

The behaviour and cholinesterase inhibitory activity of fenthion and its products by light and chlorination

Maiko Tahara, Reiji Kubota, Hiroyuki Nakazawa, Hiroshi Tokunaga and Tetsuji Nishimura

ABSTRACT

We established a method for quantitative analysis of fenthion (MPP) and its related compounds in water samples, using solid-phase extraction and liquid chromatography/mass spectrometry. With this method, the values of the limit of quantification ranged from 0.2 to 100 ng l⁻¹. Using this method, we examined the fate of MPP in water and the products produced by light irradiation and chlorination. MPP decreased gradually and reached 50% of the initial concentration after 48 hours in water. In particular, MPP-sulfoxide was formed. With light irradiation, MPP decomposed immediately into MPP-sulfoxide, *O,O*-Dimethyl S-[3-methyl-4-(methylthio)phenyl]phosphorothioate and other compounds. With chlorination, MPP decomposed into MPP-sulfoxide, MPP-sulfone, and their oxons. The concentration of oxons increased in a time-dependent manner. In their effects on organisms, MPP, MPP-sulfoxide and MPP-sulfone showed weak inhibitory activity to cholinesterase, whereas their oxons showed strong activity. It is feared that MPP and its products exist in environmental water and are produced by the disinfection treatment process. Comprehensive evaluation of the toxicity of MPP and its related compounds is important in order to understand the effects of MPP on ecosystems and human health.

Key words | ChE activity, chlorination, light irradiation, MPP, oxidized products, water

Maiko Tahara
Reiji Kubota
Hiroshi Tokunaga
Tetsuji Nishimura (corresponding author)
Division of Environmental Chemistry,
National Institute of Health Sciences,
Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501,
Japan
Tel.: +81-3-3700-9346
Fax: +81-3-3700-9346
E-mail: nishimur@nihs.go.jp

Hiroyuki Nakazawa
Department of Analytical Chemistry,
Faculty of Pharmaceutical Sciences,
Hoshi University,
Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501,
Japan

INTRODUCTION

Fenthion (MPP) is an organophosphorus pesticide used in modern agriculture primarily as an insecticide for paddy fields. Monitoring of pesticides in natural water used as a source of drinking water has been performed at various places in Japan. Results showed that MPP was detected in natural water closely situated to paddy fields. In addition, the MPP-related compounds, such as MPP-sulfoxide and MPP-sulfone, were also detected in natural water without use as pesticide (Wang *et al.* 1987; Nagafuchi *et al.* 1994; Tsuda *et al.* 1998). Compounds in natural water are affected by environmental conditions such as irradiation by sunlight, the concentration of hydrogen ions and oxygen, microorganisms, and so on. They are also modified and oxidized through the disinfection processes of water treatment plants. Similarly, pesticides in the environment may also be affected by

environmental and artificial factors. There is concern about the fate of pesticides in water sources, and their effect on ecosystems and human health (Tsuda *et al.* 1997).

In this work, we describe the methods developed for simultaneous quantitative analysis of MPP and related compounds in water samples using solid-phase extraction and liquid chromatography/mass spectrometric detection. We then examined the fate of MPP in water treated by light irradiation and chlorination. Because organophosphorus pesticides commonly inhibit nervous system cholinesterase (ChE) activity, resulting in adverse effects on organisms (Jokanović 2001), we investigated the effect of MPP and its products on ChE activity using an *in vitro* bioassay (Tahara *et al.* 2005), in order to assess how human health might be affected.

EXPERIMENTAL

Chemicals and reagents

MPP and MPP-sulfoxide were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). MPP-sulfone, MPP-oxon, MPP-oxon-sulfoxide, and MPP-oxon-sulfone were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany).

Standard solutions were prepared individually in acetone at concentrations of 1,000 mg l⁻¹ for MPP and MPP-sulfoxide, and 100 mg l⁻¹ for MPP-sulfone. The three oxon solutions were purchased at 10 µg ml⁻¹ in acetonitrile solution. All standard solutions were stored at -20°C. The working solutions were freshly prepared for every use by dilution of the standard solution with acetonitrile and/or 0.15% acetic acid, as necessary.

High quality acetone, acetonitrile, acetic acid, sodium hypochlorite solution, and L(+)-ascorbic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd. Laboratory water was purified by a Milli-Q gradient A10 and Elix with EDS polisher system water-purification (Millipore, Bedford, Massachusetts). Methanol was not used in this study, because transesterification of organophosphorus pesticides may occur in methanol (Hong & Pehkonen 1998).

Solid-phase extraction

Compounds in water samples were extracted and concentrated with solid-phase extraction (SPE) cartridges. The cartridges were equilibrated with 5 ml acetonitrile and 5 ml water, respectively. Extraction of water samples was carried out with a 10 ml min⁻¹ flow rate using an automatic concentrator, Sep-Pak Concentrator Plus (Waters, Milford, Massachusetts). Air was then passed through the cartridges for 5 min. The compounds were eluted from the cartridges with 5 ml acetonitrile. The eluted solutions were concentrated to less than 0.3 ml under a gentle nitrogen stream, and for liquid chromatography/mass spectrometry (LC/MS) samples 0.15% acetic acid was added to a final volume of 1.0 ml. The final solution for LC/MS analysis was composed of 0.15% acetic acid/acetonitrile (v/v = 7:3).

Standard solutions in acetonitrile were spiked into 500 ml purified water, for final concentrations of 50 ng ml⁻¹ for

MPP, 0.1 ng ml⁻¹ for MPP-sulfoxide, 1 ng ml⁻¹ for MPP-sulfone, 0.25 ng ml⁻¹ for MPP-oxon, 1 ng ml⁻¹ for MPP-oxon-sulfoxide, and 0.5 ng ml⁻¹ for MPP-oxon-sulfone. The recovery of compounds from water samples was performed using three cartridges: Oasis HLB Plus Extraction Cartridge, Sep-Pak Plus PS-2 Cartridge, and Sep-Pak Plus C18 Cartridge (Waters). The blank consisted of 500 ml of purified water.

Analysis with LS/MS

The target compounds were analysed by LC/MS for qualitative and quantitative analysis.

LC was carried out using an Agilent 1100 series (Agilent, Waldborn, Germany) instrument equipped with a Rheodyne Model 7750 injector. The analytical column was Zorbax Eclipse XDB-C18 (Agilent), 4.6 mm i.d. × 250 mm, 5 µm particle size. The column oven temperature was 40°C. Mobile phases were 0.15% acetic acid (A) and acetonitrile (B) with the following gradient programme: maintaining 70% A for 5 minutes; by a linear gradient from 70% A at *t* = 5 minutes to 30% A at *t* = 20 minutes; maintaining 30% A for 5 minutes. The flow rate was set to 1.0 ml min⁻¹ and the injection volume was 10 µl. The MS system was an Agilent 1100 series (Agilent) quadrupole equipped with an electrospray ionization (ESI) source. The instrument was operated in scan mode and the positive and negative ionization mode of selected ion monitoring (SIM) mode. The operating conditions for ESI were nebulizer gas (nitrogen) 60 psi; drying gas (nitrogen) flow 10 l min⁻¹; gas temperature 350°C. Capillary voltages were 4,000 V for positive and 2,000 V for negative. The fragmentor voltage was kept at 200 V. The scan mode was 50–500 *m/z*.

Extraction of MPP and its products from water

MPP standard solution was added to purified water to a final concentration of 0.001 mg l⁻¹. Strict pH adjustment was not performed but the extraction was conducted in neutral conditions. After stirring at room temperature for 5 minutes, a 500 ml sample was taken for the original water sample, reaction time at 0 hour. With stirring at 20°C, 500 ml samples were taken at 1, 2, 4, 6, 24 and 48 hours. MPP and its products were extracted by SPE. The operations were done at room temperature, around 25°C.

Light irradiation

Photolysis experiments were performed in purified water using an original laboratory photoreactor. An ultraviolet (UV) GL6 lamp (National, Osaka, Japan) with electrical power at 6 W and maximum wavelength of 254 nm was located at the centre of the reactor. The characteristics of this lamp were suitable to evaluate the effect in a narrow wavelength range because about 90% of the energy is concentrated in 254 nm spectrum. MPP solution of concentration $1 \mu\text{g ml}^{-1}$ was put in a standard rectangular quartz cell (1 cm pathlength) and placed at a distance of 17 cm from the light source. MPP solutions were irradiated by UV light (254 nm) for 10, 20, 30, 45, 60, 90 and 120 seconds in the short irradiation experiment, and for 1, 2, 5, 10, 20 and 30 minutes in the long irradiation experiment. Sample solutions were analysed directly by LC/MS.

A 250 mg l^{-1} MPP solution was irradiated by UV light for 0.5, 1, 1.5, 2, 2.5, 3 and 4 hours for the detection of ChE inhibitory activity. The products were also analysed by LC/MS direct injection.

Chlorination

We examined the behaviour of MPP and its products in chlorine water to investigate the effect of chlorination on MPP in water treatment plants, using sodium hypochlorite solution, which was generally used as a disinfectant providing an effective barrier to many pathogens, especially bacteria at treatment plants.

The chlorination experiment for the examination of MPP behaviour was carried out at low MPP concentration having regard to the real-world situation. The preparation of samples for the evaluation of chlorination products was performed at high MPP concentration on the basis of the sensitivity of bioassay and the yield of products.

MPP standard solution was added to purified water to a final concentration of 0.001 mg l^{-1} . After stirring at room temperature for 5 minutes, a 500 ml sample was taken for the original water sample, reaction time at 0 hour. A sodium hypochlorite solution was then added to a final concentration of free chlorine of 1 mg l^{-1} . With stirring at 20°C , 500 ml samples of solution were taken at the reaction times of 5, 15, 30, 60 and 120 minutes for the short exposure experiment, and 1, 2, 4, 6, 24, 48 and 72 hours for the long exposure experiment.

A 1 ml solution of sodium ascorbic acid (10 g l^{-1}) was added to the sample solutions to eliminate chlorine. MPP and its products were extracted by SPE. The operations were done at room temperature, around 25°C .

Sodium hypochlorite solution was added to an aqueous solution of 0.01 mg l^{-1} MPP, to a final concentration of free chlorine of 5 mg l^{-1} . The solution was maintained at 20°C for 0.5, 1, 2, 4 and 24 hours. Chlorine was eliminated in the sample solutions by sodium ascorbic acid. MPP and its products were concentrated 250-fold by SPE.

Evaluation of ChE activity

Stock solutions of ChE (Wako Pure Chemical Industries, Ltd) dissolved in water ($1,250 \text{ IU l}^{-1}$) and 5-methyl-2-thenoyl-thiocholine-iodide (MTTC) (2.0 mM) were prepared. A 0.25 mM chromogen solution of 5, 5'-dithiobisnitrobenzoic acid (DTNB) was prepared in 0.1 mol l^{-1} phosphate buffer (pH 7.4). All chemicals were purchased from Wako Pure Chemical Industries, Ltd. They were stored at 4°C . The sample solutions were prepared in water. A solution of ChE and each appropriate sample were uniformly mixed in a ratio of 4:1, so that each sample contained 7 mIU ChE. MTTC substrate solution ($63 \mu\text{l}$) was added to $7 \mu\text{l}$ of each sample containing ChE in a 96 microwell plate, and $280 \mu\text{l}$ of the DTNB chromogen solution was added. The plate was incubated at 37°C for 7 minutes, and the absorbance was measured at 405 nm using an Ultrospec Visible Plate Reader II 96 (Amersham Biosciences, Tokyo, Japan). All experiments were performed in triplicate wells.

The mechanism of colour development is as follows: active ChE enzymatically cleaves the substrate MTTC to release thiocholine. The released thiocholine reacts with the chromogen DTNB to generate a yellow product, quantifiable at 405 nm by UV absorption, and which is impeded when ChE activity is inhibited (Karahasanoglu & özand 1967; Tahara *et al.* 2005).

RESULTS AND DISCUSSION

Calibration curves and limit of detection by LC/MS

The following six compounds were targeted for examination: MPP, MPP-sulfoxide and MPP-sulfone (containing an

oxidized thio-methyl group); MPP-oxon, MPP-oxon-sulfoxide and MPP-oxon-sulfone (three oxon forms containing P = O moiety oxidized P = S moiety of the characteristic structure for organophosphorus pesticides).

The experiments were performed using two methods: liquid chromatography with mass spectrometric detection (LC/MS) or gas chromatography with mass spectrometric detection (GC/MS). As a result of the comparison of sensitivity for detecting MPP and five related compounds, we selected the LC/MS method. The analytical conditions established for LC/MS were as shown above in the Experimental section. The target compounds were analysed in the positive and negative ionization SIM mode for qualitative and quantitative analysis by detection of the signal from the more abundant daughter ions. The daughter ion was identified in the scan mode during the acquisition of the mass spectrum. The selected ion and ionization modes are summarized in Table 1. Calibration curves were determined from the results of measurements of seven concentrations of standard solutions in the SIM mode. Standard curves show excellent linearity with correlation coefficients higher than 0.999 for all six compounds. This indicates that the established analytical conditions performed well in quantitative analysis of these compounds.

The value of limit of detection (LOD) was calculated as three times the standard deviation of the slope of the calibration curve. LOD values obtained using LC/MS for MPP, MPP-sulfoxide, MPP-sulfone, MPP-oxon, MPP-oxon-sulfoxide and MPP-oxon-sulfone were 10, 0.02, 0.2, 0.05, 0.2 and 0.1 ng ml⁻¹, respectively. Concentration ranges

and LOD values for the six compounds are summarized in Table 1. With LC/MS, low concentrations of the six compounds were measured at high accuracy.

Limit of quantification and recovery test by LC/MS

The value of the limit of quantification (LOQ) was determined at 10 times the value of the standard deviation and the lowest concentration that provided relative standard deviations (RSDs) of 10% or less in the recovery test. LOQ values obtained were 50 ng ml⁻¹ for MPP, 0.1 ng ml⁻¹ for MPP-sulfoxide, 1 ng ml⁻¹ for MPP-sulfone, 0.25 ng ml⁻¹ for MPP-oxon, 1 ng ml⁻¹ for MPP-oxon-sulfoxide, and 0.5 ng ml⁻¹ for MPP-oxon-sulfone.

The results of a comparison of recovery tests on extracting six compounds from tap water using three different types of solid-phase extraction cartridge showed that average recovery by the Oasis HLB Plus was 60.0–90.4% (RSD 1.2–9.8%), Sep-pack PS-2 58.3–83.9% (1.0–10.1%) and Sep-pack C18 39.8–86.1% (0.5–10.0%). There were discrepancies in recovery rates among the three cartridges. Oasis HLB Plus was selected to extract all the target compounds in these experiments, because it obtained satisfactory recovery rates for simultaneous analysis of all tested compounds.

The behaviour of MPP in water

MPP was added to purified water at a final concentration of 0.001 mg l⁻¹, and the behaviour of MPP and its products in water was examined at reaction times of 1, 2, 4, 6, 24 and 48

Table 1 | Analytical conditions in SIM mode of MPP and related compounds by LC/MS (P: positive mode)

Compound	MW	Monitor ion	Retention time (min)	Range (ng ml ⁻¹)	Correlation coefficient	LOD (ng ml ⁻¹)	LOQ (ng ml ⁻¹)
MPP	278	279 P	18.3	10–1,000	0.999	10	50
MPP-sulfoxide	294	295 P	13.3	0.02–5	0.999	0.02	0.1
MPP-sulfone	310	311 P	16.9	0.2–20	0.999	0.2	1
MPP-oxon	262	263 P	16.2	0.05–10	0.999	0.05	0.25
MPP-oxon-sulfoxide	278	279 P	4.0	0.2–20	0.999	0.2	1
MPP-oxon-sulfone	294	295 P	6.6	0.1–20	0.999	0.1	0.5

hours. The solution pH was about 6.0 after addition of MPP and was not changed during the reaction time. Though MPP itself decreased gradually, MPP-sulfoxide was formed immediately in water and its concentration increased in a time-dependent manner. At 48 hours, MPP-oxon-sulfoxide and MPP-oxon-sulfone were detected at low levels (Figure 1). The concentration of MPP and its products was calculated using the standard curve determined by the value of the peak area obtained by SPE (Figure 2). After 24 hours, 70% of MPP remained, and 5% of MPP changed to MPP-sulfoxide. After 48 hours, 50% of MPP remained and 30% was changed. We could not detect residual MPP. It was speculated that the residual might have decomposed to other products. Chemical hydrolysis played an important role in the behaviour of MPP in an aqueous environment.

The behaviour of MPP exposed to UV

The photochemical transformation of MPP in water was studied after irradiation with UV light, because compounds in natural water are irradiated by sunlight. No change was observed under dark conditions within the timescale of these

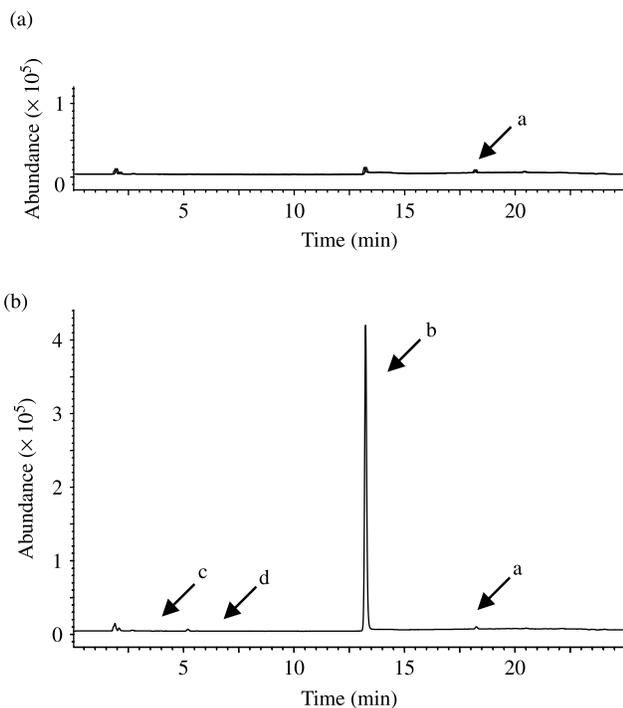


Figure 1 | Chromatograms of MPP and its products in water (a) 0h, (b) after 48 h. a: MPP, b: MPP-sulfoxide, c: MPP-oxon-sulfoxide, d: MPP-oxon-sulfone.

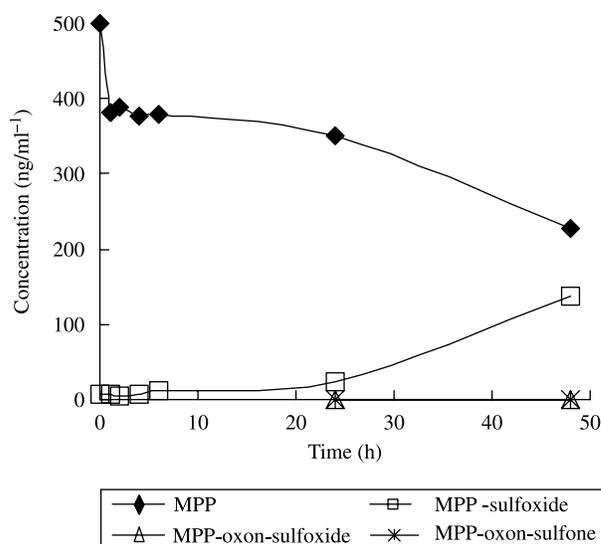


Figure 2 | Behaviour of MPP in water.

experiments. Following irradiation, MPP itself disappeared rapidly, and four main photoproducts were confirmed on the chromatogram (Figure 3). Some photoproducts of MPP have already been reported (Chukwudebe *et al.* 1989; Minelli *et al.* 1996; Huang & Mabury 2000; Hirahara *et al.* 2005; Torrisi & Sortino 2004). Two products among them were found in purified water. One identified product was MPP-sulfoxide, according to mass spectral information. It was directly produced by the oxidative reaction of MPP. The other product, detected at a 17.6 minute retention time, showed the formation $M + H^+ = 279$. It was presumed to be *O*, *O*-Dimethyl *S*-[3-methyl-4-(methylthio)phenyl]phosphorothioate by the fragment ions of the mass spectrum (Figure 4). It was formed by the isomerization of thiono-thiolo (e. g. $RO-P = S \rightarrow RS-P = O$) (Lacorte & Barceló 1994; Torrisi & Sortino 2004; Zamy *et al.* 2004). It involves the lowest excited singlet state of the pesticide and a σ cation as the key intermediate in the photodecomposition of MPP (Torrisi & Sortino 2004). Both products were detected at their highest concentration at 1 minute (Figure 5). These products were also confirmed by light irradiation using a chemical lamp (6 W, maximum wavelength 352 nm). Some minor peaks were present. However, we were not able to elucidate their structure from mass fragment information. The area values of these peaks were small compared with that of the main peak.

Although the strength of the UV wavelength range of sunlight is usually weak, the solar spectral intensity is typically

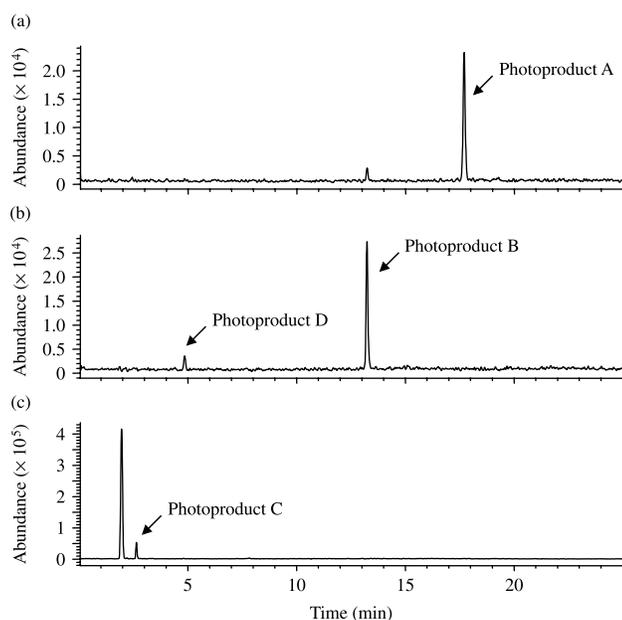


Figure 3 | Chromatograms of MPP and its products irradiated for 120 s. (a) m/z : 279, positive mode, (b) m/z : 295, positive mode, (c) m/z : 141, negative mode.

sufficient to break down chemical bonds of the molecule. There is a report that MPP degrades much faster under sunlight conditions than in darkness (Lartiges & Garrigues 1995). Therefore, there is concern that these compounds are formed in the environment.

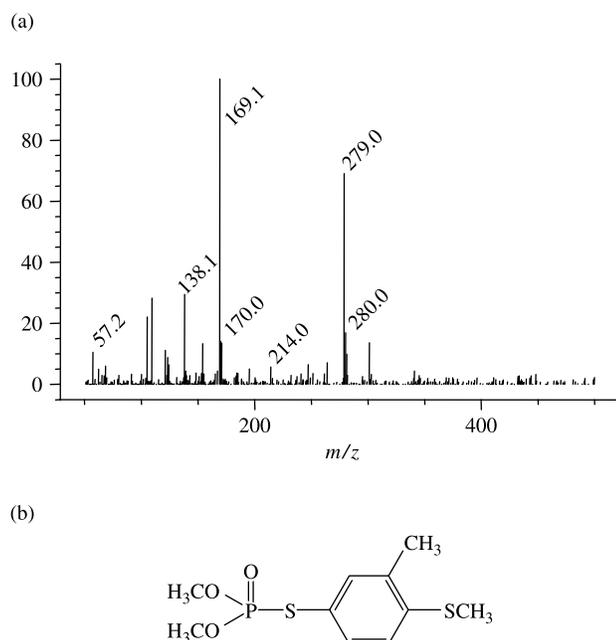


Figure 4 | Photoproduct A. (a) MS spectrum, (b) chemical structure.

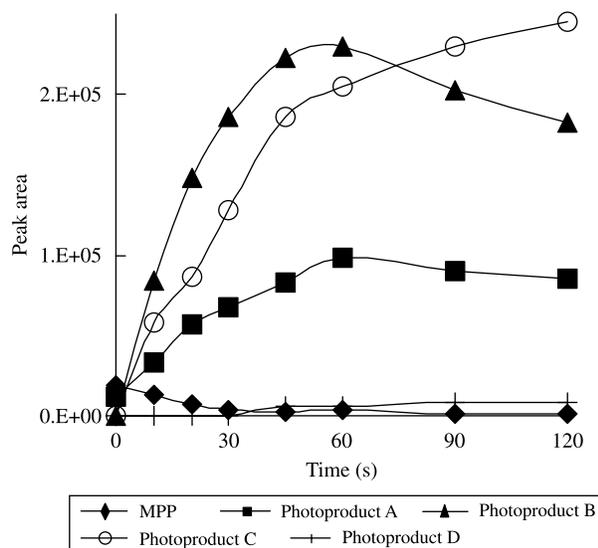


Figure 5 | Time-dependent behaviour of MPP and its products treated by light irradiation.

MPP behaviour under conditions of chlorination

MPP standard solution was added to purified water to a final concentration of 0.001 mg l^{-1} . A 500 ml sample of the solution was taken as the original water sample, reaction time at 0 h, after stirring at room temperature for 5 minutes. A sodium hypochlorite solution was added so that the concentration of free chlorine was 1 mg l^{-1} . The solution pH was about 6.0 after addition of MPP to purified water and changed to about 8.0 when the chlorine was added. However, it was resulted to get to 6.0 with the reaction time. In water containing chlorine, MPP was undetectable within 5 minutes after contact with chlorine. MPP-sulfoxide and MPP-sulfone were detectable immediately, and increased in parallel with the decrease of MPP. The concentration of these products peaked at 5 and 15 minutes, respectively. Each compound was then gradually converted to its oxon form (Figure 6). The rates of conversion from MPP-sulfoxide and MPP-sulfone to their oxon forms were slow in comparison with the rate of conversion from MPP to MPP-sulfoxide and MPP-sulfone. In this experiment, MPP-oxon was undetectable. As a result of chlorination in the long exposure experiment of 1, 2, 4, 6, 24, 48, 72 and 96 hours, MPP-oxon-sulfoxide almost disappeared by 48 hours. MPP-oxon-sulfone concentration peaked at 24 hours and maintained the same concentration level until 48 hours. The concentration of free chlorine was 0.79 mg l^{-1} after 48 hours.

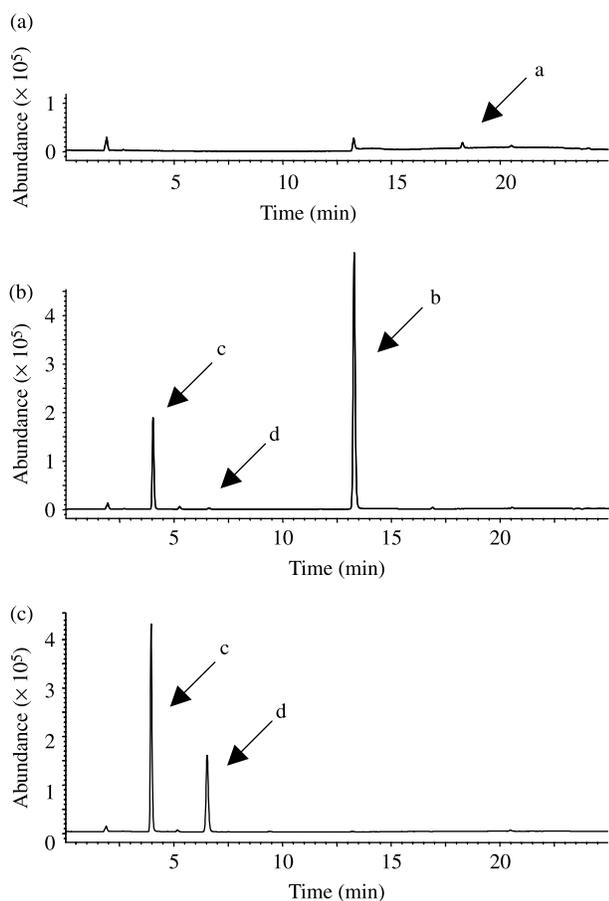


Figure 6 | Chromatograms of MPP and its products after chlorination. (a) 0 min, (b) 5 min, (c) 120 min. a: MPP, b: MPP-sulfoxide, c: MPP-oxon-sulfoxide, d: MPP-oxon-sulfone.

The concentration of MPP and detected products in the short exposure experiment was calculated using the standard curve determined by the value of peak areas obtained by SPE (Figure 7). The results indicate that under chlorination conditions, MPP changed to related compounds and converted primarily into MPP-oxon-sulfoxide after 48 hours.

If MPP exists in sources of drinking water, and is not eliminated sufficiently at water purification plants, it will come into contact with chlorine. MPP is rapidly oxidized to MPP-sulfoxide and MPP-sulfone, and their oxons may persist in drinking water.

ChE inhibition activity

It is known that ChE, a key neuroregulatory enzyme, is targeted and inhibited by organophosphorus pesticides and

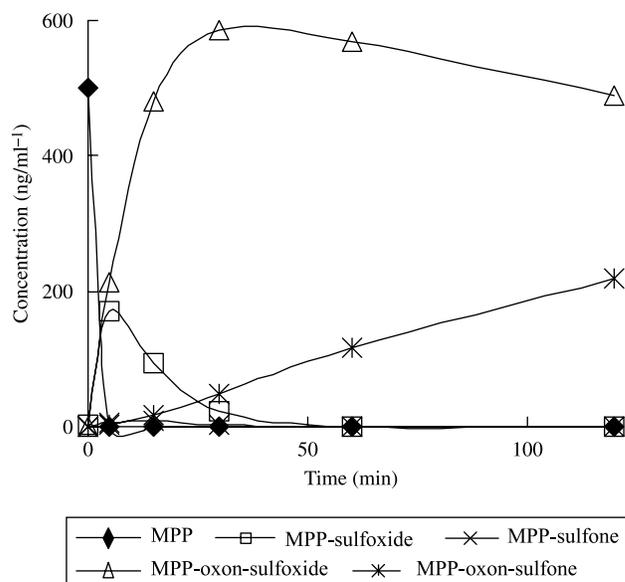


Figure 7 | Time-dependent behaviour of MPP and its products after chlorination.

their active metabolites, causing acute toxicity (Rodnitzky 1975; Soliman *et al.* 1982; Nagymajtényi *et al.* 1988). In this study, ChE inhibition activity was examined by a previously established *in vitro* method that uses MTTC as an indicator of ChE activity, in order to evaluate the effect of MPP and related compounds on organisms.

MPP, MPP-sulfoxide and MPP-sulfone showed weak inhibitory activity. However, the oxon forms showed a high inhibitory effect at ng levels. The inhibition by oxons

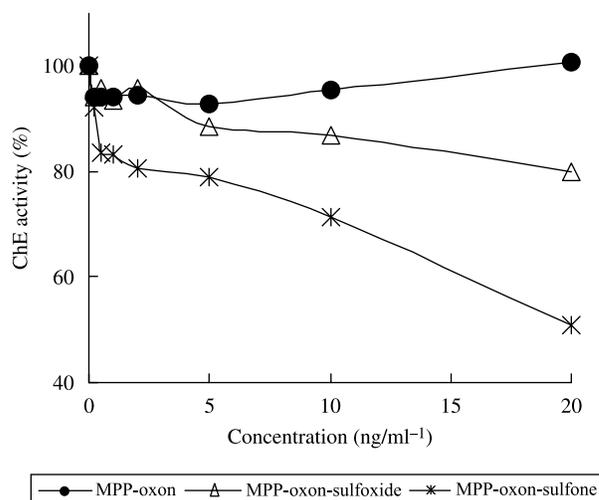


Figure 8 | Comparison of ChE inhibitory activities. The concentration at the beginning of the reaction was defined as 100%.

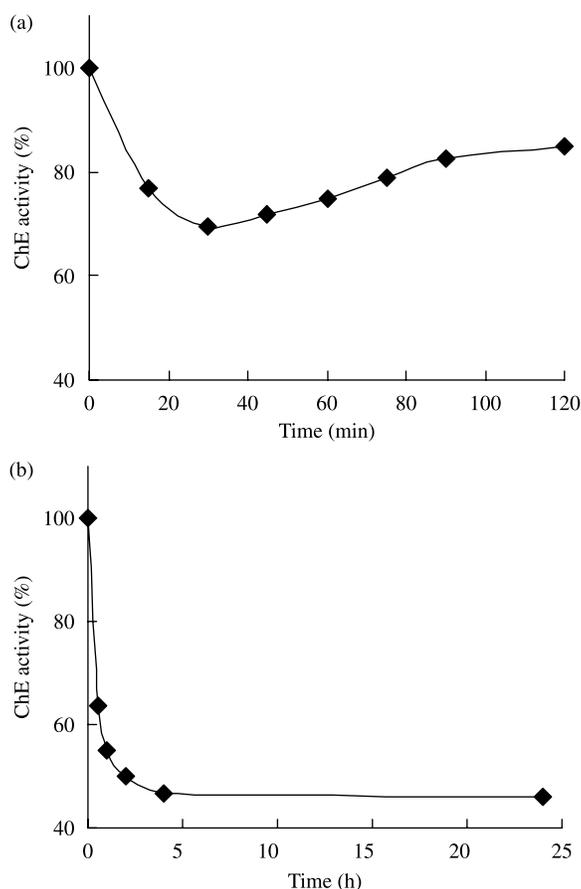


Figure 9 | Effect of reaction products mixture on ChE activity. (a) light irradiation, (b) chlorination. The activity at the non-treated MPP solution was defined as 100%.

strengthened with the degree of oxidation, in the following order: MPP-oxon, MPP-oxon-sulfoxide and MPP-oxon-sulfone (Figure 8). Concentrations of chlorpyrifos oxon, diazinon oxon, EPN oxon and fenitrothion oxon causing 20% inhibition were, respectively, 1.1, 8.9, 140 and 330 ng ml⁻¹ (Tahara *et al.* 2005); and concentrations for MPP-oxon-sulfoxide and MPP-oxon-sulfone were 5.4 and 0.32 ng ml⁻¹, respectively. The inhibitory activities of MPP-oxon-sulfoxide and MPP-oxon-sulfone are high compared with other oxons of organophosphorus pesticides. Therefore, the potential for adverse effects of MPP is high, because MPP is changed to MPP-oxon-sulfoxide and MPP-oxon-sulfone.

We also studied the ChE inhibition activity of MPP solutions irradiated by UV or treated with chlorine. The treated solutions may contain mixtures of MPP and its reaction products. The results in solutions from both treatments showed

strong ChE inhibitory activity in comparison with the non-treated MPP solutions (Figure 9). Photodegradation reactions may result in the formation of products with a high acute toxicity. The next step would be to isolate photodegradation products and elucidate their toxicity.

CONCLUSIONS

The present work has shown that MPP converts easily to the oxidized compounds, MPP-sulfoxide, MPP-sulfone and their oxons by photo-irradiation and by treatment with chlorine in water. In an aquatic environment, MPP may be changed by passing through different physical, chemical and biological processes. It is important to control MPP and its reaction products, in order to protect human health and the ecosystem, because these compounds have adverse effects on organisms.

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