2012

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Recommended Citation

Claborn, David M., Skylar A. Martin-Brown, Sanjay G. Sagar, and Paul Durham. "A rapid and inexpensive bioassay to evaluate the decontamination of organophosphates." Army Medical Department Journal July-September (2012).

A Rapid and Inexpensive Bioassay to Evaluate the Decontamination of Organophosphates

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ABSTRACT

An inexpensive and rapid bioassay using adult red flour beetles was developed for use in assessing the decontamination of environments containing organophosphates and related chemicals. A decontamination protocol was developed which demonstrated that 2 to 3 applications of 5% bleach solution were required to obtain nearly complete decontamination of malathion. The bioassay was also used to screen common household cleaners as potential decontaminating agents, but only 5% bleach was effective at improving survival of insects on steel plates treated with 25% malathion. A toxic degradation product (malaoxon) was detected using gas chromatography/mass spectrophotometry; this toxin affected the decontamination efficacy and resulted in continued toxicity to the beetles until subsequent decontaminations. The bioassay provides evidence to support the use of red flour beetles as a sensitive, less expensive method for determining safety levels of environments contaminated with malathion and other toxins, and may have application in the study of chemical warfare agents.

The organophosphates and closely related chemicals include not only important insecticides, but also potent chemical warfare agents (CWA) that have been used by militaries and terrorists alike. Important organophosphate insecticides include malathion, parathion, and chlorpyrifos which are used in a broad range of insect control programs from agriculture to public health. Among the related CWA are the neurotoxins sarin, tabun, and VX. Decontamination of areas containing these chemicals may be necessary following the inappropriate use of insecticides or following a terrorist attack with a CWA. For instance, in the early 1990s, several illegal applications of methyl-parathion, a potent agricultural insecticide, were used to control cockroach infestations in human residences in Ohio, Mississippi, Louisiana, Illinois, and Mississippi.[1] More than 4,500 homes were affected and 2 children were killed. More recently, resurging bed bug populations in the United States led to the illegal use of malathion, carbaryl, and cypermethin in over 70 houses in New Jersey, eventually requiring varying levels of decontamination.[2] Similar decontaminations have also been required after improper termiticide applications in military housing.[3,4] The threat to public health presented by inappropriately used insecticides is obvious, but the CWA are also of interest to people working in homeland security and national defense. The intentional use of sarin in the Tokyo subway system in 1995 is an example of terrorists' use of CWA. That one event resulted in 12 deaths and approximately 5,000 injuries, including injuries to first responders.[5]

Decontamination of the CWA has been defined as the "process of making any person, object or area safe by absorbing, destroying, neutralizing, making harmless, or removing chemical…agents."[6] Consistent with this definition, early attempts at CWA decontamination included washing with soap and water, absorbing with Fuller's earth, and simply leaving the chemicals to weather naturally over time. Actual chemical degradation of the toxin often relied on harsh chemicals such as calcium oxide and chlorine dioxide. New decontaminating compounds have been developed that are more effective or more environmentally friendly, including organophosphorous acid anydrolase (a hydrolyzing enzyme), and decontaminating foam with hydrogen peroxide. Much of the research required to quantify CWA decontamination requires sophisticated instrumental analytical techniques such as liquid or gas chromatography, which involves expensive equipment and trained personnel.[7-9] Extensive reviews of analytic detection and monitoring techniques are provided by Witkiewicz et al[10] and Kientz.[11] Such techniques are considered definitive but may provide

All authors state that they have no conflicts of interest involving the research reported in this manuscript and do not have investments or business with the companies mentioned. Mention of any company or product should not be seen as an endorsement by Missouri State University or any branch of the US military. This study was approved by the Institutional Review Board of Missouri State University.

only indirect measures of the biological toxicity. Often such processes document the breakdown of the target chemical into degradation products that are also toxic, though perhaps much less so than the original toxin. As Munnecke[12] stated, "the true change in toxicity of a pesticide containing medium can only be measured by conducting pertinent in vivo bioassays…." The same is probably true for CWA.

An inexpensive and rapid bioassay would be a useful screening tool with which to assess potential decontaminating agents for subsequent, more definitive testing by chemical analysis. Such a screening test would also be useful in quantifying or confirming changes in biological toxicity as a result of decontamination efforts. This article describes such a bioassay using an easily maintained insect colony. The bioassay is primarily intended to be a rapid screening tool. It is based on a previously published study by the first author and colleagues that demonstrated the detoxification of insecticides by certain types of paint used on ships.[13] In that study, the red flour beetle, *Tribolium castaneum* (Herbst), was used as a test organism on painted and unpainted steel plates treated with three different pesticide formulations. The mortality rate of beetles exposed to some of the insecticides was lower on painted steel plates when compared to unpainted plates. This study used a similar technique as a bioassay to investigate the level of decontamination of an organophosphate insecticide that has also been used as a CWA simulant.

MATERIALS AND METHODS

Development of Bioassay

The red flour beetle (RFB) was selected as a test organism with which to develop a rapid, inexpensive, and sensitive bioassay for evaluation of organophosphate decontamination. The RFB was used because it is easy to rear and handle in the laboratory, and has a long history of use in insecticide tests.[13] A colony of insects was obtained from the USDA Stored Products Laboratory in Manhattan, Kansas, in April 2011. The insects were raised in 946.4 ml canning jars on a diet of whole wheat flour and baker's yeast (10:1 mixture). The center of the jar lid was replaced with an unbleached coffee filter and held in place with the ring. A new colony was started every week by moving a few adults and a spoonful of flour with larvae into a new jar with the flour:yeast mixture. The jars were kept at room temperature (21°C to 24°C) in a dark cabinet with an open pan of water to provide moisture. All of the insects used for these experiments were from colonies started on April 15 or April 20, 2011.

Unpainted steel plates (20 cm by 20 cm) were cleaned with ethanol and allowed to air dry. The plates were treated with a 1-ml aliquot of 25% malathion. A commercial formulation of 50% malathion (Spectracide, Chemsico, St. Louis, MO) was used as the stock solution and was further diluted to 25% with xylene, which was also the diluent in the commercial formulation. This percentage was used because greater concentrations produced nonuniform dispersal on the plates as evidenced by oily droplets after decontamination. At 25% concentration, the malathion could be applied uniformly and it dried enough to allow the insects to move freely on the surface. The 1-ml aliquot was dripped onto the plate from a pipette, then spread evenly with a dry, 2.54 cm wide nylon bristle brush, covering the surface area of one side of the steel plate. Applications were made under a hood and the plates were allowed to dry for 48 hours. The plates then received the first decontamination.

All decontaminations were applied with an air brush (Iwata Revolution, Iwata-Medea Inc, Portland, OR) to prevent any unintended physical removal of the malathion that might occur with a brush or other application technique. The propellant for the air brush was 1,1-diflouroethane in a pressurized can. Decontaminant applications were all done in 1-ml aliquots. Potential decontaminants included:

- Standard household bleach (5% sodium hypochlorite), Clorox Bleach, The Clorox Company, Oakland, CA
- 95% ethanol
- Lysol All-Purpose Cleaner (active ingredient 3.2% lactic acid), Reckitt Benckiser Inc, Parsippany, NJ
- Simple Green Concentrate, Sunshine Makers Inc, Huntington Beach, CA
- Pine Sol Concentrate (active ingredient 8.7% pine oil), The Clorox Company, Oakland, CA

Bleach and ethanol were both selected because they are commonly used for insecticide decontamination, and bleach is a standard decontamination agent for nerve agent weapons.[9] Lysol, Simple Green, and PineSol are common household cleaners that could be used by residents of a contaminated facility to clean an insecticide-contaminated area. Ethanol and bleach were used as decontaminants in all tests, but only one of the 3 household cleaners was used in each of 3 replications. The decontamination schedule is shown in Table 1.

In each replication, the plates were decontaminated 3 times consistent with the schedule in Table 1. After each decontamination, the plates were allowed to dry

for 48 hours before the insect bioassay was performed. An untreated, nondecontaminated control was used for each replication as well as a control that was treated only with 1 ml of 5% bleach and another treated with ¾ ml of the xylene diluent. In replication No. 1, Lysol was compared to bleach and alcohol; in replication No. 2, Simple Green was substituted for Lysol, followed by Pine Sol in replication No. 3. Three to 4 plates received each treatment/decontamination combination in each of the 3 replications. A separate bioassay was run to compare the toxicity of each of these household cleaners to an untreated (no malathion) control.

For the bioassay, 10 RFB adults were counted into small disposable Petri dishes and allowed to starve overnight. They were observed the next day to ensure that they were still alive, then one side of the Petri dish with the RFB inside was inverted on the steel plates, exposing the insects to the treated surfaces. After one hour, the plates were inverted, collecting the RFB back into the Petri dish and the insects were observed for toxic effects. All of the insects in each dish were immediately observed and placed into one of the following 3 categories:

Category 1: Alive (moves when prodded with a probe).

Category 2: Knockdown (moribund but showing some movement of legs or head)

Category 3: Dead (total lack of movement even when prodded with a probe)

The lids were replaced on the Petri dishes and the insects were allowed to sit undisturbed on the bench top until they were evaluated again for toxic effects 24-hours postexposure. This process was performed after each of the 3 decontaminations. At the end of each bioassay, the tested insects were destroyed and not used for subsequent bioassays.

Statistical Analysis

Data were analyzed using the PROC ANOVA procedure in the SAS 9.2 software (SAS Institute Inc, Cary, NC). The models were used to describe the effects of decontamination on survival (those classified as alive) at 1-hour postexposure and at 24-hours postexposure. Another category of some movement combined the categories of alive and knockdown. Means of survival and survival with knockdown were compared using the Tukey's HSD (honest significant difference) test.

Table 1. Application and bioassay schedule for decontamination of malathion with common household cleaners.

Day 0	25% malathion applied to plates
Day 2	First decontamination
Day 4	First bioassay; second decontamination of plates
Day 6	Second bioassay; third decontamination of plates
Day 8	Third bioassay

Comparison to Standard Chemical Assay

The described steel plate assay was used to compare to a standard analytical process using gas chromatography/mass spectrophotometry (GC/MS). Twelve steel plates were set up as in the assay as previously explained, and then smaller steel plates (5.08 cm by 5.08 cm) were placed in the center of each plate as coupons. The coupons were taped onto the larger steel plates with only a small edge of the laboratory tape extending onto the coupon. This was done to prevent treatments from contaminating the underside of the coupon. The plates and coupons were then treated with 1-ml aliquots of 25% malathion and dried for 24 hours. Four of the coupons were then removed, placed in glass jars, and transported to the chemistry laboratory at the Jordan Valley Innovation Center, Missouri State University, for analysis. At that time, the remaining plates with coupons received a single decontamination with 5% bleach identical to that described earlier. After 24 hours, 4 more coupons were removed for chemical analysis, and the remaining plates with coupons received a second decontamination treatment. Following another 24 hours, two of the coupons were removed for analysis and the last two plates with coupons received a third decontamination treatment. After another 24 hours, the bioassay was performed on the larger steel plates, placing the Petri dishes on treated (malathion) and decontaminated (bleach) areas next to the sites where the coupons had been removed.

Reagents and Materials. Malathion and malaoxon PESTANAL analytical standards were purchased from Sigma-Aldrich (St Louis, MO). Optima grade acetone was purchased from Fisher Scientific (Fair Lawn, NJ). Stock standards of 150 µg/ml and 100 µg/ml were freshly prepared in acetone each week and stored at 4ºC in opaque Nalgene bottles (Fisher Scientific). Calibration standards (0.1 µg/ml - 150 µg/ml) were also prepared weekly in acetone from the stock standards by serial dilution in acetone and stored at 4ºC in opaque Nalgene bottles.

Sample Extraction. The sample extraction procedure was adapted from Rogers et al.[14] The coupons were removed from the treated steel plates and placed in 250 ml glass straight-sided jars (Fisher Scientific) to which 80 ml of acetone was added. The samples were then sonicated for 30 minutes. After sonication, 1 ml was removed from the jar and added to an autosampler vial for GC/MS analysis. A single extraction cycle proved to be sufficient for the steel coupons.

GC/MS Analysis. A Varian 450 GC (Varian Medical Systems Inc, Palo Alto, CA), coupled to a Varian 320 triple quadropole mass spectrometer was used for the analysis. The instruments were interfaced to a computer running Varian MS workstation version 6.9.1 for instrument control and data processing. Instrument conditions were similar to those used in Rogers et al.[14] The column used for separation was a Zebon ZB-1701 with 5 mm Guardian guard column (Phenomenex, Torrance, CA). The column dimensions were 30 mm by 0.25 mm by 0.15 µm film thickness. A 1 µl sample was injected in splitless mode at 200ºC. The GC oven was programmed as follows: 100ºC hold for 2 minutes, increased to 180ºC at a rate of 10ºC/minute, increased to 220ºC at a rate of 5ºC/minute, increased to 260ºC at a rate of 20ºC/minute and held at 260ºC for 2 minutes. Total run time was 22 minutes. Helium was the carrier gas with a flow rate of 0.8 ml/minute. The mass spectrometer was operated in electron impact mode. The transfer line and ion source temperatures were set to 280ºC and 230ºC, respectively. Retention times were determined and parent ions were verified in full scan mode. Quantification and qualification ions were selected and collision energies were determined experimentally by tandem mass spectrometry. Analyte specific information is shown in Table 2.

Peak areas of standards were plotted using a quadratic function with weighting scaled by the inverse of analyte concentration. A minimum of 6 points was required for an acceptable calibration curve. Both calibration curves had correlation coefficients of $r^2 > 0.990$.

Statistical Analysis. Mean concentrations were calculated with the PROC MEANS procedure in SAS 9.2 for both malathion and malaoxon on the coupons. Results were graphed using a Microsoft Excel 2010 spreadsheet.

RESULTS

Bioassay Development

Survival on plates that did not receive an application of malathion or a decontaminant was not different from plates that received only a xylene application (0.75 ml) or 1 to 3 applications of 5% bleach ($r^2 = 0.01$, $P = .65$). This finding indicated negligible toxicity of the diluent (xylene) and the standard decontaminant (5% bleach). Due to the lack of toxicity on these plates, the control throughout the study was subsequently defined as plates that did not receive a malathion application or any decontamination treatment, or that received only a bleach or xylene application. Similarly, survival on plates that were treated only with the decontamination agents of Lysol, Pine Sol, Simple Green, and ethanol was not significantly different from an untreated (no malathion) and undecontaminated control.

Survival levels on plates treated with malathion but which were not decontaminated were consistent throughout the multiday study for each replication. The last bioassay on each replication was run 8 days after the initial application of malathion, but survival levels on control plates on the last bioassay was not different from that of the first or second bioassays in each replication ($r^2 = 0.002$, $P = .97$). This finding provides evidence that in the protected environment of the laboratory, malathion was not degraded and it remained active throughout the duration of the biological testing.

Table 3 displays the survival of 10 RFB confined for one hour on steel plates treated with malathion, then decontaminated with 1, 2, or 3 treatments of 5% bleach solution. Bleach was used as the standard decontamination treatment for this study. To monitor changes in the level of toxicity to each treatment, survival levels were measured one hour and 24 hours after initial exposure, and as a combined measurement of survival and knockdown (some movement) 24 hours after initial exposure. A means separation test indicated that the first application of a decontaminant on Day 2 of the experiment did not result in significant detoxification. This finding was consistent with all 3 measures of toxicity. However, after a second application of the bleach solution on Day 4, survival was significantly increased on the plates as measured by the bioassay on Day 6. This finding was also consistent with all 3 measures of toxicity. After a third decontamination of the plates on Day 6, the survival level as measured on Day 8 was slightly greater, but was not significantly different from that of the second decontamination. Survival levels after the second and third decontaminations were not significantly different from that on plates that did not receive an application of malathion except as measured by simple survival (not knockdown) at 24 hours. That measure indicated a difference in survival on malathion-treated plates that received only 2 decontaminations as compared to plates that received no malathion but did receive bleach applications. Of the 3 measures of toxicity, the measure of "some movement" explained the greatest

Table 2. Analyte information for gas chromatography/mass spectrophotometry analysis.

Analyte	MW	Parent Ion	Retention time (min)	Quantification Ion (m/z)	Qualification Ion (m/z)	Collision Energy
Malathion	330.36	331	17.2	285	173	5 eV
Malaoxon	314.30	315	17.7	173	127	6 eV

MW indicates molecular weight.
m/z indicates mass to charge ratio.

Table 3. Postexposure percentage of surviving *Tribolium castaneaum* after confinement for one hour on 20 cm by 20 cm steel plates which had been treated with 1 ml of 25% malathion, then decontaminated sequentially with 1, 2, or 3 applications (1 ml) of 5% bleach solution.

Number of decontamination treatments	n	Survival 1-hour postexposure % (SD)	Survival 24-hours postexposure % (SD)	Some movement* 24-hours postexposure % (SD)
0	36	59.7 (26.4)a	30.8 (22.9)a	30.8 (22.9)a
1	12	44.2 (28.1)a	12.5 (31.0)a	24.2 (35.0)a
2	12	82.0 (28.0)b	55.0 (40.5)b	85.8 (18.3)b
3	12	96.7 (4.5)b	80.0 (34.9)b,c	97.5 (4.5)b
Bleach only	27	97.7 (4.4)b	98.8 (3.3)c	98.8 (3.3)b
r^2		0.40	0.62	0.70

n indicates the number of steel plates that received the designated treatment.

Note: Values in a column followed by the same letter are not significantly different (Tukey test, *P*=.05).

*Combined category including insects that were alive or knocked down.

amount of variation in the model as demonstrated by an r^2 value of 0.70.

None of the household cleaners appeared to decrease toxicity, with only bleach demonstrating a decontaminating effect as demonstrated by the bioassay. Table 4 compares the standard (5% bleach) to common household and laboratory cleaners in their capacities to decontaminate malathion. The measures of toxicity were the same as used earlier. All treatments for each decontaminant were combined for comparison in Table 4. There was decreased survival on plates treated with Pine Sol and Simple Green alone as compared to the nondecontaminated control, a finding that is inconsistent with the control studies that indicated no toxicity due to the decontaminating agents alone. Survival on bleach-treated plates was significantly greater than on plates decontaminated with any of the other agents.

GC/MS assay

To correlate data from the bioassay experiments to the degradation of malathion, GC/MS was used to measure the amount of malathion and malaoxon, the oxidative byproduct of the decontamination of malathion. Because malaoxon is toxic, the concentration of this chemical was also determined in the GC/MS assay. Concentrations of malathion and malaoxon after 0 to 3 decontaminations are graphically depicted in the Figure. The presence of malaoxon as a toxic byproduct of the oxidation of malathion continued after 1 and 2 applications of bleach decontaminant, but was completely removed after a third application Three decontaminations resulted in almost complete degradation of malathion.

COMMENT

The simple bioassay demonstrated in this study provides a quick screening mechanism that can be used to investigate factors affecting the decontamination of neurotoxic chemicals, particularly the organophosphates. It allowed the identification of an effective application rate of bleach for use as a decontaminating agent. This is perhaps the greatest utility of the bioassay. When the toxic agent was 25% malathion, about twice as much 5% bleach by volume was required to significantly improve survival of RFB on malathion treated plates. Nearly complete decontamination was obtained by 3 subsequent applications of bleach with each application being the same size by volume as the 25% malathion. This rapid assessment of efficacy can be useful when putting together decontamination protocols for toxic agents, especially because it measures actual biological toxicity. Further research is necessary to determine if lower concentrations of the bleach decontaminant or smaller aliquots might be effective in repeated decontaminations. The concentration used in this study (5%) was very high and would not be suitable for use in many situations.

Table 4. Postexposure percentage of surviving *Tribolium castaneaum* after confinement for one hour on 20 cm by 20 cm steel plates which had been treated with 1 ml of 25% malathion, then decontaminated sequentially with a common household cleaning product.

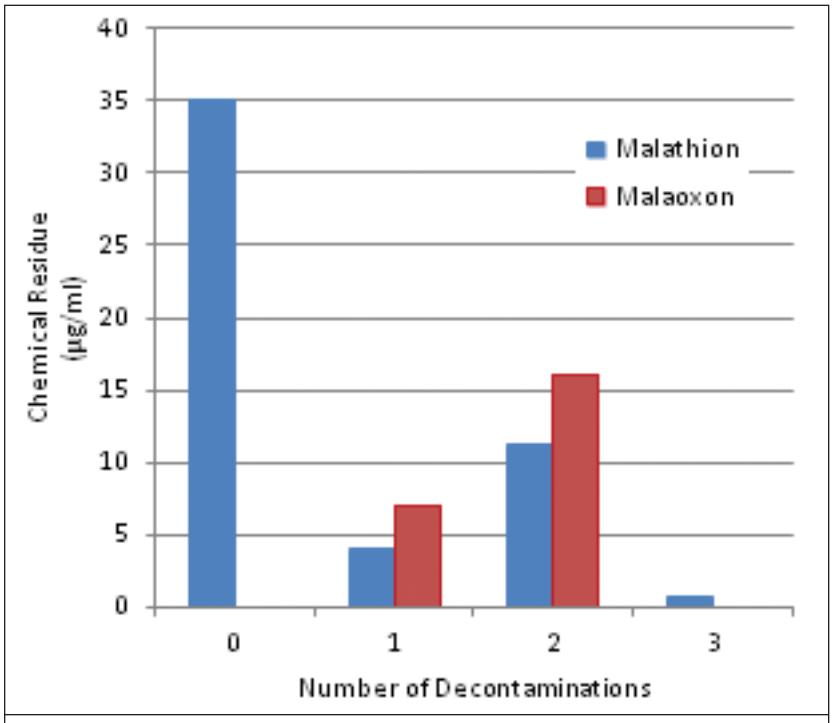
Decontaminant	n	Survival 1-hour postexposure % (SD)	Survival 24-hours postexposure % (SD)	Some movement* 24-hours postexposure % (SD)
No decontamination	98	8.1 (20)a	9.5 (20.0)a	30.8 (22.0)b
Pine solvent	9	3.3 (5.0)a	1.1 (3.3)a	3.3 (5.0)a
Simple Green	12	8.3 (11.0)a	1.7 (3.9)a	15.0 (23.5)a,b
Ethanol	23	3.9 (6.6)a	16.0 (25.0)a	37.8 (32.0)b
Lysol	12	18.3 (25.1)a	20.0 (26.9)a	41.7 (31.6)b
Bleach (5%)	24	61.2 (38.4)b	67.0 (39.0)b	91.7 (14.3)c
No malathion/no decontamination	37	96.6 (7.7)b	95.2 (12.0)b	98.6 (3.5)c
r^2		0.85	0.80	0.79

n indicates the number of steel plates that received the designated treatment.

Note: Values in a column followed by the same letter are not significantly different (Tukey test, *P*=.05).

*Combined category including insects that were alive or knocked down.

Chemical analysis of the residual toxin on decontaminated plates confirmed that a significant amount of malathion remains on the plates after one decontamination; the concentration of degradation byproducts was also increased. When 25% malathion was decontaminated with 5% bleach, nearly complete decontamination of the surface was achieved with 3 treatments, a finding consistent with both the bioassay and the GC/MS analysis. The presence of a toxic byproduct of decontamination (malaoxon) was demonstrated by both the chemical analysis and suggested by the bioassay, demonstrating the need for validated protocols for decontamination processes.



Mean malathion and malaoxon residuals (µg/ml) on steel plates after 0, 1, 2, or 3 applications of 5% sodium hypochlorite (bleach) solution applied with an air brush.

A rapid screening of household cleaners using the RFB bioassay failed to identify any additional decontaminating agent other than the common bleach solution already known to be an effective decontaminant. Other cleaners like Simple Green and Pine Sol might be useful in the physical removal of the agent, but do not demonstrate a reduction in toxicity of malathion as determined by the insect bioassay. The results of the bioassay, however, were not always straightforward. The increased toxicity on malathion-treated plates decontaminated with Simple Green and Pine Sol was unexpected. These undiluted substances are slightly viscous. Perhaps this physical characteristic impedes the insect's movement or covers the spiracles leading to asphyxiation. Alternatively, the cleaners may break down protective characteristics of the insect cuticle, thereby increasing the insect's susceptibility to the toxin. Insects exposed to plates that received only Pine Sol or Simple Green applications, but no malathion, did not elicit greater mortality than did plates that had received no application. This suggests that these 2 cleaners may somehow synergize the action of the malathion, though this possibility would require more research to confirm. Another interesting finding in this screening was the lack of efficacy of ethanol as a decontaminating agent against malathion. Ethanol has been used as a decontaminant for other insecticides, specifically organochlorines,[4] but did not show any efficacy against an organophosphate.

The benefits of this bioassay include its rapidity, very low expense, and its actual measurement of biological toxicity. The latter is important given that analytical chemistry-based measures can quantify the breakdown of the target chemical, but may fail to measure the toxicity of degradation products. No expensive equipment is required and this bioassay could actually be performed in a field situation with only minor modifications. This type of bioassay provides almost immediate results and can easily be adapted to test a variety of surfaces such as concrete, wood, and tile. It can also be used to study the impact of environmental variables such as temperature, humidity, and insolation on the decontamination of toxic chemicals. However, this bioassay does not identify the mode of toxic action, nor does it rule out the possibility of other forms of toxicity such as endocrine disruption or carcinogenicity. Since this bioassay does not identify the mechanism by which the insects are killed, it is not a replacement for the standard analyses involving analytical chemistry. Also, the surfaces to be tested must be dry. Wet surfaces lead to concentration of the toxins or decontaminants and wetting of the insect cuticle, both of which can cause inconsistent measures of toxicity. This phenomenon was particularly observable with the Lysol applications and may limit the utility of the bioassay for such decontaminants.

Future research using this bioassay will include investigations of decontamination efficacy on various surface types, extended screening of potential decontamination agents, and evaluation of environmental factors such as temperature and humidity on decontamination processes. Although the current screening was done with

decontamination of an insecticide on steel plates as a model, this bioassay may also serve as a method to study the decontamination of a variety of toxic environments such as facilities that have been contaminated during inappropriate termiticide applications, chemical warfare agent attacks by terrorists or national militaries, or even houses contaminated by the illegal manufacture of methamphetamines. It would be most useful when used as an initial screening tool as it is not a replacement for the more comprehensive and expensive analytical tests.

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