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Prevalence and Persistence of Organophosphate and Carbamate Pesticides in Cambodian Market Vegetables

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Abstract: The prevalence of pesticide residue contamination in market vegetables, and the rate of pesticide disappearance from field vegetables, were assessed as indicators of the health risk to vegetable consumers in Cambodia. A total of 245 leafy vegetable and long bean samples from multiple markets in Phnom Penh were screened using cholinesterase-inhibition assays, and indicated that between 15% (long bean) and 95% (white-stemmed kale) of market vegetables contain detectable levels of organophosphate/carbamate (OP/C) pesticides. OP/C levels varied significantly between vegetable types, and between individual vegetable sellers in the markets. Methylparathion, which is banned and highly toxic, but widely used in Cambodia, was not detected in 30 subsamples analyzed with HPLC. In test plots, methylparathion rapidly disappeared after spraying, reaching levels below maximum residue limits within 10 days. Field trials with water spinach indicated dithiocarbamates also have a short half-life (approximately three days), but that overall OP/C levels on crops depend on the specific pesticides used, and on the specific field conditions. These results point towards the need for a regular monitoring programme in Cambodia to assess the extent of pesticide contamination on vegetables, and to give guidance on strategies for limiting pesticide exposure to vegetable consumers.

Key words: Pesticides**,** organophosphates, carbamates, market vegetables, methylparathion, Cambodia.

Introduction

Many developing countries have increased the use of agrochemicals, including pesticides, in an effort to increase food security (Carvalho, 2006; Ecobichon, 2001). Pesticide use is particularly prevalent in tropical regions where farmers see it as a quick and easy solution to deal with the large number of insect pests and plant diseases (Carvalho, 2006). Cambodia presents a good example of this trend; pesticide use has dramatically increased as the country has become more stable following several decades of civil war, and as governmental and non-governmental organizations work towards national goals of increasing agricultural productivity. Although detailed documentation of pesticide use is lacking due to the past instability in the country, a 2001 survey reported 84% of farmers using pesticide treatment (AVRDC, 2002), and a 2004 survey found that 97% of pesticide traders report a steady increase in pesticide sales (CEDAC, 2004).

Unfortunately, the rapid increase in pesticide use has occurred in an environment of weak enforcement of regulations, poor education of farmers and pesticide dealers, and variable quality of pesticide products (EJF,

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2001). Despite a sub-decree banning the use of pesticides in WHO Class 1a and 1b categories (#69, Standards and Management of Agricultural Materials), banned and highly toxic pesticides such as methylparathion are widely available in Cambodian markets (CEDAC, 2000; CEDAC, 2004). This availability is coupled with a widespread lack of knowledge on proper pesticide usage by both farmers and pesticide dealers. Popular pesticides are usually imported from neighbouring countries and therefore labelled in Thai or Vietnamese rather than Khmer. Farmers and dealers alike usually cannot read these directions (CEDAC, 2000), and pesticides are therefore often used at inappropriate times, at excessive concentrations, and/or for the wrong pests.

Organophosphates and carbamates (OP/C) are two categories of pesticides commonly found in Cambodia (CEDAC, 2000). In a 2004 survey, five out of the eight most common pesticides found in pesticide dealer shops were OP/C, with the most common pesticide being the organophosphate methylparathion (CEDAC, 2004). Some OP/C break down faster in their environment than other categories such as organochlorines (Sharom et al., 1980) but can still present both short-term and accumulated health risks to the health of farmers and consumers (Wesseling et al., 2002). A variety of analytical techniques, from simpler enzyme inhibition tests to more complex chromatographic techniques, can be used to detect OP/C. This ability to use multiple measurement techniques facilitates the establishment of monitoring programmes in countries with lower capacity for intensive pesticide monitoring.

A large proportion of Cambodians work in the agricultural sector, and thus are at risk of exposure to pesticides. Leafy vegetable crops grown for sale in local markets are particularly vulnerable to insect attack, and pesticide use on these crops is therefore proportionally higher compared with other crops such as rice (CEDAC, 2000; CEDAC, 2004). Although the widespread use of pesticides on vegetable crops makes it likely that vegetables sold in Cambodian markets could contain pesticide residues, to date there is very little information available on actual levels of pesticides. Pesticide measurements generally require special skills and equipment, and Cambodia has lacked the capacity to conduct regular monitoring. Thus, despite the obvious need to assess pesticide exposure in Cambodia, critical information on the prevalence of pesticide residues is lacking. The goals of this study were: 1. To assess the feasibility of using several pesticide measurement technologies in the Cambodian context, 2. To determine the prevalence of pesticide residues in market vegetables, and 3. To examine the persistence of several commonly used pesticides. The results of this study suggest future strategies for how pesticide monitoring can assist in the reduction of pesticide residues in market vegetables.

Methods

Several experimental techniques were chosen to fit the availability of materials and the goals of the individual experiments. Experiments and the techniques used in measurements are summarized in Table 1. Cholinesterase tests were used to broadly screen for pesticides, as they were easier to perform and less expensive. HPLC measurements were used to give more quantitative information as desired on specific pesticides.

Sample Collection

Vegetables for analysis were selected based on availability, and on which were at the greatest risk for containing pesticide residues based on frequency of pesticide use for crops. For the market surveys, long bean and six leafy vegetables (cabbage, kale, thin stem kale, white-stemmed kale, curly-leafed kale and Chinese broccoli) were selected as vegetables for which pesticide use is particularly intensive during production (CEDAC, personal communication). In the first round of market screening tests (using the GT test kit and HPLC measurements of methylparathion), a total of 122 samples were collected from three urban markets (Psar Kandal, Psar Doeum Kor and Psar Chbar Ampov) during the August through October period of 2006. A second round of market tests was carried using the semi-quantitative Abraxis cholinesterase test, in which 123 samples were taken from four markets (Psar Doeum Kor, Psar Neak Meas, Psar Chba Ampov and Psar Koengkang Koing) during June and July of 2008. Vegetables were immediately washed with water as they would normally be washed for cooking in homes, and then kept frozen (-20°C) until analysis (in the first round of screening), or analyzed immediately (in the second round of screening).

For the studies of methylparathion behaviour in test plots, kale and green choy sum (edible rape) were grown on the grounds of the Royal University of Phnom Penh. Samples were harvested at various times prior to, and after spraying of methylparathion, and then frozen (-20^{o}C) until analysis. Methylparathion was sprayed on plants using a regional formulation (Thailand) that was purchased at Oreusey market in Phnom Penh. Pesticide was mixed and sprayed in the same manner as that used by farmers.

Table 1: Summary of experimental techniques

	Test	*Qualitative or quantitative results*	*Source of sample collection*	*Extraction*	*Detection*
1.	First round of OP/C screening for market vegetables	Qualitative	Three markets in Phnom Penh	GT Test kit extraction solutions	GT Test kit (cholinesterase inhibition)
2.	Second round of OP/C screening for market vegetables	Semi-quantitative (based on diazinon equivalents)	Four markets in Phnom Penh	Surface dislodgable extraction with methanol	Abraxis kit (cholinesterase inhibition), colour quantified with absorption spectrophotometry
3.	Test for methyl-parathion in market vegetables	Quantitative	Three markets in Phnom Penh	QuEChERS	HPLC
4.	Methylparathion disappearance kinetics in test plots	Quantitative	Vegetables grown in test plots at the Royal University of Phnom Penh	QuEChERS	HPLC
5.	OP/C disappearance in field trials of water spinach	Semi-quantitative (based on diazinon equivalents)	Farmers' fields in wetlands (Boeng Chueng Ek)	Surface dislodgable extraction with methanol	Abraxis kit (cholinesterase inhibition), colour quantified with absorption spectrophotometry

For the studies of pesticide behaviour on water spinach (also known as morning glory; *Ipomoea aquatica*) in farmers' fields, samples were gathered directly from the wetland field and processed immediately without washing. Fields were selected in which farmers were spraying OP/C pesticides; farmers were also surveyed to determine the type of pesticide, and to estimate the mixing and application rates. Since trials occurred in June and July, which is the start of the wet season, the number of rain events was monitored during the trial period. All fields were located in Boeng Cheung Ek, a wetland area on the southern edge of Phnom Penh supporting a large peri-urban production of aquatic vegetables that are sold in city markets.

Sample Preparation

Samples for the first round of screening were prepared using the extraction solutions and techniques from the GT-test kit (Bangkok, Thailand). For the second round of screening, and for the water spinach trials in farmers' fields, total dislogeable (surface) pesticides were analyzed. Extraction of external OP/C was carried out in a process described by Abraxis (Abraxis LLC, 2008). Briefly, stem and leaves of each sample (20 g) were placed in a plastic bottle and extracted with 40 mL methanol (99%). The extracted sample was filtered once or twice with a vacuum pump using 0.2 µm mixed cellulose ester filters, and two or three times with a syringe filter using the same type of membranes. Once a clean extraction was achieved, the sample was diluted with water 1:1 and vortexed. When residual discolouration remained, appropriate dilutions were made with methanol (49.9%). Most water spinach samples were prepared in duplicate.

Samples for HPLC analysis of methylparathion were extracted using the QuEChERS technique (Schenck and Hobbs, 2004), which resulted in total (internal and surface) pesticide content. Briefly, a 10 g sample of vegetable was blended, then vortex mixed with 10 ml acetonitrile, 1 g NaCl, and 4 g $MgSO_4$. The sample was shaken for 3-4 min, then centrifuged for 5 min at 1500 rpm. A 4 ml subsample of the supernatant was evaporated to less than 1 ml. Clean-up was performed with a dual layer graphitized carbon black/primary secondary amine (GCB/PSA) solid phase extraction (SPE) column (Supelclean ENVI-Carb II/PSA). The SPE column was prepped by adding 1-2 cm $MgSO_4$ to the top of the column and washing with one column volume of 3:1 acetone/toluene. The concentrated vegetable extract was passed through SPE column dropwise and the solution discarded. Pesticide bound to the SPE column was then slowly eluted with a 3:1 acetone/toluene mix. Eluent was evaporated to 1 ml using a 50°C bath, then filtered using a syringe filter (0.45 mm, Durapore PVDF, Sigma-Aldrich). The processed sample was stored at 4°C until HPLC analysis.

Sample Analysis: Cholinesterase Inhibition

Screening of market vegetables in the first round was achieved with the GT pesticide test kit (Bangkok, Thailand). The presence of pesticide residue was indicated by a darker colour in the solution. Responses were visually categorized as equal to negative control ("no positive response"), positive but less than the critical control (and therefore "safe"), or positive and at or above the critical control ("unsafe").

In the second round of market vegetable screening, and in the water spinach field trials, OP/C were quantified with a cholinesterase inhibition test from Abraxis LLC (Warminster, PA, USA). All extracted samples were tested in duplicate. Upon completion of the assay, sample absorbance was measured at 405 nm using an automated microplate reader. Since the assay has different sensitivities for different specific pesticides, exact concentrations cannot be calculated unless the identity of the pesticide is known. Concentration was therefore calculated based on a diazinon standard curve, and all values are expressed as "diazinon equivalents".

Sample Analysis: HPLC

Detection of methylparathion was achieved with HPLC using a Shimadzu LC-10AD head pump, and a dual channel detector (Shimadzu SPD-10A UV/Vis detector). Samples were injected into a 20 µl injection loop; the separation solvent was methanol/water (70:30) pumped at a flow rate of 1 ml min^{-1} through a C18 column (Agilent Zorbax 4.6 × 250 mm) held at 35°C. All pesticide standards were purchased from AccuStandard Inc. (New Haven, Connecticut, USA). Methylparathion was measured at 273 nm, and had a retention time of approximately 9.5 min. Organic cabbage, green bean, green choy sum and kale purchased from a local specialty market and used as negative controls indicated that there was no peak overlap from the vegetable extracts with the methylparathion peak.

Measurement of quality control parameters indicated a linear dynamic range up to at least 20 µg/ml, and a minimum detection limit of 0.3 µg/ml solution (3.14 × standard deviation for seven replicates of 1 µg/ml standards) which corresponded to a detection limit of 0.04 mg/kg wet mass in a vegetable sample. Recovery was 80-100% (N = 5, organic vegetable samples spiked with 4 µg/ml methylparathion during extraction). Quality control measurements therefore verified the HPLC methodology for quantifying methylparathion in vegetable samples.

Statistical Analysis

Two-way analysis of variance (ANOVA) was performed using Microsoft Excel analysis tools. Half-lives for the bi-exponential decay in the methylparathion test plot trials were calculated using a backstripping procedure (Newman, 2010).

Results and Discussion

Survey of Market Vegetables using Screening Tests

In the first round of market vegetable screening, all four kinds of vegetables tested showed a high percentage of positive responses for pesticide residues (Table 2), suggesting widespread presence of pesticide residues on vegetables sold in Phnom Penh markets. Positive responses for pesticide residues was most common in cabbage, and had the highest percentage (34.1%) in the unsafe range. Long bean shows the fewest responses for pesticides residue, with no samples showing unsafe levels.

In the second round of market vegetable testing, the screening test indicated the presence of pesticide residues in 88% of curly-leafed kale, 95% of white-stemmed kale and 93% of Chinese broccoli samples. Average contents (mg diazinon equivalents per kg wet weight) were 9.2 ± 6.2 (N = 41) in curly-leafed kale, 11.7 ± 6.2 (N = 38) in white-stemmed kale and 10.2 ± 5.8 (N = 44) in Chinese broccoli. The test results cannot be compared directly to established levels of concern such as that reported by the WHO for two reasons: 1. values represent surface

Table 2: First round of market vegetables tested for OP/C

	Number of samples	*No positive response for pesticide residues*		*Positive response for pesticide residues, but amount is less than critical ("safe")*		*Positive response for pesticide residues, at or above critical level ("unsafe")*	
		Number	*%*	*Number*	*%*	*Number*	*%*
Cabbage	44	13	29.5	16	36.4	15	34.1
Long bean	33	28	84.9	5	15.2	0	0
Thin stem kale	19	13	68.4	4	21.0	2	10.5
Kale	26	13	50	9	34.6	4	15.4

pesticides, and total pesticide content is therefore likely to be higher than these reported values, and 2. the specific pesticides present in the vegetables were unknown and concentrations therefore could not be calculated based on a calibration curve of the actual pesticides present (hence expressing concentrations as "diazinon equivalent"). None-the-less, the results of both the first and second market surveys do indicate the widespread presence of some pesticide residues on vegetables.

A subsample of eight vegetable sellers who sold all three vegetable types was selected to test for statistical differences between vegetable types and sample source (seller) (Figure 1). Two-way analysis of variance indicated strongly significant differences in pesticide content dependent on both vegetable type and sample source (p<0.001 for both). The second round of screening therefore confirmed that pesticide residues are more prevalent in some types of vegetables, as was also indicated in the first round of screening. Furthermore, the level of pesticide residues clearly depends on the source of vegetables – some sellers had vegetables that were more contaminated than others. The data do not indicate whether this was a difference in handling of the vegetables by the seller (e.g. washing), or a difference in crop treatment by the farmer (e.g. frequency of pesticide spraying).

Taken together, the two rounds of screening in vegetables purchased from major food markets in the Phnom Penh region suggest that 1. consumers are regularly exposed to pesticide residues in local vegetables, and 2. there is significant variation in levels of pesticide residues. These results are consistent with the widespread use of pesticides on vegetables (CEDAC, 2004) and suggest the need for more intensive monitoring of market vegetables.

Targeted Analysis of Market Vegetables for Methylparathion

Given that screening tests indicated the presence of pesticide residues in market samples, 30 vegetable samples that screened positive for residues were tested with HPLC to assess levels of one specific pesticide: methylparathion. Despite being officially banned, methylparathion is one of the most widely used pesticides in Cambodia (CEDAC, 2000; CEDAC 2004), and is one of the most toxic pesticides (WHO Class Ia, extremely hazardous; IPCS, 1993). Methylparathion also has established HPLC techniques for quantification (e.g. Funch, 1981). In the 30 subsamples tested (cabbage, kale and long bean; N = 8, 10, 12, respectively; collected from separate sellers in three markets), none had detectable methylparathion. Figure 2 shows example results for four samples of long beans, demonstrating the absence of a methylparathion peak in the extracts.

These results indicate that contamination of market vegetables with methylparathion residues is not widespread, despite its frequent use on vegetable crops. This highly toxic pesticide may not present the greatest

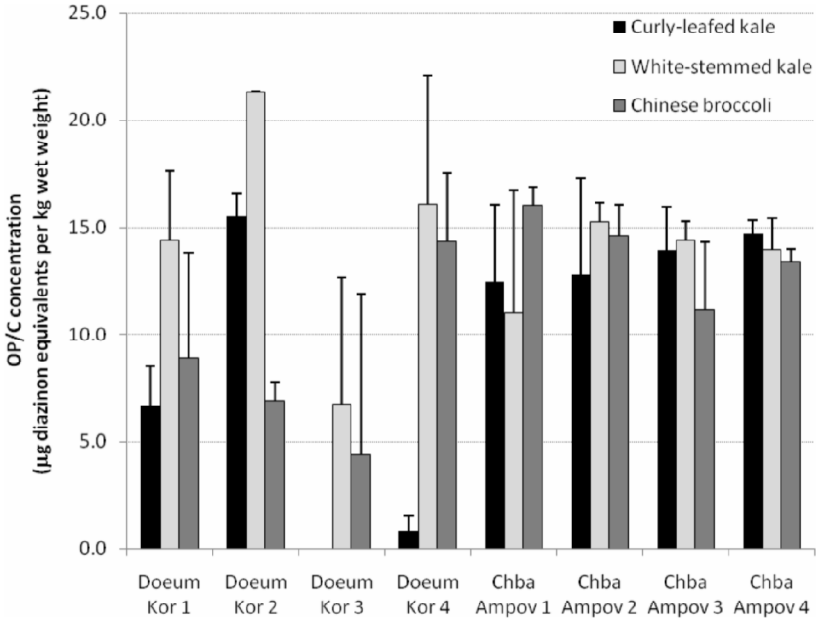

Figure 1: Second round of market vegetables tested for OP/C. Four sellers were chosen from each of the two markets (Doeum Kor and Chba Ampov). Data are means ± standard deviation (N = 3 for each).

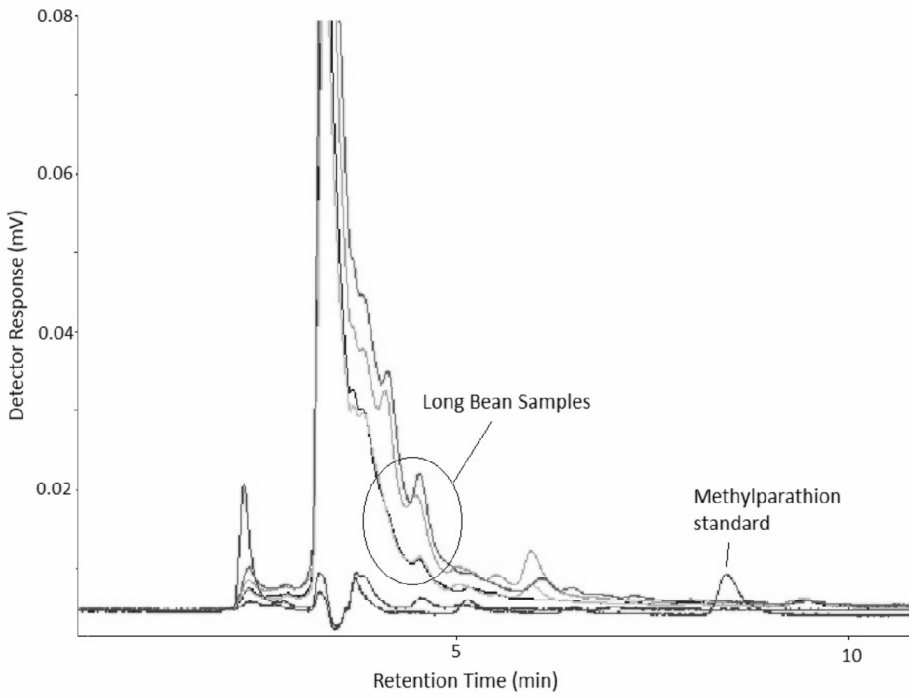

Figure 2: Example analysis of four long bean samples for methyl parathion, showing no peaks in long bean extracts at the retention time for methylparathion.

health risk to consumers, relative to other pesticides that are more persistent. The high percentage of samples indicating positive for OP/C contamination in the initial screening tests was apparently due to the presence of one or more pesticides other than methylparathion. It is important to note that these results assess the risk for consumption of vegetables, and do not address risks to farmers. Significant health impacts typically occur during the pesticide spraying process (e.g. Kishi et al., 1995), and farmers who spray methylparathion are likely still at a high risk for health impacts.

These results also demonstrate the utility of combining screening tests with more targeted analyses, thus giving an overall monitoring plan that is comprehensive, but at the same time less resource intensive. In this case, the use of the inexpensive and quick cholinesterase inhibition test provided information on which samples to use in the more expensive and time-consuming HPLC tests.

Methylparathion Degradation in Test Plots

The lack of methylparathion in samples suggested that this pesticide may rapidly breakdown after application, or otherwise disappear from the plant (e.g. washed off by rains). Test plots were therefore constructed in which vegetables were sprayed, and the timecourse of methylparathion content in harvested samples was followed. In two trials, each with two vegetable types, methylparathion rapidly disappeared from samples (Figure 3). The timecourse of disappearance from samples was characteristic of a bi-exponential decay, suggesting that two separate processes or compartments determined the kinetics of pesticide breakdown or dissipation. The first, more rapid, process had a half-life of between 0.31 and 0.64 days. The second process was obviously slower (calculated half-life of 1.4 to 6.0 days), but methylparathion was at a low enough levels by this point that there was more variability in the data. Knio et al. (2000) saw a similar initial rapid decline followed by more slow decline for dithiocarbamates on vegetables. Degradation appeared slightly more rapid during cloudy and rainy weather of the second trial hours, compared to the sunny weather of the first trial (Figure 3). These results are consistent with other studies showing a rapid decomposition of methylparathion when applied to vegetable crops (IPCS, 1993), a generally low persistence in tropical regions (Adhya et al., 1987), and a decrease in pesticide persistence during rainy periods (Bhanti and Taneja, 2007).

Two important strategies for reducing pesticide exposure are suggested by these data. First, the rapid disappearance of methylparathion suggests that a wait period of approximately 10 days after spraying is sufficient for methylparathion to reach levels that are considered low enough to be safe for human consumption

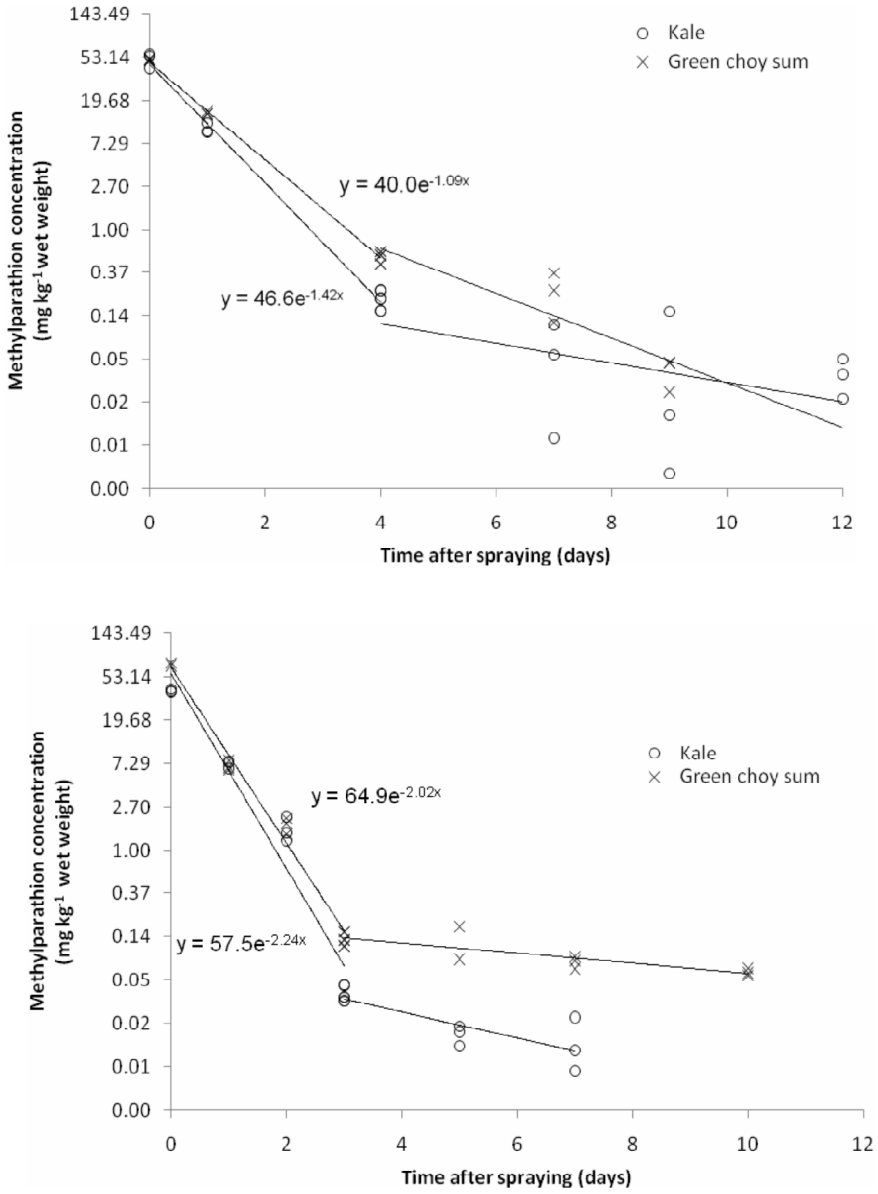

Figure 3: Total methylparathion content during two trials with test plots of kale and green choy sum, plotted on a log *y* axis. In the first trial (upper panel), weather was dry and sunny for the entire 12-day period. In the second trial (lower panel) weather was intermittently cloudy and rainy.

based on FAO/WHO maximum residue limit of 0.05 mg/kg for cabbage (FAO/WHO, 2010). Second, the pattern of methylparathion disappearance emphasizes the need to consider both toxicity and persistence when assessing health risks to consumers.

Farm Trials for OP/C Sprayed on Water Spinach

The clear trend in methylparathion disappearance suggested similar patterns might be found in other pesticides which are regularly sprayed on crops. Similar persistence studies were therefore performed in which samples were taken from actual production fields for water spinach. These fields were being sprayed by farmers with an assemblage of pesticides that are normally used on these aquatic vegetables. Surveys indicated that two of the farmers sprayed their fields primarily with dithiocarbamates, fungicides which are contact pesticides and as such are intended to remain on the plant surface to prevent growth of fungi. Concentrations of dithiocarbamates were variable in different sections of sprayed fields (e.g. the two samples from Farm A on day 5), but showed a general decrease

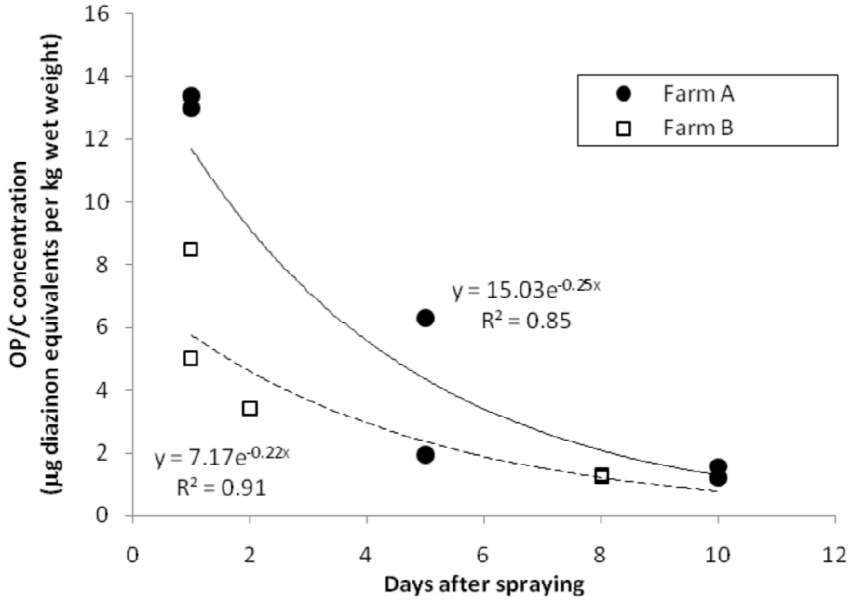

Figure 4: Concentrations of OP/C in water spinach fields using only dithiocarbamates (Farm A: mancozeb, maneb, propineb, zineb), or primarily dithiocarbamates with one organophosphate (Farm B: maneb, propineb, monocrotophos). Nine rain events occurred during the 10-day trial period for farm A, and two rain events occurred during the 8-day trial period for farm B.

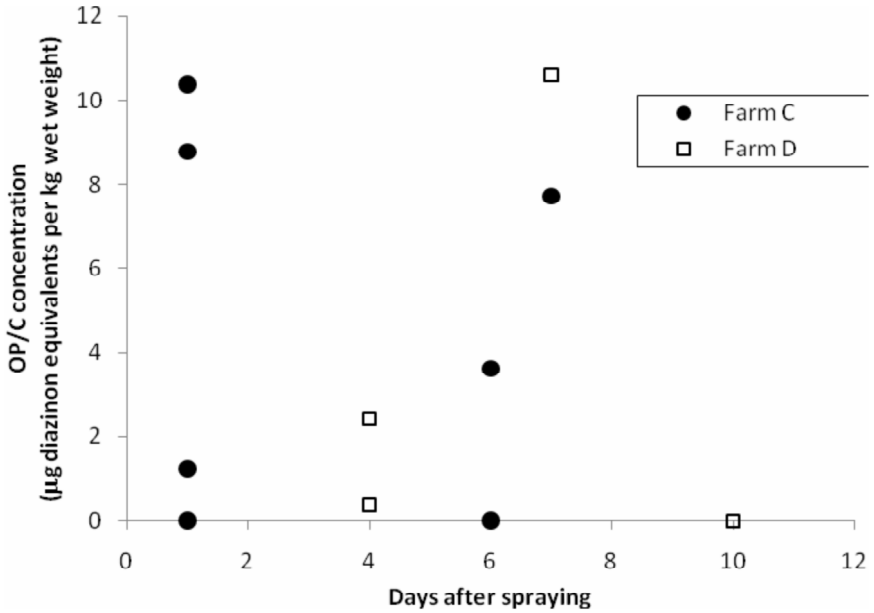

Figure 5: Concentrations of OP/C in water spinach fields using only organophosphates (Farm C; dichlorvos, monocrotophos), or an unknown mixture (Farm D). Five rain events occurred during the 10-day trial period for farm C, and three rain events occurred during the eight-day trial period for farm D.

in concentration and could be fitted to an exponential decay model (Figure 4). The half life was calculated as 2.8 and 3.2 days for farms A and B, respectively, consistent with previous reports of low persistence for dithiocarbamates (Knio et al., 2000).

Unlike the trials in fields sprayed with dithiocarbamates, no consistent trends were observed in fields sprayed solely with organophosphates, or with an unknown mixture. Levels of pesticides were highly variable both between days after spray, and between

samples taken from different areas of the field (Figure 5). For instance, organophosphate levels at one day after spraying on Farm C varied from 0 to 10.4 µg diazinon equivalents per kg vegetable wet mass. In both farms C and D, relatively high levels of pesticides were still present in samples taken on day 7, suggesting a longer period is needed for these pesticides to disappear from vegetables. These results suggest that variability in spraying and local conditions in fields create large differences in levels of pesticides after spraying, consistent with other studies showing that pesticide persistence is highly site- and compound-specific (e.g. Bondarenko et al., 2004). In addition, organophosphates used on Farm C (dichlorvos and monocrotophos) were systemic pesticides, suggesting that pesticides that are taken up in plants may show less predictable patterns of degradation or dissipation.

Conclusions

Assessing the health risk of the increased pesticide use in Cambodia requires both monitoring the routes of exposure, and quantifying that exposure to groups at risk. Three important conclusions from this study have implications for monitoring of pesticide exposure risk for consumers of market vegetables in Cambodia. First, the current study indicates that pesticide residues are widespread in market vegetables. Consumers are likely exposed to these pesticide residues, although the screening tests used in this study do not indicate the level of health risk associated with the residues in market vegetables. This suggests that a regular sampling programme should be established to monitor the extent and magnitude of residue contamination. Selection of specific vegetables and purchasing from specific sellers in markets can reduce exposure to pesticide residues. Second, in prioritizing health risks, these results demonstrate the need to consider both toxicity and persistence in assessing the impacts associated with residues on vegetables. Methylparathion, although widely used and highly toxic, was not present on the samples tested in this study, likely due to its consistent and rapid rate of disappearance from crops. However, field trials with a variety of other pesticides emphasize that general rates of pesticide degradation are variable and highly dependent on both the type of pesticide, and the specific field conditions. Finally, this study demonstrates a pesticide monitoring strategy that is appropriate for contexts with lower resources and capacity, such as that in Cambodia. Screening of vegetable samples with simpler test methods (such as the cholinesterase inhibition assay) coupled with targeted analysis of samples of concern can provide an optimal combination of information about prevalence and magnitude of residues, at a lower investment of resources and time.

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