Chemosphere 261 (2020) 127743

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Effect of chlorination on anti-acetylcholinesterase activity of organophosphorus insecticide solutions and contributions of the parent insecticides and their oxons to the activity

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HIGHLIGHTS

- Malathion (MA) and methidathion (ME) were transformed to oxons during chlorination.
- Anti-AChE activity of oxons was much higher than that of parent insecticides.
- Upon chlorination, activities of MA and ME solutions increased and then decreased.
- Activity of chlorinated samples was fully explained by parent and oxon activities.
- Oxons were the only active transformation products generated during chlorination.

ARTICLE INFO

Article history:
Received 2 June 2020
Received in revised form
10 July 2020
Accepted 14 July 2020
Available online 21 July 2020

Handling Editor: Jian-Ying Hu

Keywords:
Diazinon
Dimethoate
Drinking water
Malathion
Methidathion
Toxicity

ABSTRACT

Organophosphorus insecticides are known to be partly transformed to their respective oxons during the chlorination step of drinking water treatment. For most organophosphorus insecticides, the toxicological endpoint for determining acceptable daily intake levels is inhibition of acetylcholinesterase (AChE). Like the parent insecticides, oxons also inhibit AChE, so the presence of oxons in drinking water is also evaluated. However, no attention is paid to the possible presence of transformation products (TPs) other than oxons. In the present study, we determined whether the anti-AChE activity observed for chlorinated solutions of the organophosphorus insecticides malathion and methidathion could be solely attributed to the parent compounds and their oxons. Upon chlorination, both malathion and methidathion were immediately transformed to their oxons; the maximum transformation ratios were 60% and 30%, respectively, indicating that at least 40% and 70% of these compounds were transformed into other TPs. Before chlorination, malathion- and methidathion-containing solutions exhibited little to no anti-AChE activity, but the solutions showed strong activity after chlorination. The contributions of the parent insecticides and their oxons to the activities of the chlorinated samples were calculated from the concentrations of the compounds in the samples and dose–response curves for chemical standards of the compounds. For both the malathion-containing solution and the methidathion-containing solution, the calculated anti-AChE activities were almost the same as the observed activities at every chlorination time. This suggests that the observed activities could be attributed solely to the parent insecticides and their oxons, indicating that other TPs need not be considered.

1. Introduction

Around the globe, large amounts of pesticides have been applied to agricultural fields to control crop-damaging insects and weeds. Organophosphorus insecticides are among the most commonly used pesticides because of their low cost, ready availability, wide-ranging efficacy, and ability to control many pest species (Tankiewicz et al., 2010). In 2017, worldwide sales of organophosphorus insecticides amounted to approximately 2.5 billion USD,

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https://doi.org/10.1016/j.chemosphere.2020.127743
0045-6535/

second only to the sales of nicotinamides (including neonicotinoid insecticides) at approximately 3.5 billion USD (Phillips, 2019). However, organophosphorus insecticides are toxic to humans. The toxicity is induced by inhibition of acetylcholinesterase (AChE) in synapses and on the membranes of red blood cells, which results in accumulation of acetylcholine (ACh) and overstimulation of ACh receptors in synapses of the autonomic nervous system and neuromuscular junctions (Eddleston et al., 2008). According to the Joint FAO/WHO Meeting on Pesticide Residues (WHO, 2020), USEPA (2017) and the Food Safety Commission of Japan (FSCJ, 2020), the toxicological endpoint for determining the acceptable daily intake levels and chronic reference doses of most organophosphorus insecticides is inhibition of AChE.

After being applied to agricultural fields, organophosphorus insecticides flow into rivers during rain events (Wee and Aris, 2017; Derbalah et al., 2019; Xu et al., 2020); and the resulting contaminated river water can be drawn into downstream drinking water treatment plants. Because organophosphorus insecticides are quite difficult to be removed by conventional drinking water treatment processes consisting of coagulation, sedimentation, and sand filtration (Stackelberg et al., 2007; Ormad et al., 2008; Matsushita et al., 2018), these compounds are present in the treated water when it is disinfected with chlorine prior to release into the distribution system. Upon exposure of the insecticides to free chlorine, oxidation of the P═S bond to a P═O bond results in transformation of some of the molecules into their oxons (Duirk et al., 2009; Kamel et al., 2009; Li et al., 2016). Under the circumstances that transformation products (TPs) produced in the environment and water treatments recently attracts increasing attention worldwide (Yin et al., 2017; Kiefer et al., 2019; Zahn et al., 2019), attention is paid not only for the organophosphorus insecticides but also for their oxons in drinking waters, because the oxons inhibit AChE activity as well as their parent insecticides do (Eddleston et al., 2008).

The European Union Drinking Water Directive sets the maximum allowable concentration for any individual pesticide at 0.1 μg/L, and this value applies not only to the active substance but also to its relevant TPs (European Commission, 2018). For this purpose, a TP is defined as a compound that is formed either in organisms or in the environment and that "has intrinsic properties comparable to the parent substance in terms of its biological target activity, or…poses a higher or comparable risk to organisms than the parent substance or…has certain toxicological properties that are considered unacceptable" (EN, 2009). The oxons of organophosphorus insecticides are included in the regulation because they have the same mode of action as their parent insecticides and are produced and detected in environmental water as well (Duttagupta et al., 2020). According to the Japanese drinking water quality guideline, the oxons of 10 organophosphorus insecticides (butamifos, chlorpyrifos, diazinon, EPN (*O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate), fenitrothion, fenthion, isofenphos, isoxathion, malathion, and prothiofos) and the parent insecticides must be quantified in finished water.

However, the percentage of the parent insecticide that is transformed into the oxon, the transformation ratio, is usually less than 100% (Onodera et al., 1995; Li et al., 2016), which means that the remainder of the insecticide is transformed into other TPs. In fact, Magara et al. (1994) reported that chlorination of diazinon produces not only diazinon-oxon but also diethyl phosphate, dichloroacetic acid, and trichloroacetic acid. Even though non-oxon TPs may inhibit AChE, these TPs are unlikely to be taken into account in the European Union regulation and are not taken into account in the Japanese drinking water quality guideline (exceptions include the sulfoxide, sulfone, oxon-sulfoxide, and oxon-sulfone of fenthion, because they are known to inhibit AChE (Tahara et al., 2008)).

Oxons are generated not only by chlorination but also by other water treatment processes, such as ozonation (Wu et al., 2009), UV irradiation (Zamy et al., 2004; Čolović et al., 2010), UV irradiation in the presence of H_2O_2 (Li et al., 2015), and UV irradiation in the presence of TiO_2 (Bavcon Kralj et al., 2007; Calza et al., 2008). Bavcon Kralj et al. (2007) reported that AChE was inhibited by a UV/TiO_2-treated malathion-containing solution and implied that the inhibition was due to the oxon of malathion, on the basis of the fact that the anti-AChE activity of the solution depended on the oxon concentration. However, these investigators did not determine whether the pure oxon at the concentrations present in the irradiated samples showed the same inhibitory activity against AChE as did the irradiated samples. Onodera et al. (1995) concluded that AChE inhibition by a chlorinated EPN-containing solution was due to the oxon of EPN, but no evidence was provided for this conclusion. Only Čolović et al. (2010) did the necessary quantitative comparison; specifically, they compared anti-AChE activity by a UV-treated diazinon-containing solution with anti-AChE activity by a chemical standard of diazinon-oxon at the same concentration as that in the UV-treated sample, and they concluded that the anti-AChE activity observed at the beginning of the treatment (after irradiation for 5 min) could be attributed to diazinon-oxon in the UV-treated sample. To our knowledge, no one has quantitatively compared observed anti-AChE activity with anti-AChE activity thought to be derived from an oxon generated during chlorination, leaving it unclear as to whether the toxicities of compounds other than parent insecticides and their oxons in drinking waters after chlorination should be considered.

Accordingly, the objectives of the present study were to investigate whether the activities of insecticides and their oxons could fully explain anti-AChE activity observed upon chlorination of solutions containing organophosphorus insecticides. First, we investigated anti-AChE activities by four organophosphorus insecticides (malathion, methidathion, diazinon, and dimethoate) and their respective oxons by using chemical standards. On the basis of the resulting data, we selected two insecticides (malathion and methidathion) for additional experiments. Solutions containing each insecticide were separately treated with free chlorine, and samples were withdrawn at predetermined intervals. The concentrations of insecticides and their oxons in the samples were determined, and their anti-AChE activities were measured. Finally, the contributions of the parent insecticides and their oxons to observed activity were evaluated by combining their concentrations in the chlorinated samples with dose–response curves generated with chemical standards. In this way, we were able to clarify whether it is reasonable to consider only parent insecticides and their oxons when assessing contamination of drinking water.

2. Materials and methods

2.1. Chemicals

Chemical standards of malathion, methidathion, diazinon, dimethoate, and their respective oxons (Fig. S1) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan) and were used as received. To avoid any effect of organic solvents on the experimental results, stock solutions of the insecticides (malathion, 29 μM; methidathion, 230 μM; diazinon, 38 μM; dimethoate, 30 μM) and oxons (malathion-oxon, diazinon-oxon, and dimethoate-oxon, 100 μM; methidathion-oxon, 1000 μM) were prepared in a Milli-Q water-based (Milli-Q Advantage, Millipore Co., Bedford, MA, USA) phosphate buffer (10 mM, pH 7.0). AChE (derived from human erythrocytes) was purchased from Merck KGaA (Darmstadt, Germany). ACh and choline (Ch) were purchased

from Fujifilm Wako Pure Chemical Corporation.

2.2. Anti-acetylcholinesterase activity assay

An *in vitro* anti-AChE activity assay was conducted with commercially available AChE and ACh according to Liu et al. (2014) with some modification as follows. Phosphate buffer (1 mM, pH 7.4), prepared by dissolving Na_2HPO_4 and NaH_2PO_4 in Milli-Q water, was supplemented with NaCl at a concentration of 150 mM. Hereafter, this buffer is referred to as the assay buffer. A working solution of AChE was prepared by adding AChE (240 units/L) to the assay buffer, and a working solution of ACh (120 μM) was prepared by adding ACh to the assay buffer.

Each sample solution was diluted to the desired concentration with the assay buffer, and then 285 μL of the diluted sample was poured into at least three wells of an ice-cooled 96-well microplate. Each sample was supplemented with 7.5 μL of the AChE working solution and then preincubated at 37 °C for 30 min, during which inhibition of the AChE by compounds in the sample was expected to occur. After the preincubation, the 96-well plates were cooled on ice, and then each sample was supplemented with 7.5 μL of the ACh working solution on ice and then incubated at 37 °C for 120 min, during which time release of Ch from ACh by uninhibited AChE was expected to occur. After the incubation, an aliquot (200 μL) of each sample was mixed with 200 μL of acetonitrile to inactivate the AChE. The concentration of Ch in the final solution was measured by liquid chromatography–mass spectrometry (LC-MS). A control (prepared with the assay buffer instead of the diluted sample) and a blank (prepared with the assay buffer instead of both the diluted sample and the AChE working solution) were also subjected to the assay procedure.

Anti-AChE activity by each sample was calculated by means of Eq. (1):

$$\text{Anti}-\text{AChE activity} = \frac{[Ch]_{control} - [Ch]_{sample}}{[Ch]_{control} - [Ch]_{blank}} \quad (1)$$

where $[Ch]_{control}$, $[Ch]_{sample}$, and $[Ch]_{blank}$ are the Ch concentrations in the control, the sample, and the blank, respectively.

2.3. Batch chlorination experiments

Malathion and methidathion were separately dissolved in 10 mM phosphate buffer (pH 7.0) at concentrations of 28 and 230 μM, respectively, in glass vials. For chlorination, these solutions were supplemented with sodium hypochlorite at mol-Cl_2/mol-C ratios of 1 and 3 for malathion and methidathion, respectively, so that chlorine was present throughout the procedure; and the supplemented samples were magnetically stirred for 5 min. Then the vials were tightly sealed with screw caps and Parafilm to prevent volatilization and allowed to stand at 20 °C in the dark for 168 h. During this period, aliquots were withdrawn at predetermined intervals, quenched with sodium sulfite to remove residual chlorine, and subjected to the anti-AChE activity assay (after 200-fold dilution with the assay buffer). In addition, the concentrations of the parent insecticides and their oxons in the aliquots were determined by LC-MS.

2.4. Quantification of the concentrations of choline, parent insecticides, and oxon

The concentration of Ch was measured with a hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific, Inc., Waltham, MA, USA) coupled with an LC system (UltiMate 3000, Thermo Fisher Scientific). A 5-μL sample of each solution was assayed by LC on a 100 mm × 2.1 mm Cortecs UPLC HILIC column (1.6-μm particle size, Waters Corporation, Milford, MA, USA). The mobile phase was a binary gradient of 100 mM ammonium formate in Milli-Q water (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 200 μL/min as follows: begin with 5% B (v/v), increase linearly to 60% B over a period of 0.75 min, hold at that percentage for 0.25 min, decrease linearly to 30% B over a period of 0.25 min, hold at that percentage for 1 min, and then decrease linearly to 5% B over a period of 3.75 min. The mass spectrometer was operated in electrospray-ionization mode (positive) with a spray voltage of 3.2 kV. The temperatures of the capillary heater and the electrospray-ionization-probe heater were 220 and 450 °C, respectively. The flow rates of the sheath gas, auxiliary gas, and sweep gas were 50, 15, and 0 units, respectively. The S-lens ratio frequency level was set to 57. The concentration of Ch was quantified in selected-ion-monitoring mode (*m*/*z* 104.1070) at a resolution of 70,000. Detection limit of Ch was 5 nM.

The concentrations of insecticides and oxons were measured with the same apparatus used to measured Ch concentration. A 5-μL sample of each solution was assayed by LC on a 50 mm × 2.1 mm Hypersil Gold column (1.9-μm particle size, Thermo Fisher Scientific). The mobile phase was a binary gradient of 2 mM ammonium formate in Milli-Q water (solvent A) and 100% methanol (solvent B) at a flow rate of 200 μL/min as follows: 1% B (v/v) for 1.5 min, increase linearly to 60% B over a period of 1 min, increase linearly to 99% B over a period of 5.5 min, hold at that percentage for 1.5 min, decrease linearly to 1% B over a period of 0.5 min, and then hold at that percentage for 2.0 min. The mass spectrometer was operated in electrospray-ionization mode (positive) with a spray voltage of 3.2 kV. The temperatures of the capillary heater and the electrospray-ionization-probe heater were 220 and 450 °C, respectively. The flow rates of the sheath gas, auxiliary gas, and sweep gas were 50, 15, and 0 units, respectively. The S-lens ratio frequency level was set to 57. The concentrations of malathion, malathion-oxon, methidathion, and methidathion-oxon were quantified in selected-ion-monitoring mode (*m*/*z* 331.0433, 315.0662, 302.9691, and 286.9920, respectively) at a resolution of 70,000. Detection limits of malathion, malathion-oxon, methidathion, and methidathion-oxon were 3, 2, 0.3, and 2 nM, respectively.

3. Results and discussion

3.1. Comparison of anti-acetylcholinesterase activities of insecticides and oxons

First, we assayed the anti-AChE activities of chemical standards of the four parent insecticides and their respective oxons (Fig. 1). Among the insecticides, only malathion showed relatively strong anti-AChE activity; methidathion and diazinon showed only slight activity, and dimethoate showed no activity at all in the tested concentration range. In contrast, all the oxons showed dose-dependent anti-AChE activity, and in all cases the oxons were more active than the respective parent insecticides. To compare the activities of the compounds, we calculated median half maximal inhibitory concentrations (IC_{50}) by using logistic regression analysis (Table 1). The IC_{50} values of the parent insecticides were more than 3 orders of magnitude higher than those of their respective oxons, indicating that the oxons were >1000 times as active as the parent insecticides. This tendency is consistent with published data obtained by means of *in vitro* assays (Tahara et al., 2005; Krstić et al., 2008; Čolović et al. 2010, 2011), although the IC_{50} values were different among literature probably due to the differences in experimental conditions as shown in Table S1. The activities of the

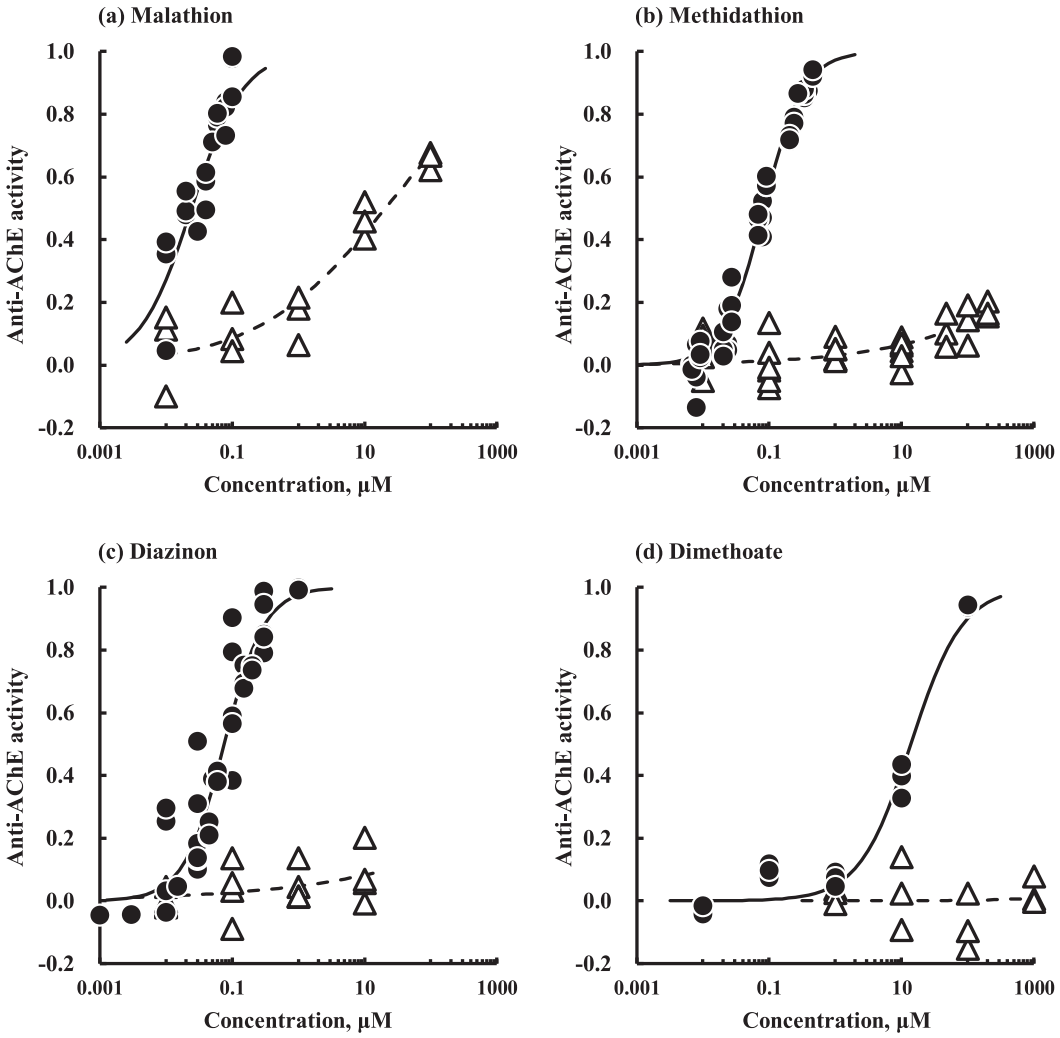

Fig. 1. Concentration dependence of anti-AChE activity by pesticides (white triangles) and their oxons (black circles).

Table 1
Comparison of IC_{50} values (in μM) for organophosphorus insecticides and oxons used in this study.

	This study		Published literature	
	Parent	Oxon	Parent	Oxon
Malathion	20	0.024	370 [a]	2.4 [a]
Methidathion	(23,000)	0.084		
Diazinon	(120,000)	0.075	>33 [b], 87 [c], >200 [d]	<0.35 [b], 0.051 [d], 0.068 [c]
Dimethoate	–	14		

The values given in parentheses were obtained by extrapolation and thus lack accuracy.
[a] Krstić et al. (2008).
[b] Tahara et al. (2005).
[c] Čolović et al. (2010).
[d] Čolović et al. (2011).

compounds differed considerably and decreased in the order malathion-oxon > diazinon-oxon ≈ methidathion-oxon ≫ dimethoate-oxon ≈ malathion > methidathion ≈ diazinon > dimethoate. Notably, the activity of dimethoate-oxon was as low as that of malathion, even though the former was an oxon.

3.2. Transformation of malathion and methidathion to their oxons during chlorination

We investigated the effects of chlorination on the activities of solutions of malathion and methidathion because of the relatively high anti-AChE activities of the oxons of these two insecticides. During chlorination, malathion immediately reacted with free chlorine (Fig. 2a). The malathion almost completely disappeared within 15 min. At that point, the malathion-oxon concentration was equivalent to 60% of the initial malathion concentration, and the concentration of the oxon then gradually decreased with chlorination time. The transformation of malathion to its oxon during chlorination that we observed is consistent with that previously reported (Duirk et al., 2009; Li et al., 2016).

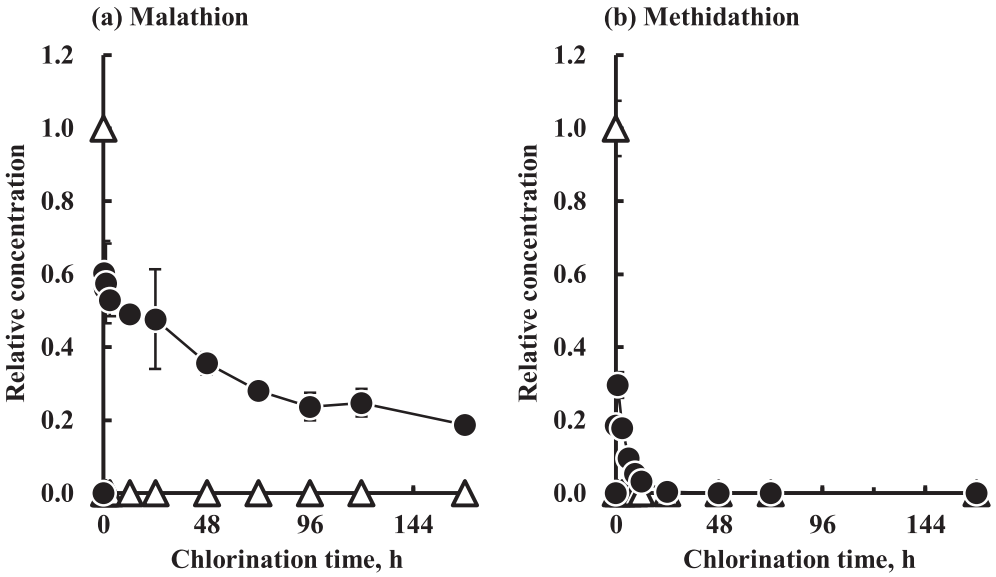

Fig. 2. Effect of chlorination time on the concentrations of (a) malathion and (b) methidathion (white triangles) and their respective oxons (black circles) relative to the initial concentrations of the parent insecticides. The initial concentrations of malathion and methidathion were 28 and 230 μM, respectively. The concentrations were measured at 0, 0.25, 0.5, 1, 3, 12, 24, 48, 72, 96, 120, and 168 h for malathion, and at 0, 0.17, 1, 3, 6, 9, 12, 24, 48, 72, and 168 h for methidathion. Error bars indicate standard deviations of three measurements.

Likewise, methidathion reacted with free chlorine immediately, disappearing almost completely within 10 min, and methidathion-oxon was produced by the reaction, as has previously been reported (Duirk et al., 2009; Kamel et al., 2009). However, the ratio of the oxon concentration to the initial methidathion concentration (30%) was half the corresponding ratio for the malathion oxon. The lower transformation ratio for methidathion may have been due partly to differences in the initial insecticide and free chlorine concentrations: both concentrations were necessarily higher for methidathion than for malathion because the anti-AChE activity of methidathion-oxon was lower than that of malathion-oxon. This possibility was confirmed by an experiment showing that when the initial concentrations of methidathion and free chlorine were decreased to 33 nM (from 230 μM) and to 1 mg-Cl_2/L, the ratio increased to 83% (Fig. S2).

3.3. *Effect of chlorination on anti-acetylcholinesterase activity by insecticide-containing solutions*

Before chlorination, the malathion-containing solution slightly inhibited AChE (Fig. 3a). Upon exposure to free chlorine, the anti-AChE activity of the solution rapidly increased and then gradually decreased with chlorination time. In contrast, the methidathion-containing solution showed no activity before chlorination (Fig. 3b), but upon exposure to free chlorine, the solution showed high activity; then the activity rapidly decreased, and no activity was observed after 24 h of chlorination. Our results are consistent with previously reported chlorination-induced increases in the anti-AChE activity of solutions containing other organophosphorus insecticides, such as EPN (Onodera et al., 1995) and fenthion (Tahara et al., 2008). Our findings clearly show that both malathion

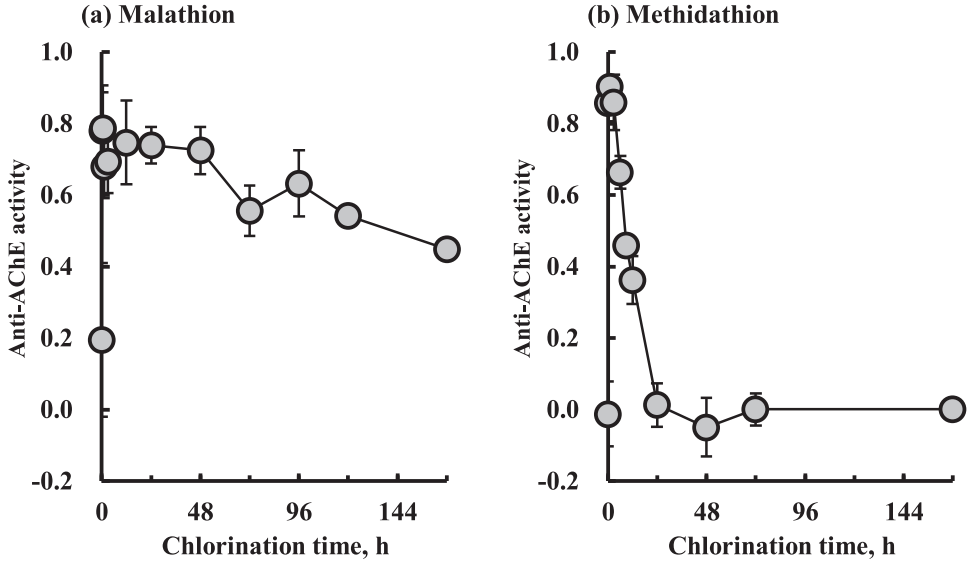

Fig. 3. Effect of chlorination time on anti-AChE activity by (a) malathion and (b) methidathion. Error bars indicate standard deviations of three measurements.

and methidathion were converted to their respective TPs and that some of the TPs showed anti-AChE activity. During chlorination, the parent insecticides were transformed to their oxons (Fig. 2), and the oxons were more active than the parent insecticides (Fig. 1). Accordingly, transformation of the insecticides to their oxons probably contributed to the increase in activity.

However, as mentioned above, only 60% of the initial malathion and 30% of the initial methidathion were transformed to their oxons, which means that the remainder of the initial amounts of the two insecticides were transformed into TPs other than oxons. Duirk et al. (2009) reported that organophosphorus insecticides are oxidized to their oxons by HOCl but are hydrolyzed by OCl^-. In fact, we detected dimethyl phosphate, a hydrolyte of methidathion-oxon, during chlorination of the methidathion-containing solution (Fig. S3). This compound may also have been produced from malathion, judging from the fact that both insecticides have an *O*,*O*-dimethyl dithiophosphate group (Fig. S1). Therefore, we assayed the anti-AChE activity of a chemical standard of dimethyl phosphate; the assay showed that this TP had low anti-AChE activity (Fig. S4) and thus that it did not contribute to the observed activity of the chlorinated solutions. Nevertheless, other, as-yet-unidentified TPs with anti-AChE activity may have contributed to the observed activity.

3.4. Contributions of parent insecticides and their oxons to anti-acetylcholinesterase activity by chlorinated insecticide-containing solutions

Because the dose–response curves for the activities of the parent insecticides and their oxons were not linear (Fig. 1), the inhibition was not dose-additive; that is, the activity of a solution containing both the insecticide and its oxon could not be calculated simply by summing the individual activities of the insecticide and its oxon. To estimate the total anti-AChE activity induced by the parent insecticides and their oxons in the samples, a mixture containing the same concentrations of the parent insecticides and their oxons as those in the chlorination samples should be prepared and then subjected to the anti-AChE activity assay (Krstić et al., 2008; Čolović et al., 2011). However, before chlorination (chlorination time = 0 h), the solutions contained only the parent insecticides and no oxons; whereas after chlorination, the solutions contained only the oxons and no parent insecticides. Therefore, we could determine the anti-AChE activity of the parent insecticide from the data for the sample before chlorination, and we could determine the activity of the oxon from the data for the sample after chlorination. For the unchlorinated solutions, we calculated the activity of the parent insecticide from its concentration in the initial sample (Fig. 2) and the dose–response curve (Fig. 1), being sure to take into account the dilution factor in the anti-AChE activity assay (200-fold). For the solutions after chlorination, we used the same procedure to calculate the activity of the oxon at every chlorination time.

Comparison of the observed and calculated anti-AChE activities (Fig. 4) revealed that the observed activity of the malathion-containing solution before chlorination was roughly the same as the activity calculated as described above, indicating that the observed activity of the unchlorinated solution was attributable malathion. After treatment with free chlorine, the observed activity was almost the same as the calculated activity at every chlorination time, which clearly indicates that the observed activity was due to malathion-oxon and that the other, as-yet-unidentified TPs did not inhibit AChE. As mentioned in the introduction, the Japanese drinking water quality guideline requires both malathion and its oxon to be measured. Our current findings indicate that this guideline is reasonable for regulating malathion in drinking water.

Likewise, the lack of anti-AChE activity of the methidathion solution before chlorination is consistent with the activity calculated from the methidathion concentration in the sample and the dose–response curve (Fig. 4b). After treatment with free chlorine, the observed and calculated activities were still almost the same at every chlorination time. This comparison clearly indicates that the activity of the chlorinated solution was due only to methidathion-oxon and not to any other TPs. The current Japanese drinking water quality guideline does not require quantification of the oxon of methidathion, even though methidathion must be quantified; the reason for the difference between the two compounds is unclear. Our results suggest the oxon of methidathion should be quantified.

Krstić et al. (2008) reported that antagonistic inhibition effects were observed when AChE was simultaneously exposed to malathion-oxon and isomalathion, both of which had anti-AChE activity, under relatively high concentrations. Therefore, in the present study, some unidentified TPs having anti-AChE activity

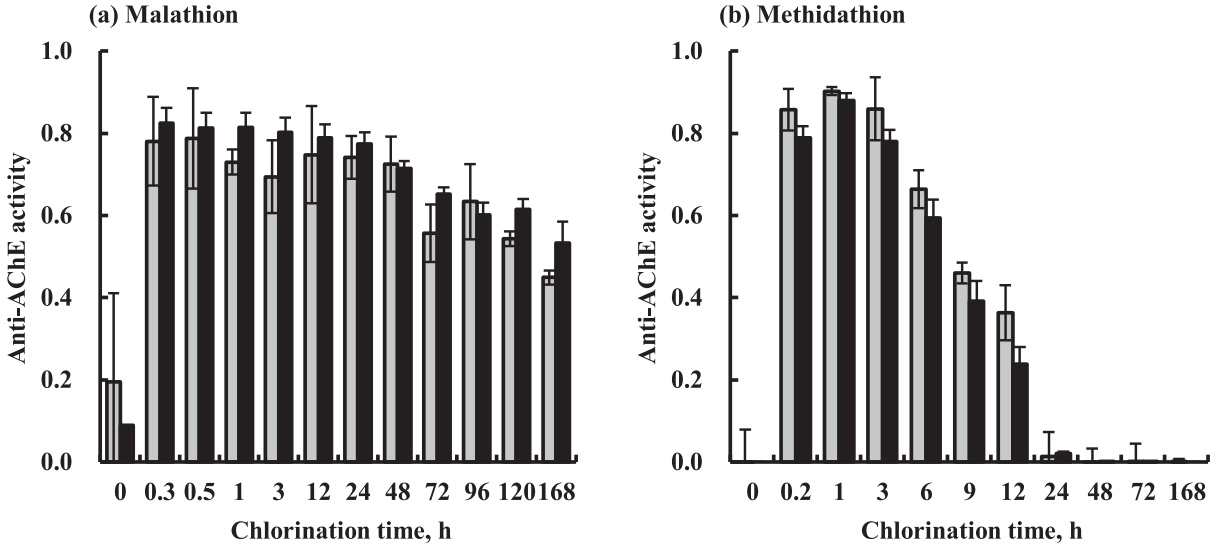

Fig. 4. Comparison of chlorination time dependence of (gray) observed anti-AChE activities of chlorinated solutions and (black) activities calculated from insecticide/oxon concentrations and the dose–response curves for (a) malathion and (b) methidathion. Error bars indicate standard deviations of three measurements.

might have been produced, but their contributions to the observed anti-AChE activity might not have been recognized because of the antagonistic effect with oxons having strong anti-AChE activity.

The crop protection market has changed over the past two decades. Specifically, Europe and North America decreased their shares in 2018, and the shares attributable to Asia and Central and South America increased. The increase was driven in large part by developing countries in the latter regions (Phillips, 2019). Explosive population growth in these developing countries will increase the need for food production and thus will most likely further increase the market shares attributable to Asia and Central and South America, and African countries may follow them. Our findings revealed that the anti-AChE activities of malathion- and methidathion-containing solutions after chlorination are due solely to the parent insecticides and their oxons. Accordingly, if developing countries already regulate or plan to regulate malathion and methidathion, we recommend that the oxons also be regulated.

4. Conclusions

1. The anti-AChE activities of the oxons of the four insecticides evaluated in this study were >1000 times the activities of their respective parent insecticides. Anti-AChE activity decreased in the order malathion-oxon > diazinon-oxon ≈ methidathion-oxon ≫ dimethoate-oxon ≈ malathion > methidathion ≈ diazinon > dimethoate.
2. Both malathion and methidathion immediately reacted with free chlorine, and their respective oxons were produced. The concentrations of the oxons decreased with increasing chlorination time.
3. Solutions of malathion and methidathion showed little or no anti-AChE activity before chlorination. However, chlorination resulted in a rapid increase in the activities of the solutions, but thereafter the activities decreased with increasing chlorination time.
4. The activities of the chlorinated malathion- and methidathion-containing solutions were attributable solely to the parent insecticides and their respective oxons. No other TPs were shown to inhibit AChE.
5. If any countries have already incorporated or plan to incorporate malathion and methidathion into regulatory guidelines, the oxons of these two insecticides should also be included.

CRediT authorship contribution statement

Taku Matsushita: Conceptualization, Methodology, Formal analysis, Visualization, Writing - original draft, Project administration. **Yuki Fujita:** Investigation. **Kei Omori:** Investigation. **Yuxiang Huang:** Investigation. **Yoshihiko Matsui:** Writing - review & editing, Supervision, Funding acquisition. **Nobutaka Shirasaki:** Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was supported in part by a Health and Labor Sciences Research Grant (no. 19LA0501) from the Ministry of Health, Labor, and Welfare of Japan and by a grant from the Fuso Innovative Technology Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127743.

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