PROBLEMS OF RESIDUE-FILM BIOASSAY WITH DROSOPHILA MELANOGASTER

By

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1. Introduction

A bioassay unit was set up as a fast, reliable, relatively simple and inexpensive measuring technique of toxicological properties of insecticides. In some respects this method is superior to the more sophisticated and expensive chemical analysis, especially in studies of degrading or metabolizing processes which influence the toxic potential of the parent toxicant . Good use can be made of bioassay in residue analysis.

The principle of bioassay is to compare responses of test insects to treated samples with those of a series of standards under the same conditions. Responses to toxicants are usually defined as a certain moribund stage of the test insect.

Many factors affect the insect response. Therefore the testing procedure has to be standardized and a standard series has to go along with every test in order to obtain reproducible results.

As bioassay has been used increasingly over the last two decades a lot of publications have amassed which are summarized in a number of reviews (e.g. 1, 2, 4,6, 7, 8, 9). Testing the performance of our bioassay unit yielded some results which are reported in the sequence of the testing procedure.

2. Bioassay procedure

Drosophila melanogaster was chosen as test insect because it can be reared and handled easily (3). The Evin-strain has been inbred for six years. This genetically homogeneous population is considered highly uniform in its physiological make-up as long as their rearing conditions are maintained along the lines of a certain set of rules. Temperature and humidity of the rearing room are set at 27±2°C and 70–80% r.h. Nutrition and population densityare regulated in the following way. The food consists of 125 gr. corn

flour+6 gr.Agar-Agar+62 gr. sugar+7 gr. yeast which is distributed equally into 6 jars of 450 ml capacity. 12 females and 8 males are put into each container which is covered by a piece of cloth. The drosophils are immobillized by CO_2 for a few minutes to sex and transfer them.

Petri dishes of 9 cm diameter were chosen as test containers. The toxicants were dissolved in low boiling solvents (e.g. petroleum ether, acetone) whenever possible. The same solvent was used for both the treated sample and the standard. Petri dishes with the solvent only were used as check, mortalities of which were related to the mortalities of treated samples by means of the Abbott formula. Usually 5 to 6 replicates of each treated sample were tested. Each replicate consisted of 20 drosophils, 1 to 3 days old.

Generally mortalities were recorded at time intervals. LT_{50} values were calculated as average figures of the replicates and were expressed in hours as decimal units . Plotting the LT_{50} values of the standard versus their respective concentrations on log-log paper results in an eye fitted line. The LD_{50} figures of the treated sample can then be read off (5). We prefer this more tedious procedure in case of unpredictable concentrations (e.g. fast degrading deposits) to the direct calculation method of LD_{50}. If it is practical to limit the exposure period, the probits of mortalities are plotted against log. concentrations of the standard series and the LD_{50} are read off on the straight line.

3. Variability of insect response

Despite all standardization efforts a certain variability of the test insects has to be reckoned with. The magnitude of variation of the bioassay results gives an indication as to the accuracy and reproducibility of the bioassay method.

3.1 The relationship between response and concentration of the stimulus was established. Four concentration levels (ml/plate) of Nexion 40 EC were tested, each being replicated 3 to 9 times. The LT_{50} values and their variation coefficients are recorded in table 1.

Table 1. Effect of insecticide concentration on response.

	Concentration	n	LT_{50}	V.C.
1.	1.9×10^{-6}	9	2.52	15
2.	1.9×10^{-5}	6	0.88	14
3.	1.9×10^{-4}	3	0.42	4
4.	1.9×10^{-3}	3	0.35	3

LT_{50}=Time when 50% mortality is recorded. V.C.= variation coefficient

The variation of responses to higher concentrations is small. The results at the lower concentrations, which fall into the range of residue analysis are more variable, but can still be considered as satisfactory. Incidentally, the dose of 1.9×10^{-3} ml/plate is equal to 3 l/ha field dosage.

3.2 The vriability of the insect response over a period of time was checked by repeated tests. During 22 days nine tests with Nexion 40 EC at 1.9 x 10^{-6} ml/plate were carried out; each test consisted of 5 replicates. The LT_{50} figures varied considerably around the median value of 2.52 hours (Fig. 1). Although the flies were reared under the same conditions their response intensity changed from day to day. This does not influence the accuracy of the biotest result because the LT_{50} value of each test is always correlated to a corresponding standard series. The sensitivity of the determination may be impaired by such low LT_{50} figures as in the tests No. 8 and 9. However, the high values of the variation coefficients of the tests No. 1, 2, 8 and 9 seriously influenced the accuracy.

These conclusions are confined to this one insecticide. Other insecticides of different chemical properties might alter the response intensity.

4. Susceptibility of the test insect

4.1 Satisfactory susceptibility of the test insect is defined as an appropriate response pattern over a concentration range of 10^{-3} to 10^{-5} ml/plate, this is the range of macro–to microassay. And the criterion for a satisfactory response is that the LT_{50} is ranging from 0.3 to 3.0 hours. Values below and above this range are considered to be affected by too large an error.

LT_{50} figures of the upper and lower detection limits were recorded for a number of insecticide formulations. The concentration levels were prepared as acetone solutions of the insecticide with an active ingredient content as indicated in table 2.

Table 2. LT_{50} values of the upper and lower detection limits.

		Concentration range of		
No.	Insecticide	10^{-3}	10^{-5}	10^{-6} ml
1	Anthio 25 EC	(0.90–1.30 for 10^{-4})		
2	DDT 25 EC	1.80–2.30		
3	DDVP 50 EC		1.50–2.00	4.00
4	Diazinon 20 EC	0.06	0.50–0.80	0.80–4.50
5	Dimecron 20 EC	0.90	4.00	
6	Dipterex 80 WP	0.40	0.60–1.00	1.00–4.80
7	Endrin 18.5 EC		2.60–3.00	
8	Folithion 50 EC	0.35–0.40		
9	Gusathion M 20 EC	1.10–1.30	2.20–3.00	
10	Lebaycid 50 EC		0.30–1.00	
11	Lindane 20 EC	0.13–0.14	1.00–1.60	1.40–5.00
12	Malathion 95 techn.		0.37–0.40	1.80–4.40
13	Nexion 40 EC	0.30–0.38	0.60–1.00	1.40–3.60
14	Perfekthion 40 EC	(0.30 for 10^{-4})	0.50–0.60	1.20–3.10

15	Roxion 40 EC	0.70	1.60–3.50
16	Sevin 85 WP	higher than 3.00 at 10^{-2}	
17	Supracid 40 EC	0.70–0.80	1.00–3.50
18	Thiodan 35 EC	0.70–0.90	1.40–2.40
19	Toxaphen 50 EC	(4.50 for 10^{-4})	

The insecticides No. 1,3,6,7,8,9,13,14,15,17 and 18 can be assayed successfully over the concentration range 10^{-3} to 10^{-5} ml per plate, although not all the necessary figures are yet available. Several of them can even be determined in the 10^{-6} ml range, which means for residue analysis that their detection limit is lower than 0.1 ppm. The insecticides No. 2 and 5 pose a problem for the lower range and No. 16 and 19 cannot be assayed by Drosophila. The pesticides No. 4, 10 and 11 have a high toxic value for the upper dosage level; by further dilution they will meet the requirements. Drosophils are very efficient and versatile test insects. Figures which are compiled (9) for the lower limit of susceptibility are in good agreement with our results.

4.2 In residue analysis of tea leaves the susceptibility of the drosophils towards Rogor, Anthio, Nexion and Folithion was lowered to such an extent that the masking effect of the tea leaf extractives had to be removed by a clean-up procedure. The recovery data for Nexion before clean-up ranged from 0.02 to 4% for amounts of 5 to 100 ppm toxicant which were added to the acetone stripping solution. 8.7% of Folithion was recovered in a similiar test adding 1.5 ppm of that toxicant.

4 3 Sexual differences in susceptibility towards insecticides are often claimed. Females are supposed to be more resistant to contact poisons than males, e g to aldrin according to Sun (8), to nicotine (1).

The higher resistance level is attributed to weight differences of the sexes. If this assumption is correct the difference in susceptibility of the sexes would influence the accuracy of bioassay results in case populations of mixed sexes are used.

As exact figures are scarce, the susceptibility of males and females of ***Drosophila melanogaster*** (Evin strain) towards a number ot insecticides was tested. The sexes were submitted concurrently to each acetone insecticide solution (5-6 replicates). The LT_{50} values and their variation coefficients are summarized in table 3. The LT_{50} figures were compared statistically by the t-test.

Of the 20 tested insecticides, the following five produced significant differences in response on the basis of LT_{50} values: Folithion, Perfekthion and DDT as well as Anthio and Endrin. Males were more susceptible to the first three while they were more resistant to the last two insecticides than females.

The different concentrations of three of the insecticides did not affect the response pattern of the sexes. Interesting to note is the fact that in the DDT-Lindane compound the faster acting Lindane determined the type of reaction. For practical bioassay purposes males or females have to be selected for those insecticides to which the sexes show different susceptibility. Insecticides which produce no sexual differences in susceptibility can be bioassayed by mixed populations. A number of

insecticides show large differences in variation. In such cases the sex which exhibits the smallest variation should be prefered for the sake of accuracy.

Table 3. Sexual differences of susceptibility.

Insecticide	Concentration ml/plate	LT50 ♀+	V.C.	LT50 ♂	V.C.	Difference
Nexion 40 EC	$1.9x10^{-4}$	40	4.8	39	2.6	N
Nexion 40 EC	$1.9x10^{-4}$	37	15.1	39	30.7	N
Nexion 40 EC	$1.9x10^{-5}$	100	10.1	101	6.4	N
Nexion 40 EC	$1.9x10^{-5}$	59	17.0	61	13.0	N
Nexion 40 EC	$1.9x10^{-6}$	361	17.3	335	24.0	N
Nexion 40 EC	$1.9x10^{-6}$	325	4.1	357	6.2	N
Nexion 40 EC	$1.9x10^{-6}$	390	19.4	390	15.4	N
Gusathion M 20 EC	$1.9x10^{-3}$	115	26.6	132	11.9	N
Gusathion M 20 EC	$1.9x10^{-3}$	129	23.3	122	13.7	N
Roxion 40 EC	$1.9x10^{-5}$	70	11.9	72	6.1	N
Folithion 50 EC	$1.9x10^{-3}$	41	8.8	37	1.6	S
Diazinon 20 EC	$1.9x10^{-3}$	6	21.2	6	4.7	N
Diazinon 20 EC	$1.9x10^{-5}$	82	45.6	48	37.5	N
Lebaycid 50 EC	$1.9x10^{-4}$	23	4.3	24	9.9	N
Perfekthion 40 EC	$1.9x10^{-4}$	34	6.1	29	7.0	S
Malathion 95 T	$1.9x10^{-5}$	37	5.4	41	15.2	N
DDVP 50 EC	$1.9x10^{-5}$	197	42.1	156	31.0	N
DDVP 50 EC	$1.9x10^{-5}$	238	6.6	218	69.2	N
Superacid 40 EC	$1.9x10^{-5}$	72	32.9	82	35.6	N
Anthio 40 EC	$1.9x10^{-4}$	96	9.5	127	8.2	S
Dimecron 20 EC	$1.9x10^{-3}$	87	8.9	91	11.0	N
Dipterex 80 SP	$1.9x10^{-3}$	42	39.6	46	27.9	N
Metasystox R	$1.9x10^{-4}$	112	11.9	102	17.1	N
DDT 25 EC	$1.9x10^{-3}$	232	16.5	176	21.3	S
Lindane 20 EC	$1.9x10^{-3}$	13	14.6	14	13.6	N
Lindane 20 EC	$1.9x10^{-5}$	101	9.3	93	12.1	N
Lindane 20 EC	$1.9x10^{-5}$	160	29.3	121	35.5	N
DDT–Lindane 30–9	$1.9x10^{-3}$	30	17.6	26	12.1	N
Thiodan 35 EC	$1.9x10^{-4}$	75	7.0	70	10.3	N
Endrin 19.5 EC	$1.9x10^{-5}$	266	2.7	303	8.8	S
Toxaphen	$1.9x10^{-4}$	449	2.9	416	4.8	N

S = Difference statistically significant at 5% level.

N = Difference statistically not significant at 5% level.

5. Distribution of deposits

The importance of uniform distribution of the deposit is much discussed (11). Some experts feel the deposit has to cover the inner surface of the test vials evenly, other maintain it is not neccessary pointing out that the active insects will pick up equal amounts of toxicant even if the deposit is not distributed uniformly.

A Nexion 40 EC–acetone solution was distributed in two ways in the petri dishes. The whole amount was poured into the bottom part of the container (test A). In test B half of the solution was desposited in the bottom part and the other half in the upper part of the dishes. The LT_{50} values of the two tests were compared at 3 concentration levels against the same standard. (table 4)

Table 4. Effect of deposit distribution on toxicity.

	Replicates	ml/plate	LT_{50} V.C. Test A	LT_{50} V.C. Test B
1.	5	1.9×10^{-6}	2.41±21%	2.09±33%
2.	6	1.9×10^{-5}	0.66±18%	0.70±13%
3.	6	1.9×10^{-4}	0.32± 6%	0.35± 6%

There was no statistical difference (t-test) between the two kinds of distribution at the three dosage levels.

6. Aging of deposits

The aging period i.e. time interval between complete evaporation of solvent and addition of test insects should be carefully controlled, especially for more volatile insecticides (11). This also holds for medium votatile compounds. Nexion deposits were submitted to aging periods of one hour and two days at 10^{-4} and 10^{-5} ml concentration levels. The toxic potency of the deposit had decreased significantly (t–test) after two days as the LT_{50} figures showed.

7. Volatility of insecticide deposits

The loss of toxicity during the aging process is mainly due to volatilization.

7.1 Volatilization and corresponding sublimation can also cause contamination of open plates which are put in the hood for drying. Working with DDVP 50 EC dissolved in hexane we obtained erratic results. We then placed plates of a 5×10^{-2} ml concentration among untrerted petri dishes. After an evaporation time of 30 minutes all plates were bioassayad. The LT_{50} figures of the treated plates were below 0.05 hours, while those of the untreated ones ranged from 0.09 to 0.27 hours.

Consequently, the plates were put into a rack with separated compartments for drying period. The rack was connected to a wind tunnel, so that the air drought passed over each plate separately. Contamination of adjacent plates was thus prevented.

7.2 In large scale experiments sometimes not all samples can be bioassayed on the same day. The closed petri dishes are then stored for one day.

Plates which were stored for 1, 3, 6 days were tested against deposits which were prepared just before the testing. Nexion was deposited in the bottom dish at 1.9×10^{-6} ml/plate. Each test was replicated 5 times.

Table 5. Effect of storage on toxicity.

	LT^{50} no storage	LT^{50} storage of	
1	2.42	1 day	1.69
2	2.72	1 day	2.26
3	2.06	3 days	2.38
4	2.40	6 days	4.11

After one day of storage the plates showed slightly higher toxicity in comparison with those plates which were bioassayed right after preparation, while the plates stored for 3 days gave a decrease of toxicity. All differences were not significant (t-test). The toxicity decreased significantly after 6 days (table 5). The evaporating insecticide had sublimated to a large extent on the total inner surface of the container and had thus increased the toxic potential. However, after 6 days enough of the insecticide was able to escape from the petri dishes to signify its disappearance.

7.3 By volatilization and corresponding sublimation, a fraction of the original deposit which is put into the bottom part of the petri dich is redeposited also on the top part of the dish. Some of the gaseous particles escape from the container during the volatilization process, since no pair of petri dishes is tight enough to prevent it. Quantitative figures were assigned to the two effects of volatilization on the bioassay procedure.

The influence of concentration, the original deposit and the period of volatilization was tested on the model insecticide Nexion. Then the volatilization behaviour of a number of insecticides was established.

The insecticide was deposited as an acetone solution in the bottom plate of a petri dich. The concentration of the original deposit is expressed as ml of the respective insecticide formulation per plate. After a drying period of 30 minutes the bottom plates were covered and then kept for a certain volatilization period at room temperature.

At the end of that period the bottom plates were covered by other uncontaminated top plates and vice versa the top plates were covered by frech bottom plates. The 5 replicates were then bioassayed against a just prepared standard series. The toxic potential of the bottom plate (primary deposit) and that of the top plate (secondary deposit) were thus determined separately. The ratio of the two deposits was considered a Sublimstion Index in percent. The difference between the total amount of the two deposits and the original deposit was considered lost during the volatilization process. The loss of toxicant during the drying period is included in the lost fraction, The total loss is recorded in percent.

Volatilization periods of 1 hour, 2 hours and 16-19 hours at various concentration levels were tested first (table 6).

Table 6. Effect of various volatilization periods on toxicity.

Test No.	Original deposit ml/ plate	Recovery in % primary deposit	secondary deposit	Sublimation Index %	Loss %
1. Volatilization period: 1 hour					
1	$5.7x10^{-3}$	63	7	12.1	30
2	$1.9x10^{-3}$	55	3	4.5	42
3	$1.9x10^{-4}$	170	18	10.6	–
4	$1.9x10^{-5}$	140	5	3.6	–
2. Volatilization period: 2 hours					
1	$5.7x10^{-3}$	90	ca.1	0.8	9
2	$5.7x10^{-4}$	70	6	8.6	24
3	$1.9x10^{-5}$	50	14	28.0	36
3. Volatilization period: 16-19 hours.					
1	$5.7x10^{-3}$	67	ca.1	1.0	32
2	$5.7x10^{-4}$	57	6	10.5	37
3	$1.9x10^{-5}$	45	14	31.1	41

The evaporation period of 1 hour yielded erratic results. For the remarkably high recovery data of the lower concentrations no explanation can be offered. This time interval was not suitable for standardisation.

The two other evaporation periods, however, gave coherent results.

After 16–19 hours slightly more toxicant escaped from the containers than after 2 hours. The lower the original deposit was the more insecticide escaped and the higher was the sublimation index.

A number of insectisides were tested under the condition of 16-19 hours evaporation period, because the total recovery after that time was rather uniform for the three tested deposit ranges. The influence of temperature was measured at the 20°C and 45°C level. The original dosages in ml / plate refer to the formulations of the insecticides.

Table 7. Effect of volatilization on the toxicity of the deposit.

Insecticide	Original deposit ml/plate	At t °C	Recovery in % primary deposit	Recovery in % secondary deposit	Subli–mation Index	Loss %
1. Thiodan 35%EC	1.9x10-4	45	61	14	23.0	25
		20	84	16	19.0	0
2. Perfekthion 40%EC	1.9x10-4	45	40	9	35.5	51
		20	45	16	22 5	39
3. Dipterex 80%WP	1.9x10-4	45	46	13	28.3	41
		20	60	9	15.0	31
4. Diazinon 20%EC	1.9x10-4	45	6	4	58.3	90
		20	50	12	24.0	38
5. Nexion 40%EC	1.9x10-4	45	33	5	15.1	62
		20	43	4	10.0	53
6. Lebaycid 50%EC	1.9x10-3	45	65	2	29.1	33
		20	75	1	16.0	24
7. DDVP 50%EC	1 9x10-3	45	0.3	0 05	–	99
		20	50	1	2.4	49
8. Malathion 95% techn.	1.9x10-5	45	70	14	20.0	16
		20	76	15	19.7	9

Unfortunately, different dosages of the various insecticides had to be tested. Since the total recovery data do not differ greatly over the concentration range of 10-3 to 10-5, they can be compared for the insecticides within one temperature range. However, the sublimation index is greatly influenced by the dosage.

It was planned to test all insecticides at the dosage level of 10-4 ml/plate. For different reasons the doses had to be changed. DDVP is so volatile that at that dosage no secondary deposit could be recovered

Of Lindane 20 EC nothing could be recovered at 45°C and at 20°C, the sublimation index was 37.5. On the other hand DDT 25 % EC yielded no secondary deposit at an original deposit of 9.5x10-3 ml/plate.

The influence of temperature on the evaporation of various insecticides is indicated by the recovery data. DDVP, Diazinon, Perfekthion and Dipterex escaped significantly more from the test container at 45°C than at 20°C (t–test); the higher loss at 45°C of the other insecticides was not significant (table 7).

Malathion, Thiodan were more persistant than Lebaycid and Dipterex; they were followed by perfekthion, Nexion and Diazinon; DDVP was by far the least persis-

tant insecticide of all. On the other hand Diazinon and Perfekthion have a much bet- better sublimation capacity than Dipterex, Thiodan and Nexion.

The volatility problem has to be taken care of by strict standardisation of the bioassay procedure. Incidentally, these findings on volatility and sublimation are also valuable for understanding of the insecticide behaviour in the field.

8. Influence of deposit surface on toxicity

The kind of deposit surface and the exposure temperature have been found to be of importance. Nexion 40 EC deposits were tested on surfaces of plastic sheet and glass at 20 and 45°C. The original deposit was in the range of 2 1/ha field dosage.

The deposits degraded much faster on glass than on plastic sheets. After three days the glass surface exhibited almost no toxicity at the 45°C level and only 18% of the original deposit was found at the 20°C level. The toxicity of the deposits on plastic sheet did not change much for the first 6 days, on the 8th day the toxicity had increased about 50%. From then on it decreased gradually to about 20% (20°C) and 5% (45°C) of the original deposit on the 20th day.

9. Summary.

In bioassaying dry film residues one encounters a number of factors which affect the response pattern of the test insect and the behaviour of the deposit. For the sake of accuracy and reproducibility of the biotest method the significance of these factors have to be understood and the method has to be standardized accordingly.

The rearing conditions of the test insect have to be controlled in order to obtain populations of highest possible degree of homogeneity. Despite all efforts the response pattern will always vary to a certain extent. The higher the concentration of the toxicant, the smaller is the variation in response. The high variability of reaction from day to day requires a standard series for each test. Since the sensitivity at low concentrations is sometimes below the accuracy requirements other factors of the biotest procedure must be improved, e.g. choosing of the more susceptible sex, lowering the volatilization loss, adding of oil to the toxicant, introducing a clean-up step in residue analysis to account for the masking effect of the extractives.

Drosophila is very efficient and versatile in assaying a large number of insecticides over the concentration range of micro - and macroassay. There was no sexual difference in the response pattern towards 15 of the 20 tested insecticides. Males were more susceptible to three and less susceptible to two of the 20 insecticides.

A soon as the toxicant solution is poured into the petri dish a number of physico-chemical processes start. First the solvent evaporates accompanied by a small fraction of toxicant. The higher volatile the insecticide compound is , the more of it leaves the petri dish. With the very volatile compounds, contamination of adjacent dishes might occur during the drying period.

The flies in the petri dishes are submitted to contact as well as to fumigant poisoning. They pick up toxicant particles from the dry deposit and at the same time they receive a secondary deposit on their whole body. Due to volatilization, part of the gaseous toxicant sublimates on all parts of the container and on the insect. The redeposited toxicant on the wall of the container enhances the toxic effectiveness. Thus, it makes no difference whether the insecticide is deposited evenly over the whole surface of the container or only over a part of it. More important for the bioassay procedure in the fact that part of the volatilized fraction of the deposit escapes from the container. Nevertheless the containers can be stored for a certain time without significant loss. If this process impaires the sensitivity of the method, the volatilization has to be minimized. The aging period of deposits must be kept to a minimum and has to be equal for both the samples and the standard series . The same applies to the volatialization period; it should be as short as possible in order not to loose much toxicant. Other properties of highly volatile insecticides can be utilized to the same effect, for instance the adsorption on and chromatogrphic separation by filter paper (10).

Temperature also influences the toxicological behaviour of the deposit . And deposit surfaces are not interchangable in one and the same test series as seemed advantageous for a certain experimental design.

Applying strict standardisation to the bioassay procedure the method yielded fair results in residue analysis as recovery data showed. Detremination of residues of Malathion, Nexion and DDVP on cucumber and melon are being carried out. The safe interval between spraying and harvest will be established for these endangered food items because they are sprayed and harvested continuously.

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