell via the S compartment of that grid cell. The irrigation water in the W compartment comes from the R compartment of the same grid cell or from the grid cell that contains the intake gate (R compartment) for the paddy field. Vertical flows from all compartments except the R and S compartments are downward.

A set of differential mass-balance equations describing the dynamics of a solute (herbicide) and water in each compartment was formulated, based on the law of conservation of mass for the herbicides and the water. In the hydrology (water flow) part of the model, the rates of lateral water flow into ($Q_{W,in}$) and out of ($Q_{W,out}$) the W compartment are described as functions of the water level (h_W) in the compartment:

$$Q_{W,in} = A a_{W,in} \max(0, h_{W,0} - h_W) + A q_W \quad (1)$$

$$Q_{W,out} = A a_{W,out} \max(0, h_W - h_{W,0}) \quad (2)$$

The water depth in the paddy field (h_W) is artificially controlled at various levels according to the growth stage of rice and the weather conditions. The desired water level in the rice paddy field ($h_{W,0}$) and the spill-over irrigation flow rate (q_W) are input variables, which are determined by the rice farming schedules.

Vertical flow from W compartments ($Q_{W,Y}$) is described as a function of water level in the rice paddy field; this water goes into the X compartment beneath the W compartment

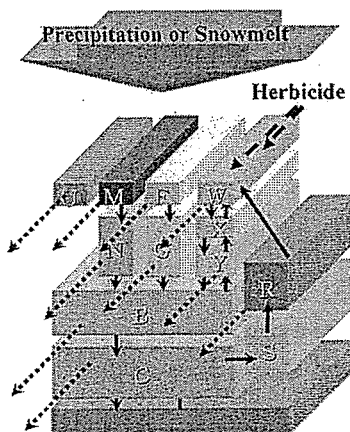


Figure 1 Compartments in a grid cell (R: river, S: river bed, W: paddy field, U: urban area, M: mountain, F: upland field)

in the same grid cell

$$Q_{w,v} = a_{w,v} A \left(\frac{h_w}{h_{w,0}} \right) \quad (3)$$

The rates of lateral flow (Q_H) from the M, F, and U compartments are described by the Manning equation:

$$Q_H = \frac{A}{B} h \frac{1}{n_M} h^{2/3} I^{1/2} \quad (4)$$

The rates of lateral interflow from X, Y, N, G, B, and C compartments are described as a function of the water level in the compartment and the slope of the compartment:

$$Q_H = a_H I(A/B)h \quad (5)$$

Vertical flows from the X, Y, M, N, F, G, B, and C compartments are described as percentages of each water content, which is equivalent to the water level relative to the compartment height:

$$Q_V = a_V A(h/h_0) \quad (6)$$

The Manning equation is also used to describe the flow rate in the R compartment:

$$Q_R = \frac{A}{L_R} h \frac{1}{n_M} h^{2/3} I^{1/2} \quad (7)$$

For solute movement between compartments, advection and diffusion are considered. Solute advectons are given as the product of the concentration and water flow rate calculated from Eqs. (1)–(7). However, the maximum real concentration for each solute is limited by its solubility in water, so any amount of herbicide over the solubility limit must exist in the solid phase and is not subject to movement. The rate of solute movement by diffusion between compartments is given by the linear driving force model:

$$q_D = A \frac{D}{L} (C_1 - C_2) \quad (8)$$

Within a compartment, both the solute concentration and the water level are assumed to be uniform, each represented by a single variable. For example, rainfall is assumed to mix completely and uniformly with herbicides in the paddy-field-water compartment (W compartment). If a compartment consists of multiple subelements (soil–solid and soil–water), a dynamic equilibrium exists between the dissolved and sorbed fractions at the solid–water interface. These phases are assumed to be in equilibrium at all times; sorption processes are considered to be instantaneous and are described by a single constant (the solid–water partition coefficient) in the linear equilibrium relationship. Hence, once the concentration in one phase is known, the concentration in the other phase can be calculated. Degradation of herbicides in each compartment follows first-order kinetics. The processes of herbicide uptake by plants and herbicide evaporation into the atmosphere were not considered in this model. The flow rate coefficient in each type of compartment (W, X, etc.) is assumed to be a single value (for each compartment) throughout the entire set of grid cells in the basin. These assumptions were made to reduce the total number of hydrologic parameters, even though the target river basin was divided into numerous grid cells, which contributed to preventing too much uncertainty in determining the model parameter values.

Site description and model application

Two river basins were selected to test the model and to analyze the effects of uncertainties in agricultural work schedules on modeled predictions of herbicide concentrations: the Chikugo River basin (1,884 km²; Figure 2) and the Oirase River basin (667 km², Matsui *et al.*, 2002). The Chikugo basin includes rice paddy fields (261 km²) cultivated by 22,860 farmers, and the Oirase basin includes rice paddy fields (92 km²) cultivated by 3,400 farmers. The Oirase River basin was divided into 667 grid cells (each 1 km²), for a total of 8,004 compartments. A set of 16,008 equations was solved to describe the movements of water and herbicides in the river basin. The Chikugo River basin was divided into 1,884 grid cells. Because the Chikugo River basin includes several dams, where river flow rates are artificially controlled, model calculations were conducted for the catchment area of each dam. The model equations were solved as a system of ordinary differential equations by Gear's stiff method from the IMSL MATH/LIBRARY.

Application of the compartment model to the river basin requires geographic data: the altitude of each compartment was determined from Geographic Information System (GIS) data (The Geographical Survey Institute, Japan, 1999), and water flow directions between compartments were determined based on the direction of the steepest gradient. The GIS data (The Geographical Survey Institute, Japan, 1990) were also used to calculate the areas of the compartments (paddy field, river, forest, etc.) in each grid. However, the GIS data available were old and may not reflect current land utilization. The area of paddy fields, which is the most important geographical information in this research, was corrected with data published by the local governments (Census of Agriculture Japan, 1995 and 2000), which include data on the percent of rice paddy area removed from cultivation due to compulsory adjustments in production. The fallow paddy fields were regarded as upland field compartments.



Figure 2 Probability distribution of farming schedules (histogram) and an allocation pattern of farming schedules in a target river basin (each farming schedule shown as a colored bar in the histogram was allocated to compartments randomly)

Hydrologic model inputs and system parameters

The amount of precipitation in each grid cell was estimated by interpolating the observed data from observation points in and around the target area and applying three-dimensional corrections for variations in altitude and location. Evapotranspiration was estimated by the method of Brutsaert and Stricker (1979) from data on air temperature, wind velocity, duration/intensity of sunshine, and celestial declination. For the Oirase river basin, the effects of snowfall and snowmelt were estimated by a temperature index method (Ikebuchi *et al.*, 1984, 1985).

The hydrologic (water flow) model requires 11 parameters optimized. The values of the vertical flow rate coefficients in the W and X compartments ($a_{w,v}$) were determined to be a typical value for the field percolation rate of water in paddy fields, 0.01 m s^{-1} . The spill-over flow rate (q_w) was estimated to be 0.02 m s^{-1} . Irrigation and drainage rate coefficients of rice paddy fields ($a_{w,in}$ and $a_{w,out}$) were inputs as 5 and 2 d^{-1} , respectively, after talking with a farmer, considering the structures and dimensions of outlets of several rice paddy fields, and also actually measuring the drainage flow rate of a paddy field. The values of the remaining parameters were searched by iteration to give the best fit to the observed river flow rate within the minimum error. In addition to this best-fit criterion, the parameters were set so as to give no annual long-term water loss in the C compartment. The hydrologic parameters of the model were successfully calibrated, with the result that they fit the observed stream flow data to the model simulation with reasonable accuracy.

Monte-Carlo generation of rice cultivation schedule

Figure 3 shows the irrigation schedule recommended by a local government, which was used to determine the input data for water depth and irrigation rate. The irrigation schedule is set according to the rice-transplanting date. To maintain the water depth in a field during various periods of rice growth, the water consumed due to evapotranspiration and percolation is replaced by irrigation. The irrigation and drainage schedule can be adjusted for herbicide dusting and ambient temperature. For example, after herbicide dusting, drainage is halted for 5 days. The water depth is also changed according to whether the ambient temperature is high ($>20^\circ\text{C}$) or not. Therefore, the input rice farming schedule can be based on the dates of rice transplanting and herbicide dusting. For a large paddy field cultivated by numerous farmers, however, the schedule of agricultural tasks in the entire paddy field is not homogeneous: rice transplanting and herbicide dusting are not each performed on a single date. The transplanting season continues for several weeks, and each herbicide is dusted once within a certain period after transplantation is finished.

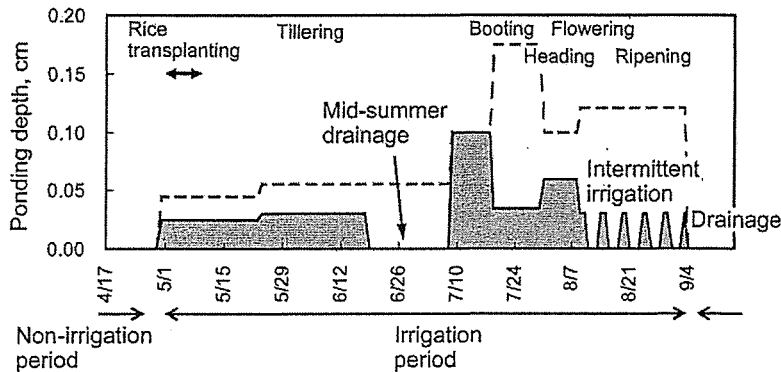


Figure 3 A pattern of irrigation (solid line for ordinary temperature and dashed line for high temperature)

For example, dusting with the herbicide mefenacet is done once, between 5 and 15 days after transplantation.

Modeling the agricultural schedules of each farmer after detailed data acquisition is ideal but too troublesome to be feasible. However, due to the large scale of the study area and its cultivation by many farmers, it should be possible to consider agricultural tasks as random events within defined periods of time, which can be estimated from data published by local governments. Two hundred farming work schedules (combinations of dates for rice transplanting and herbicide dusting) were created for each herbicide; the probability of occurrence of each work schedule is indicated by the histogram in Figure 2. For example, in about 5% of the rice paddy fields, rice seedlings were transplanted on June 23 and herbicide was dusted on July 2. Farming schedules were allocated to the paddy field compartments of the grid cells in the river basin randomly, within an expected occurrence probability (the histogram in Figure 2 shows an allocation pattern for the herbicide mefenacet). A total of 100 schedule-allocation patterns for each herbicide were created and used as input to the modeling. For comparison, model predictions with deterministic input were also conducted, for which a single farming schedule for rice transplanting and herbicide dusting was used throughout the entire river basin (the dates of the highest bar in the histogram in Figure 2). The amounts of herbicides consumed in the target river basin were estimated from marketing information on the sales of commercial herbicide products.

Monte-Carlo generation of degradation rates and soil-water partition coefficients of herbicides

Many factors (aerobic/anaerobic conditions, soil-sediment organic content, etc.) affect herbicide decomposition and its partition between soil and water. Because of a lack of information regarding the reaction environment in the field, however, it is impossible to quantify the specific decomposition rate and sorption equilibrium in each grid cell of the model, so these values are subject to various kinds of uncertainties. Although some reports include values for the decomposition rates or half-lives, the reported ranges of these rate coefficients are very wide, partly owing to variability in the reaction conditions. Therefore, a single reported value is not appropriate for representing decomposition rates in a whole area. It is more reasonable to assume that all rate parameter uncertainties are random. Using the Monte-Carlo method, a degradation rate coefficient for each herbicide in each compartment was randomly selected from values in the ranges of the reported values. The solid-water sorption coefficient of each herbicide was treated the same way. For comparison, we repeated the model calculation using the average of reported values as deterministic parameter value inputs.

Predicting herbicide concentrations

Herbicide concentrations were measured 5 days a week during 1999 and 2000 in the Chikugo River and once a week during 1995 and 1996 in the Oirase River. After the hydrological system parameters were calibrated, the hydrological and solute models were solved simultaneously by substituting solute input data, yielding predicted concentrations of herbicides in the river waters. Model-based predictions from the Monte-Carlo inputs were made, starting with each of the 100 schedule-allocation patterns. Figure 4 shows the probability distribution for the concentration of the herbicide pretilachlor on one day in 1999 in the Chikugo River. The predicted concentrations are distributed broadly, due to the Monte-Carlo generation of the farming schedules and herbicide parameters. The highest 95th percentile concentration was 7 times the lowest 5 percentile concentration. Figure 5 shows time variations in the pretilachlor concentration. About half the observed

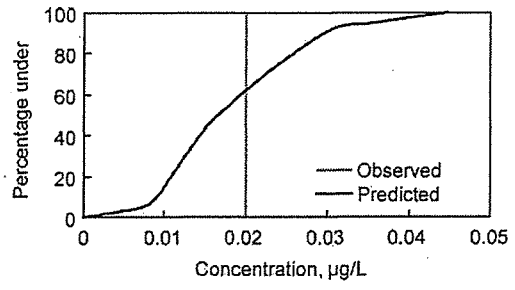


Figure 4 Probability distribution of predicted pretilachlor concentration on June 9, 1999

data points are within the 1%–99% probability range of the predicted concentrations. Although the herbicide dusting date and the amount applied are important factors in predicting the concentrations, our modeling did not consider the amount and date of herbicide dusting by individual farmers. Moreover, the simulations were conducted without optimizing the herbicide decomposition/sorption parameters. In light of these limitations, we consider the model prediction for pretilachlor to be reasonably successful. As shown in Figure 5, compared with the prediction using Monte-Carlo inputs, the prediction obtained with deterministic input yielded a rather discrete concentration variation with improper peaks.

Although prediction of pretilachlor concentration with the Monte Carlo method was good, the results for other herbicides were less successful. For example, the predicted concentration of dimethametryn was lower than that observed (Figure 6). These discrepancies could be due to poor estimates of herbicide consumption (from the sales volume) and/or of the herbicide decomposition parameter. A precise evaluation of local herbicide sales would improve the prediction. More than 60% of the predictions based on Monte-Carlo inputs were within 0.1 to 10 times the observed values, and the Monte-Carlo method reduced the percentage of ‘bad’ predictions, shown yellow in Figure 7.

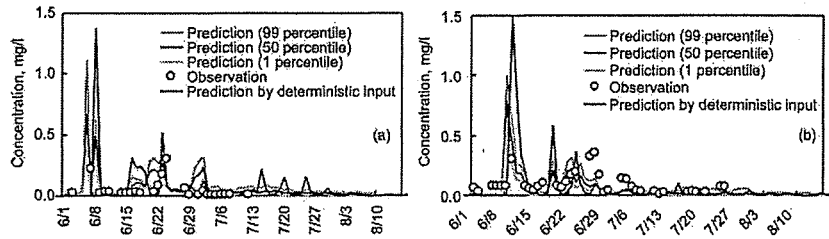


Figure 5 (a) Predicted and observed pretilachlor concentrations in Chikugo River in 1999. (b) Predicted and observed pretilachlor concentrations in Chikugo River in 1999

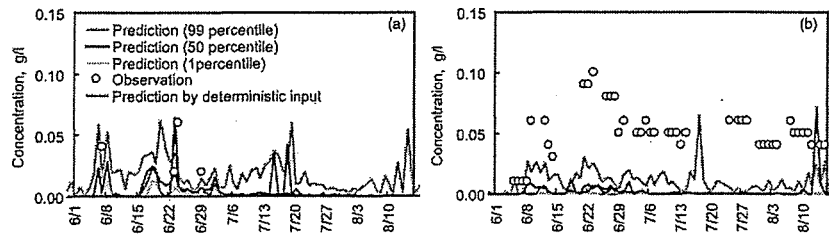


Figure 6 (a) Predicted and observed dimethametryn concentrations in Chikugo River in 1999. (b) Predicted and observed dimethametryn concentrations in Chikugo River in 2000

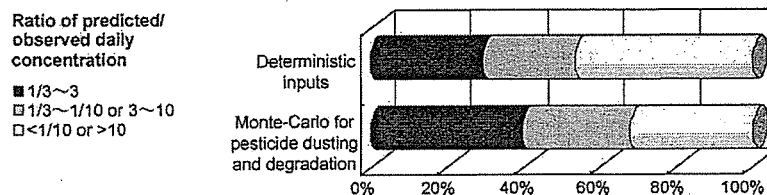


Figure 7 Comparison of model predictions with inputs generated by Monte-Carlo and deterministic methods (Chikugo River)

Conclusions

The effectiveness of the Monte-Carlo method for creating input data for agricultural work schedules and pesticide decomposition/sorption parameters was studied. The Monte-Carlo method was used to randomly allocate 200 patterns of farming work schedules to each paddy field in grid cells of a GIS-based basin-scale pesticide runoff model. The degradation rate and sorption coefficient for each herbicide in each compartment were also randomly selected from values in the ranges of reported values. Prediction of pesticide concentrations in river water by the runoff model was better with Monte-Carlo input than with deterministic input. The Monte-Carlo method alleviates the difficulty of obtaining precise data on individual farming schedules (including pesticide dusting dates) and on individual degradation rates and sorption coefficients in each soil. Once better values are available for the amounts of pesticides applied and for pesticide degradation rates and sorption parameters under actual field conditions, the GIS-based basin-scale runoff model should successfully predict river water pesticide concentrations.

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Use of cholinesterase activity as an indicator for the effects of combinations of organophosphorus pesticides in water from environmental sources

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Abstract

Organophosphorus pesticides (OPs) are commonly detected in agricultural products, animal-derived foodstuffs, and environmental samples. Until now, the focus of research has been to evaluate the adverse effect of a single OP. While each OP may be present at concentrations under recognized as “no observed adverse effect level (NOAEL)”, the combined effects of multiple OPs present at these low concentrations have not been sufficiently studied. Therefore, we developed an *in vitro* testing method to evaluate the toxicity of multiple OPs based on the degree of inhibition of cholinesterase (ChE) activity. This method requires only 10 min to complete and no specialized technology. We examined 15 OPs by this method and categorized them into three groups according to the degree of ChE inhibition. A relationship between the OPs' chemical structures and the degree of ChE inhibition emerged with the moiety $-P-O-C=N-$ showing the strongest action. The degree of ChE inhibition increased with multiple OPs, and the degree of inhibition seemed to be additive. These results demonstrate that the combined toxicity of multiple OPs present in food or environmental samples is an easily determined and toxicologically relevant measure of overall toxicity of complex OPs mixtures. It is possible to apply this testing method as a monitoring technique in water quality management in order to control OPs. As a result, this method can play the role for the potential risk reduction to the ecosystem and may contribute to the preservation of the environment.

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Keywords: Organophosphorus pesticides; Hazard assessment; Cholinesterase activity; *In vitro* method; Combined toxicity; Additive action

1. Introduction

Recently, various environmental pollutants have become a major source of concern with regards to their

potential adverse effects on humans exposed to combinations of these environmental pollutants. Water influences the health of both humans and animals and is greatly affected by water pollutants such as he organic compounds discharged from industrial, agricultural, and domestic drainage. Among the organic compounds, most concern, due to their

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extensive use worldwide and common production (Donaldson et al., 2002), are organophosphorus pesticides (OPs). Present in agricultural runoff, OPs may enter the aquatic environment and reach high concentrations downstream from discharge sites, potentially producing adverse effects in humans and wildlife (Haywood and Karalliedde, 2000; Fleischli et al., 2004).

Acetylcholinesterase (AChE; E.C. 3.1.1.7), the target enzyme of most OPs and their active metabolites, is a key neuroregulatory enzyme that is common to many species from insects to reptiles, birds and mammals. In addition, pseudocholinesterase (PChE), an enzyme present in serum, is also targeted and inhibited by OPs (Ma and Chambers, 1995, 1994; Sultatos, 1987; Sultatos et al., 1985). Toxicity of OPs is a composite effect that depends on concentration, duration of exposure, and organism size. Different organisms display different degrees of sensitivity to cholinesterase (ChE) inhibitors as OPs act as suicide substrates for AChE and PChE, irreversibly phosphorylating critical serine residues in the enzyme active site, leading to irreversible inactivation of the ChE in cholinergic synapses and neuromuscular junctions (Betancourt and Carr, 2004; Liu et al., 2002; Amitai et al., 1998; Mileson et al., 1998). To attain an acceptable daily intake (ADI) of OPs, a single pesticide is administered using a suitable animal model to assess toxicity. Unfortunately, OPs are rarely present in environmental samples as single chemical compounds, but commonly coexist as mixtures of different pesticides; thus, it is important to evaluate the total toxicity of multiple OPs in a sample using an indicator of overall potential toxicity.

While it is expensive and time consuming to evaluate the combined effects of multiple pesticides in laboratory animals, there are few efficient alternative test methods. The limited investigation of acute interactive toxicity of mixtures containing two OPs has previously been done (Karanth et al., 2004, 2001; Hazarika et al., 2003; Richardson et al., 2001). The multiple OPs we chose to evaluate can be grouped into two categories according to their structures: (1) nine chemical compounds containing a P=S moiety: butamifos, chlorpyrifos, diazinon, EPN, fenitrothion (MEP), isofenphos, isoxathion, prothiofos, and tolclofos-methyl; and (2) six chemical compounds containing a P=O moiety: acephate, dichlorvos (DDVP), edifenphos (EDDP), fosetyl, iprobenfos (IBP), and trichlorfon (DEP). In this paper, we establish a simple, inexpensive, and robust *in vitro* evaluation system suitable for hazard assessment of combinations of multiple OPs. Using 5-methyl -2-thenoylthiocholine-iodide (MTTC) as an indicator of ChE inhibition, the overall toxicity of OP mixtures, representative of those typically encountered in environmental water samples, was examined.

2. Materials and methods

2.1. Chemicals

Butamifos oxon, chlorpyrifos oxon and tolclofos-methyl oxon were obtained from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) and chlorpyrifos was obtained from GL Sciences Inc. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Laboratory water was purified using a Milli-Q gradient A10 Elix with an EDS polisher system (Millipore, Bedford, MA, USA).

2.2. Standard solutions

Stock solutions of PChE (1250 IU/L) and MTTC (2.0 mM) were prepared and stored at 4°C until further needed. A 0.25 mM chromogen solution of 5, 5'-dithiobisnitrobenzoic acid (DTNB) was prepared using 0.1 mol/L phosphate buffer (pH 7.4).

Individual standard solutions of pesticides were prepared in dimethyl sulfoxide, and each experiment was performed using seven dilutions of each standard solution. Evaluation of a single pesticide was performed using final concentrations of 10, 5, 2, 1, 0.5, 0.2, and 0.1 µg/mL. Combinations of two pesticides were evaluated by fixing the concentration of one pesticide at 0.1 µg/mL and varying the concentration of the second pesticide to 5, 2.5, 1, and 0.5, 0.25, 0.1, and 0.05 µg/mL. The pesticides that showed strong inhibition were diluted 100 fold. Combinations of three pesticides were evaluated by fixing the concentrations of two compounds at 0.05 µg/mL each, and varying the concentration of the third pesticide to 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 µg/mL. The pesticides that showed strong inhibition were diluted 5000 fold.

2.3. The evaluation of ChE activity

The active ChE enzymatically cleaves the substrate MTTC to release thiocholine. The released thiocholine reacts with the chromogen DTNB to generate a yellow product (Fig. 1), quantifiable at 405 nm by UV absorption that is impeded when ChE activity is inhibited by pesticide (Karahasanoglu and Ozand, 1967).

All experiments were done in triplicate. A 7 mIU solution of ChE and each appropriate pesticide sample were uniformly mixed in a ratio of 4:1. MTTC substrate solution (63 µL) was added to 7 µL of the mixed solution of ChE and OP in a 96 microwell plate and then 280 µL of the DTNB chromogen solution was added. The plate was incubated at 37°C for 7 min, and the absorbance at 405 nm was measured using an

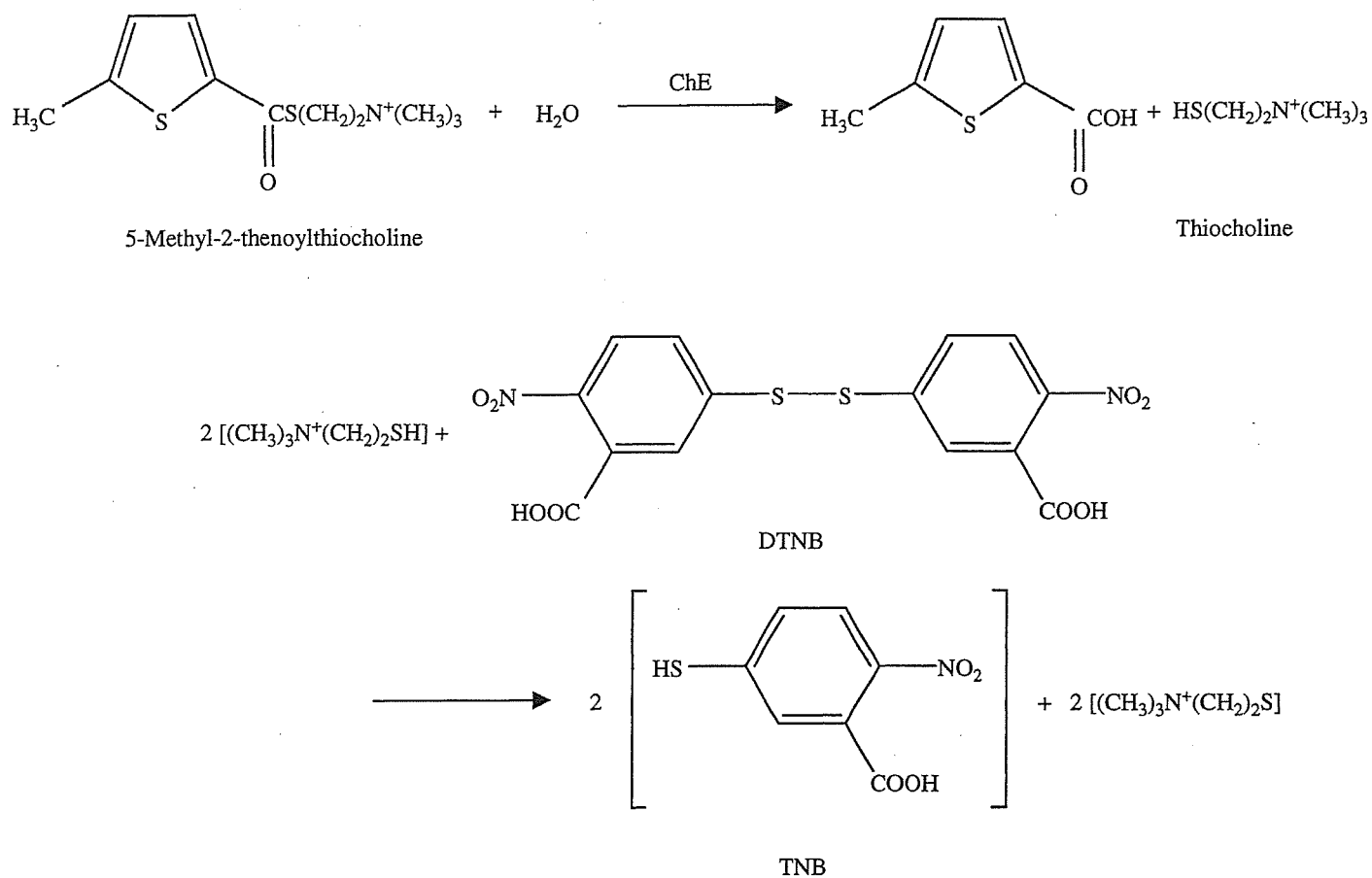


Fig. 1. The chemical reaction for generation of thiocholine and subsequent measurement of ChE activity.

Table 1
Intended use and concentration of 20% inhibition for the oxon form of nine pesticides

Group	Pesticides	Intended use	IC ₂₀ (μg/mL)
A	Chlorpyrifos oxon	I	0.0011
	Isoxathion oxon	I	0.0013
	Diazinon oxon	I	0.0089
B	Prothiofos oxon	I	0.13
	EPN oxon	I	0.14
	MEP oxon	I	0.33
C	Butamifos oxon	H	1.04
	Tolclofos-methyl oxon	G	5.09
	Isofenphos oxon	I	—

I: insecticide, G: germicide, H: herbicide.

Table 2
Intended use and concentration of 20% inhibition for six pesticides

Group	Pesticides	Intended use	IC ₂₀ (μg/mL)
A	DDVP	I	0.048
	EDDP	G	0.08
B	DEP	I	0.49
C	IBP	G	2.81
	Fosetyl	G	0.3
	Acephate	I	—

I: insecticide, G: germicide.

3. Results and discussion

3.1. Inhibition of ChE activity by 15 kinds of OPs

Parent compounds containing a P=S moiety exhibited limited inhibition (Fig. 2); however, these species undergo desulfuration to form active oxon metabolites, containing a P=O moiety. This desulfuration can occur

Ultrospec Visible Plate Reader II 96 (Amersham Biosciences, Tokyo, Japan).

The method employed typically yields a 40% background at 405 nm and IC₂₀ results (Tables 1 and 2) are calculated and corrected by setting the maximal inhibition (Figs. 3 and 4) to 100%.

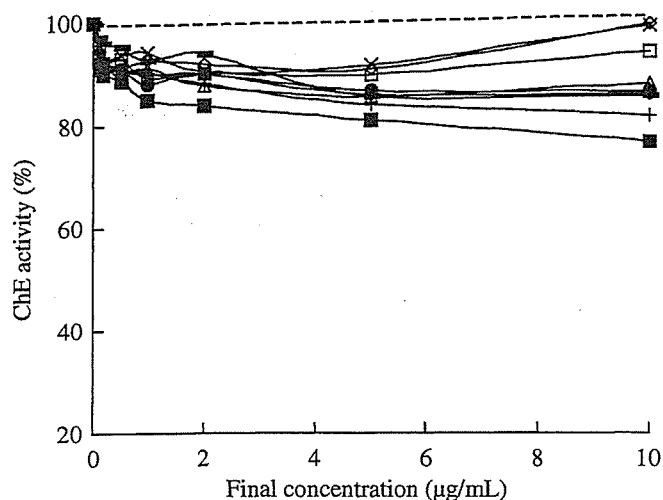


Fig. 2. ChE activity for the parent compounds of nine pesticides: —■—; isofenphos, —●—; tolclofos-methyl, —△—; butamifos, —□—; MEP, —◇—; EPN, —■—; prothiofos, —+—; diazinon, —×—; isoxathion, —◆—; chlorpyrifos.

through metabolic activation by organisms or by nonbiological oxidation in the environment (Ma and Chambers, 1995, 1994; Sultatos, 1987; Sultatos et al., 1985; Butler and Murray, 1997). Evaluation of the inhibitory activity of the oxon metabolites of these nine pesticides confirmed that the degree of inhibition was stronger than that of the parent compounds (Fig. 3). OPs were classified into three groups according to the level of ChE inhibition (Table 1). Group A showed strong inhibition of ChE with 20% inhibition (IC_{20}) at 100 ng/mL or less. Group B showed slight inhibition with IC_{20} between 0.1 and 1 µg/mL. Group C showed limited inhibition with IC_{20} exceeding 0.1 µg/mL. The degree of ChE inhibition for six pesticides containing an original P=O moiety is shown in Fig. 4, and these pesticides were similarly classified into three groups (Table 2). Of the pesticides, fosetyl inhibited ChE activity at low concentrations but the degree of inhibition did not increase with increasing concentrations (Fig. 4). Accordingly, we classified fosetyl into group C even though the IC_{20} was calculated to be 0.3 µg/mL. Consequently, the results suggest that the toxicity of OPs is due to the presence of the P=O moiety that makes the P=O pesticide more similar in structure to acetylcholine, the substrate of ChE, than the comparable P=S pesticide. However, there was no obvious relationship found between the degree of inhibition and intended use (i.e. insecticide, germicide, herbicide) of the pesticide (Tables 1 and 2).

3.2. The relation between ChE activity inhibition and the chemical structure

Strong inhibitors such as chlorpyrifos oxon, isoxathion oxon and diazinon oxon (Fig. 5) in group A

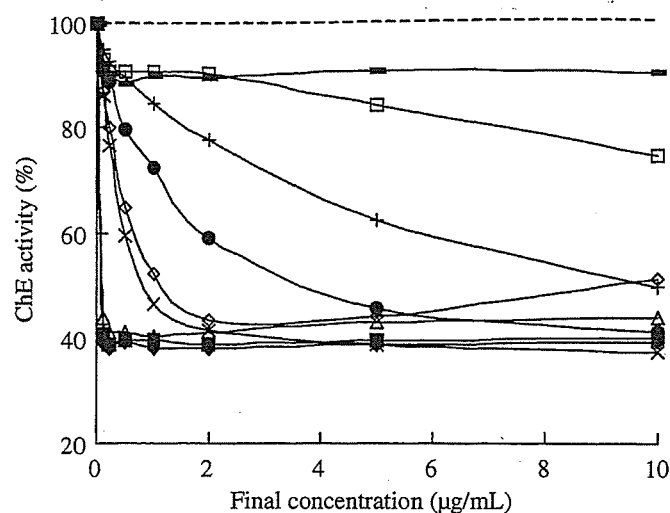


Fig. 3. ChE activity for the oxon form of nine pesticides: —■—; isofenphos oxon, —●—; tolclofos-methyl oxon, —△—; butamifos oxon, —□—; MEP oxon, —◇—; EPN oxon, —■—; prothiofos oxon, —+—; diazinon oxon, —×—; isoxathion oxon, —◆—; chlorpyrifos oxon.

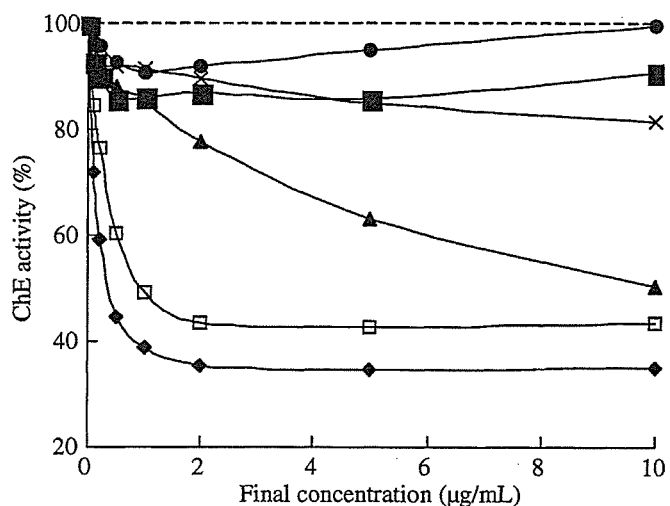


Fig. 4. The degree of ChE activity of six pesticides: —□—; acephate, —×—; fosetyl, —■—; IBP, —△—; DEP, —●—; EDDP, —◆—; DDVP.

are classified as nitrogenous heterocyclic ester thionophosphate insecticides, and this —P—O—C=N— structure may be critical to the pesticide's inhibitory activity. ChE inhibition produced by isofenphos oxon, acephate and butamifos oxon was weak; thus these compounds belong to group C (Fig. 6). They are classified as ester amidophosphates, and this structure provides selective toxicity against insects compared to mammals. Many OPs developed after the 1960s typically are of this class and present relatively low risks of toxicity for mammals. While there seems to be a relation between the structure and the degree of ChE inhibition, the precise role of the

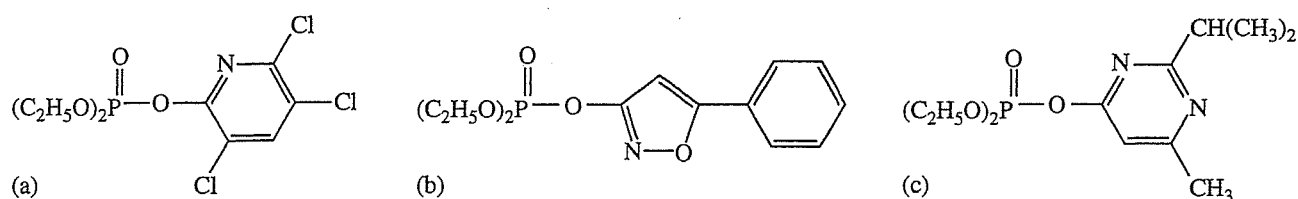


Fig. 5. Chemical structures of (a) chlorpyrifos oxon, (b) isoxathion oxon and (c) diazinon oxon.

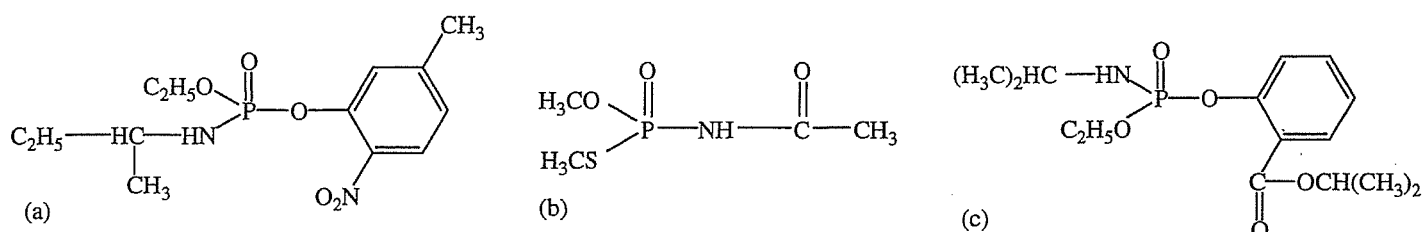


Fig. 6. Chemical structures (a) butamifos oxon, (b) acephate, and (c) isofenphos oxon.

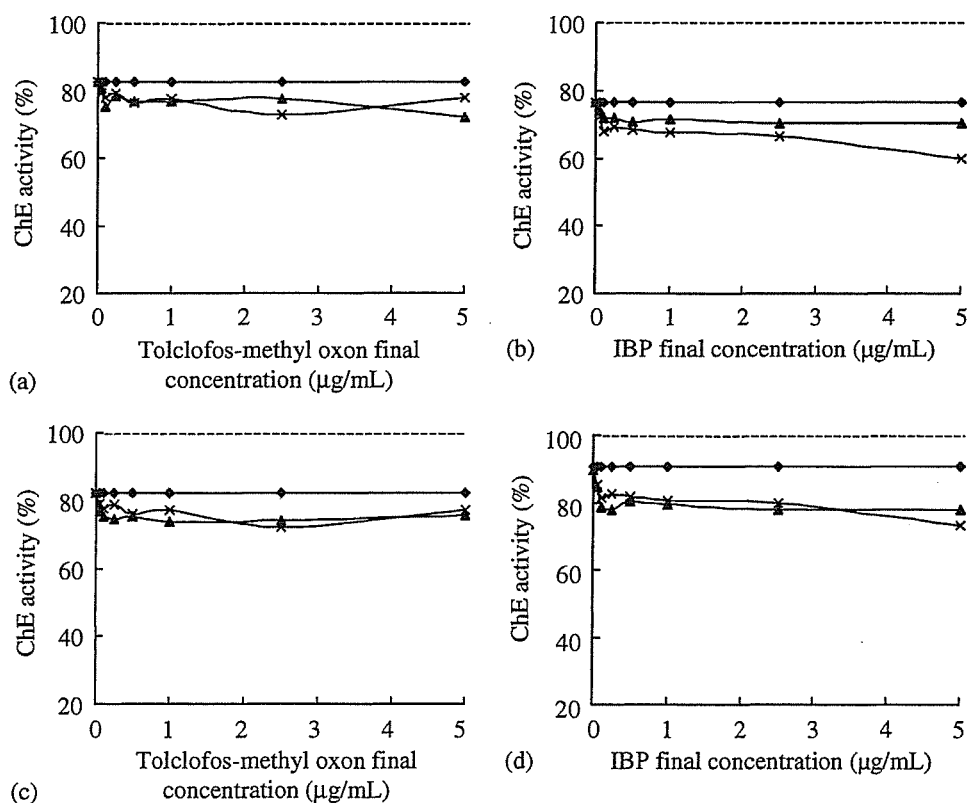


Fig. 7. The combined influence of two pesticides is shown: (a) chlorpyrifos oxon and tolclofos-methyl oxon, (b) DDVP and IBP, (c) isofenphos oxon and tolclofos-methyl oxon, (d) acephate and IBP, —◆—; X were added into the reaction at the fixed concentration, respectively, —▲—; combined influence under the condition of the fixed concentration of X and eight concentrations of Y, —■—; predicted value of the combined influence, X: (a) chlorpyrifos oxon, (b) DDVP, (c) isofenphos oxon, and (d) acephate Y: (a) tolclofos-methyl oxon, (b) IBP, (c) tolclofos-methyl oxon, and (d) IBP.

structure of the pesticide and the mechanism of ChE inhibition is unknown.

3.3. The evaluation of OPs combinations

We examined the inhibition activity of the following two-compound combinations: chlorpyrifos oxon + isox-

athion oxon, DDVP + EDDP, chlorpyrifos oxon + tolclofos-methyl oxon, DDVP + IBP, isofenphos oxon + tolclofos-methyl oxon, and IBP + acephate. No synergistic effects were observed; all combinations were additive with respect to ChE inhibition. Chlorpyrifos oxon + tolclofos-methyl oxon, DDVP + IBP, isofenphos oxon + tolclofos-methyl oxon and IBP + acephate

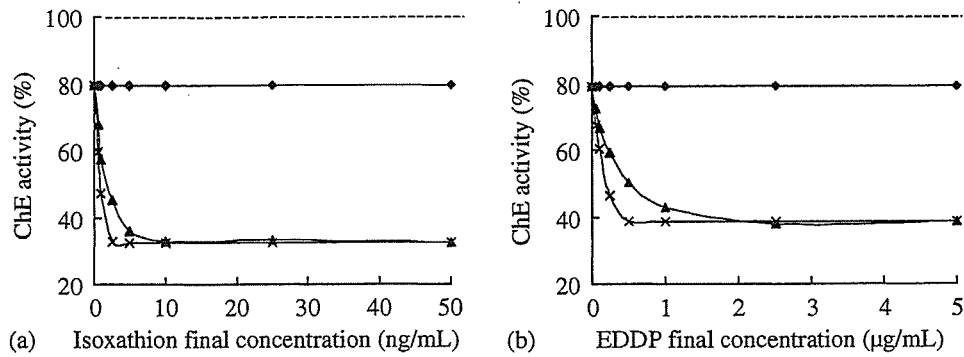


Fig. 8. The competing influence of two pesticides, including one strong inhibitor found in group A is shown: (a) chlorpyrifos oxon and Isoxathion oxon, (b) DDVP and EDDP, —◆—; X were added into the reaction at the fixed concentration, respectively, —▲—; combined influence under the condition of the fixed concentration of X and eight concentrations of Y, —■—; Predicted value of the combined influence X: (a) chlorpyrifos oxon, (b) DDVP, Y: (a) isoxathion oxon, (b) EDDP.

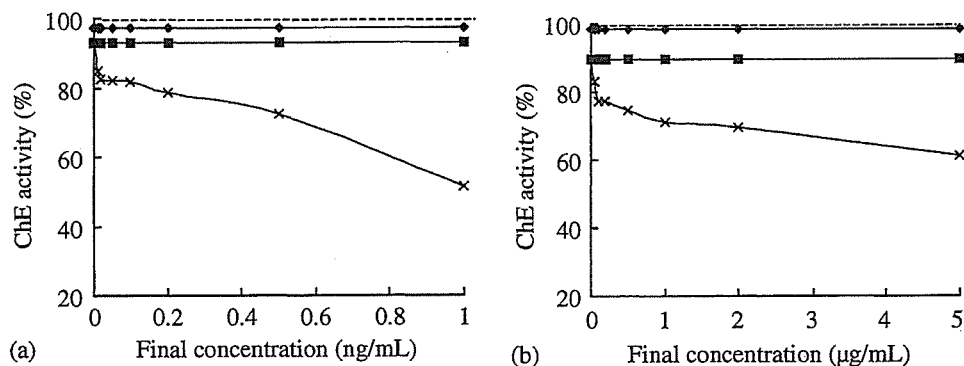


Fig. 9. The combined influence of three pesticides is shown: (a) chlorpyrifos oxon, isoxathion oxon and diazinon oxon (b) isofenphos oxon, tolclofos-methyl oxon and butamifos oxon, —◆—; X were added into the reaction at the fixed concentration, respectively, —×—; X and Y were added into the reaction at the fixed concentration, respectively, —■—; combined influence under the condition of the fixed concentration of X, Y and eight concentrations of Z. X: (a) chlorpyrifos oxon, (b) isofenphos oxon, Y: (a) isoxathion oxon, (b) tolclofos-methyl Oxon, Z: (a) diazinon oxon, (b) butamifos oxon.

showed additive effects approximately corresponding to the sum of the predicted values for individual OPs (Fig. 7). However, the total activity of chlorpyrifos oxon + isoxathion oxon and DDVP + EDDP (combinations of members from group A) showed less additive activity than that predicted from the activity of the individual components (Fig. 8).

Previously, Richardson et al. (2001) evaluated *in vitro* interaction between two OPs on ChE activity. When chlorpyrifos oxon and azinphos methyl oxon were added simultaneously to brain tissue *in vitro*, an additive effect was noted. Sequential exposure led to an additive effect at low concentrations but greater than additive inhibition at higher concentration. This study did not include the effect of three-compound combination exposures. There is no paper that examined the effect of three-compound combination exposure otherwise.

In our study, three-compound combinations, chlorpyrifos oxon + isoxathion oxon + diazinon oxon and isofenphos oxon + tolclofos-methyl oxon + butamifos oxon, were examined (Fig. 9). An additional inhibitory

effect was observed upon addition of the third compound when compared to the previously observed two-compound results. This suggests that exposure to multiple OPs might have adverse influences on human and wildlife due to an additive effect, even if each pesticide is present at concentrations under recognized NOAEL.

4. Conclusions

Our results indicate that the comprehensive evaluation of multiple pesticides is essential to assess the actual toxicity risks posed by exposure to mixtures of environmental pollutants having common biomolecular targets. In the present work, we demonstrate the utility of our simple, inexpensive, and robust *in vitro* evaluation system to examine the combined influence of OPs commonly encountered in the aquatic environment. Of note, the relative strength of ChE inhibition observed for these pesticides in our model system are well correlated with recognized values for ADI. Of course,

the adverse effect of environmental pollutants, including multiple compounds, should be evaluated in appropriate animal models. Future *in vivo* results could be compared to the results obtained using the *in vitro* method in order to gain a greater understanding of how to measure an apparent environmental exposure and establish daily toxicity monitoring. However, the reported *in vitro* method is useful for evaluating the toxicity of OPs and OP mixtures, and can be easily applied to risk assessment of complex OPs mixtures commonly observed as environmental pollutants. Consequently we suggest that this method be applied as a monitoring technique in order to preserve water quality and reduce the risk of pesticides upon the ecosystem.

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