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THE USE OF MIXED-AGE CULTURES IN THE MEASUREMENT OF RESPONSE TO PHOSPHINE

R.G. WINKS AND ELISABETH A. HYNE
CSIRO, Division of Entomology, P.O. Box 1700, Canberra, Australia

ABSTRACT

The conventional way to measure the response of insects to pesticides has been to use selected stages with stable tolerance distributions so that the data can be subjected to rigorous statistical analysis using such methods as probit analysis. This approach may have merit when comparing the efficacy of different poisons or when determining changes in tolerance associated with selection for resistance. However, it is usually not suitable for determining dosages likely to be effective in practice.

A more effective method is to use mixed-age cultures and determine the time to population extinction. A major advantage of this method is that it is not necessary to know which stage is the most tolerant; in addition, it allows for changes in tolerance during the exposure periods. However, it is essential both that material used for testing contain all developmental stages and that adequate numbers of each stage are present to ensure a satisfactory degree of repeatability. This paper describes a suitable method for determining the dosages that can be expected to be effective in practice. Data are presented both to show the relative abundance of the different stages with a number of species and strains and to demonstrate the repeatability of the method.

INTRODUCTION

Simple bioassay techniques have been a fundamental tool of toxicologists for a long time. In principle they require only that samples of the test organism be exposed to a graded series of doses of the toxicant or drug and the response observed. These data are primarily used either to establish the relative potency of different drugs or poisons or to determine variations in the response of different populations of the organism. It is implicit in these tests that they are conducted so as to make the results repeatable. For example, there are standard procedures for applying the toxicants or drugs to organisms which are based on sound quantitative principles and techniques. Likewise, it is implicit that the organisms are sampled in such a way that their response can be expected to be repeatable. Clearly, choosing a stage with a stable tolerance distribution is a distinct advantage. For this reason, adults, because of their relatively stable physiology, are usually chosen for bioassay. This

is true for insects, provided that very young and very old individuals are excluded. The tolerance of adults of a strain of *Tribolium castaneum* exposed to phosphine (PH_3) (Table 1) provides a clear example. To achieve a relatively stable tolerance distribution with immature stages, a narrow age range is selected for testing: for example, 2-d-old eggs or 15-d-old larvae. It has been shown in many cases that the tolerance of eggs, larvae and pupae changes dramatically during the development of these stages (e.g. Lindgren and Vincent, 1966; Nakakita and Winks, 1981) and measurements of response that would represent the entire stage, i.e. the full age-spectrum of these stages, would be difficult to achieve as well as relatively meaningless.

TABLE 1
The influence of age on the mortality response of adults of *Tribolium castaneum* exposed to a range of PH_3 concentrations for 6 h at 25°C, 70 % r.h.

	Mortality	
Age (weeks)	LD_{50} (mg h L^{-1})	LD_{99} (mg h L^{-1})
1–3	0.205	0.455
20–22	0.196	0.398
48–50	0.198	0.475

Data from experiments of this kind are usually subjected to a method of analysis which normalises the response, following which a linearising transformation is used to facilitate comparisons. These linearising transformations are commonly probit and logit. These techniques have been effectively used, for example, to establish the dosage variables of fumigants, i.e. the relationship between concentration and time. This approach can be taken a step further and, using the techniques developed by Bliss (1940), examined as a probit plane which embraces all of the information in a family of probit lines. Probit plane analysis yields comprehensive models describing the response of a fumigant, for example, to a wide range of dosage variables (e.g. Winks, 1984).

These techniques also provide useful methods for determining the magnitude of differences in response, such as those induced by selection for resistance, and enable comparisons to be made between species in situations, such as stored products, where pest control must deal with a mixture of different species. However, it is in the area of pest control that the conventional bioassay techniques are of little value. It can be expected that the response of the different stages of insects will vary and that the tolerance of each stage, particularly the pre-adult stages, will change during development. This variability of tolerance has been observed most dramatically in the case of PH_3, where large changes occur between the tolerant egg and pupal stages and the much less tolerant larval and adult stages. In addition, the changes in both the egg and pupal stages as they age are both

rapid and considerable (Lindgren and Vincent, 1966; Nakakita and Winks, 1981; Winks, unpublished data). Clearly, to describe the response of a single stage comprehensively it would be necessary to develop a multifactorial model that would include the variables of concentration, time and age. If the most tolerant stage were known, such a model could yield a measure of the highest dosage needed for a high kill level of this age which might then serve as a guide to the dosages necessary to achieve control of the species in a practical fumigation. While such an approach might be mentally stimulating, the time and effort needed to obtain the data would be prohibitive.

Over the years, the study of insecticides used in agriculture and related fields has been characterised by the frequent assertion that laboratory data are of little value in determining dosages required in the field. This statement is frequently quoted because it is essentially true. It is exceedingly difficult, indeed almost impossible, to simulate field conditions in the laboratory, and even if one could, it is doubtful if the results could be analysed in a meaningful way. It is usually argued that laboratory data simply provide a guide to the relative potency of different insecticides and answer the question of whether tolerance changes have occurred in field populations. The answers concerning practical dosages are usually obtained from the field via screening trials and the like. However, because of their very nature, such data lack the control and precision that can be achieved under laboratory conditions. This is because it is exceedingly difficult, if not impossible, to control such variables as weather conditions, distribution of chemicals and the rate of absorption of the chemicals by the insects (a function of their mobility and the distribution of the toxicant) in such a way that rigorous analysis, like that implicit in probit plane analysis, is possible.

In contrast, the laboratory study of the toxicity of fumigants in grain and similar commodities is much more closely aligned to field usage of these toxicants. A fundamental requirement of fumigation techniques, both in the laboratory and in the field, is that the fumigant be confined within a definable space. Moreover, the range of climatic variation within a field enclosure (such as a silo) is not as extreme as that in a field crop. Within the time frame of a normal fumigation, although there may be some variation within the grain mass, temperatures will remain relatively stable. In addition, the range over which the temperature will vary in a grain mass is small, possibly no more than 10 degrees. In a fumigation enclosure the relative humidity (r.h.) is also relatively stable, and food is present in abundance. Furthermore, the gaseous toxicant moves to the insects and not the reverse as in the case of insecticides, so in most situations, the uptake rate of toxicant is related to the concentration of fumigant in the atmosphere surrounding the insect. The mixing and distribution of fumigant within the atmosphere of the grain is also a factor. Both processes may be accelerated with active distribution techniques (Winks and Russell, 1997). It is therefore possible to develop laboratory experiments that resemble field fumigations. The major limiting factor to this approach is the insects themselves. For a laboratory experiment to be useful in the context of developing field dosages, it clearly must embrace the full range of tolerance of all the species likely to be present. This necessity led to the development of a technique using mixed-age cultures.

Mixed-age cultures have been used in the past as a means of determining response. Many of the experiments have failed because they have not satisfied the requirement of repeatability. The experiments simply took laboratory cultures and exposed them to different dosages of the fumigant. It was assumed that all insect stages were present in sufficient abundance to obtain useful and repeatable data. It would appear that often these assumptions were not well-founded.

The benefits of laboratory techniques using mixed-age cultures are considerable; they can be used to establish dosages effective in the field in a relatively short time for any number of species at selected temperatures, moisture contents (m.c.), etc. To obtain similar data from field experiments would not only require a very long time but would be logistically very difficult. Merely to evaluate the dosage variables of concentration and time would require a large number of silos all of which would need to be infested with adequate numbers of insects of essentially the same species and all the silos would need to be at about the same temperature and m.c. Part of this difficulty has been met by using test insects in cages inserted into the grain, but again, using such techniques to evaluate the efficacy of different combinations of the dosage variables, together with the effects of different temperatures and m.c., is almost impossible. It is therefore clearly questionable whether any of the reported field trials carried out to evaluate the efficacy of PH_3 using application rates of 2 or 4 or some other number of tablets per t are of any real value. Quite apart from its practical significance, the difficulty poses particular problems for fumigant registration authorities who require efficacy data to support product recommendations. The dilemma is due, on the one hand, to the requirement for commercial usage data, and, on the other, to being presented with data that are of very limited value in the context of efficacy.

A far better approach to the question of determining field dosages of a fumigant is to use mixed-aged cultures in controlled laboratory experiments. Clearly, the object of a dosage of fumigant applied in the field is to kill all stages of all species present; similarly, a mixed-age test in the laboratory should be aimed at determining the minimum dosage necessary to kill all stages of the insect species or strain under test. However, to obtain meaningful data from laboratory experiments it is essential that a number of criteria be met. Firstly, all stages must be present. Secondly, there must be an adequate number of each of the stages and particularly of the more tolerant stages. Thirdly, the method of assessment must include the full range of possible development times. Given these principles, one distinct advantage of a mixed-age culture method is that no prior information is needed concerning either the tolerance of any particular stage or the rate at which the tolerance of the various stages changes.

DETAILS OF THE METHOD

Preparation of mixed age cultures

Mixed-age cultures were established by placing 300 adults on about 1,000 g of medium in 2-L culture jars. The medium was chosen to suit the particular species. However, for species that would normally be reared in a flour-based medium, this was replaced with

one containing wheat plus broken wheat so that air flows could be achieved without difficulty during the dosing phase of the experiment. Adults were left in the culture so that there would be a continuation of egg laying, at least until the cultures were used. The cultures were selected for use at a time when it was expected that F1 adults would be starting to emerge and could be observed. At this point, all stages should have been present including teneral adults. On a few occasions cultures of the same strain, established at a later date, were added to ensure adequate numbers.

During the development of the technique the relative abundance of each stage was determined prior to the start of each test. In addition, the size of the total population was determined. To ensure the composition of cultures of *Sitophilus* spp. and *Rhyzopertha dominica* prior to use, samples of the culture medium were taken and X-rayed, following, in essence, the method described by Katz *et al.*, 1950 and Milner *et al.*, 1950. The abundance of weevil eggs was occasionally determined during the development of the method by staining egg plugs with acid fuchsin. Egg numbers of other species were checked by visual examination under a stereo microscope of samples of medium taken from the cultures. These initial checks confirmed that adequate numbers of eggs could be expected so with subsequent use of the method, checks for eggs were not continued on a regular basis.

The number of pupae was checked more regularly using the X-ray technique for *Sitophilus* spp. and for *R. dominica* and by visual examination of samples of culture medium for other species. Greater emphasis was given to determining the number of pupae since, in the case of PH_3, the pupae are frequently the most tolerant stage, and even in those cases where eggs are more tolerant, the difference is small. The estimates of numbers derived from X-ray analysis were based on the number of grains observed to contain pupae, for example, compared with the total number of grains on the X-ray plate. To facilitate counts, the sample of culture medium (grains of wheat) to be X-rayed was spread out on a metal grid attached to the envelope containing the photographic film. The data obtained in this way for one strain of *S. granarius* is given in Table 2.

Dosing technique

The cultures were dosed in the apparatus shown in Fig. 1. This apparatus consists of a number of chambers through which a constant concentration of PH_3 is passed. The concentration was prepared by accurate dilution of a measured source of PH_3 in nitrogen with air through a Brooks' Mass Flow Controller. The air was 'purified' by passing it through an activated charcoal filter inserted in the inlet to the diaphragm pump. The air was drawn from outside the laboratory to avoid laboratory 'odours'.

The r.h. of the air/PH_3 mixture was achieved by saturating the gas mixture with water at a lower temperature, determined from psychrometric data, a temperature that yielded the required r.h. when the air/PH_3 mixture was returned to the test temperature. This method achieves a reliable and constant r.h. For example to obtain an r.h. of 57%, the equilibrium r.h. for 12% m.c. in Australian wheat at 25°C (Gay, 1946), the air/gas mixture should be saturated with water at 15°C.

TABLE 2

Estimate of number of eggs and pupae in mixed age cultures of *Sitophilus granarius*, strain SG_4, used in experiments to determine times to population extinction when exposed to constant concentrations of PH_3

Date of culture set-up	Experiment began	No. of grains per culture	Grains on X-ray	No. of eggs in sample	Estimated no. of eggs in culture	No. of pupae on X-ray	Estimated no. of pupae in culture
10/01/86	28/02/86	ca. 43000	826	107	5570	26	1354
28/04/92	08/06/92	ca. 43000	355	N/A	N/A	13	1575
23/08 and 11/10/85	05/11/85	ca. 43000	937	163	7480	10	459
23/11 and 07/12/84	08/01/85	ca. 43000	660	63	4105	22	1433
15/11/85	10/01/86	ca. 43000	780	72	3969	11	606
28/12/84 and 25/1/85	26/02/85	ca. 43000	459	116	10867	11	1031
22/06/90	03/08/90	ca. 43000	464	38	3522	61	5653
15/06/84	24/07/84	ca. 43000	1133	41	1556	50	1898
02/05/86	27/05/86	ca. 43000	699	150	9227	11	677
10/01/86	28/02/86	ca. 43000	826	107	5570	26	1354
27/07/84	11/09/84	ca. 43000	560	60	4607	82	6296
23/07/90	03/09/90	ca. 43000	282	45	6862	28	4270
26/06/85	02/09/85	ca. 43000	863	62	3089	24	1196

N/A = not assessed.

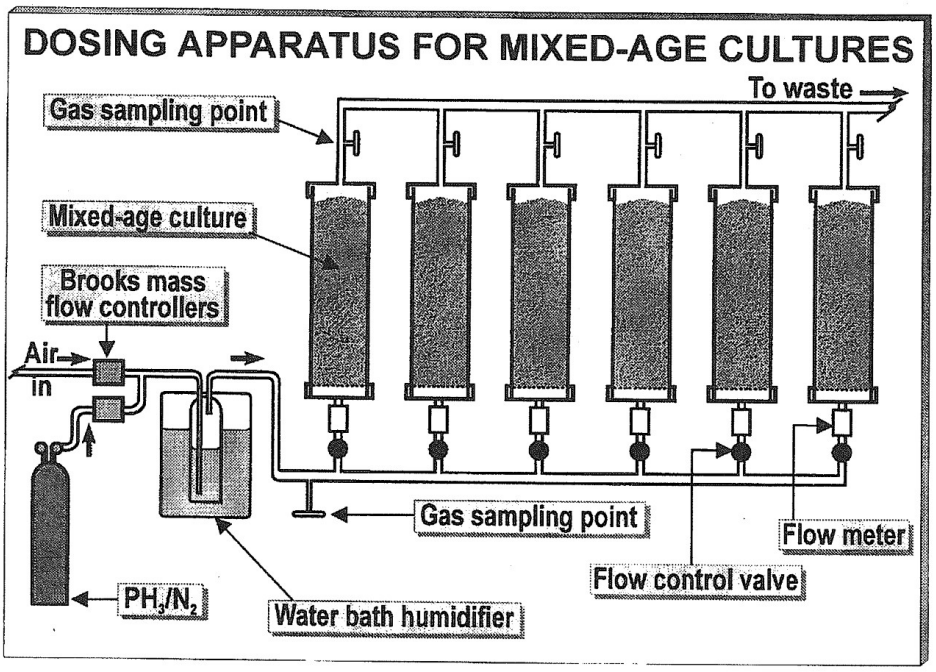

Fig. 1. Flow-through fumigation apparatus used for dosing mixed-age cultures of stored product pests with constant concentrations of PH_3.

The chambers were arranged in a bank and the flow to each controlled with a needle valve and monitored with an appropriate flow meter. Samples to check the concentration of PH_3 were drawn from the apparatus using a gas chromatographic technique based on the calibrated response of a flame photometric detector. At selected intervals (treatment times) the PH_3 flow to all chambers was stopped and samples of the culture medium taken for incubation. The samples were drawn by gently mixing the contents of the chamber, after which the required volume of culture medium was poured into a culture jar and sealed with a filter paper waxed to the rim of the jar.

Assessment of response

The samples were assessed for the presence of live adults as soon as they were drawn from the cultures, following which they were incubated and examined at approximately 8 weeks and 16 weeks. Observations at two intervals are not essential, but if only one interval is chosen, it must be long enough to ensure that all possible survivors have completed their development with allowance made for possible PH_3-induced delays. Observations at 8 weeks enabled decisions to be made concerning appropriate dosing times for subsequent experiments to be started before the full incubation period for the current experiment had elapsed.

The object of the method was to determine the minimum treatment time at a given concentration that would achieve complete kill of all stages present. This time provided an estimate of the *time to population extinction* (Winks and Hyne, 1994) and was determined from the first dosage interval that produced no survival (emergence), only provided that at least one subsequent sample or dosing interval also yielded no survival. Table 3 gives the response assessment in such a test. The data were examined graphically in the form of split-column plots (Fig. 2). The tops of the light or upper sections of the columns show the exposure time of the first sample from which no survivors were recorded (*time to population extinction*) and the tops of the dark or bottom sections of the columns show the exposure time of the previous sample from which survival was recorded.

In the early stages of developing the technique, the level of CO_2 in the effluent from the test chambers was considered. It was thought that when insect respiration ceased and the level of CO_2 in the effluent dropped to ambient levels, this would indicate population extinction, thus providing an indicator which would give a reasonable estimate of time to extinction. The CO_2 data obtained is provided in Fig. 3. It may be seen that there was poor correlation between the CO_2 levels and times to extinction from

TABLE 3

An example of the data recorded to determine times to population extinction of strains exposed to constant concentrations of fumigant in mixed-age culture experiments

	Exposure time (d)					
Strain	2	4	7	14	21	28
S. oryzae strain LS2						
No. survivors at end of treatment	194	0	0	0	**0**	0
No. emerged at 8 weeks	ca. 1000	ca. 1000	ca. 300	1	**0**	0
No. emerged at 16 weeks	N/A	N/A	N/A	0	**0**	0
S. granarius strain SG4						
No. survivors at end of treatment	190	0	0	**0**	0	0
No. emerged at 8 weeks	ca. 1000	ca. 500	32	**0**	0	0
No. emerged at 16 weeks	N/A	N/A	ca. 500	**0**	0	0
S. granarius strain SG46						
No. survivors at end of treatment	67	0	0	**0**	0	0
No. emerged at 8 weeks	ca. 1000	272	63	**0**	0	0
No. emerged at 16 weeks	N/A	N/A	N/A	**0**	0	0
S. zeamais strain SZM9						
No. survivors at end of treatment	119	0	0	0	**0**	0
No. emerged at 8 weeks	ca. 1000	ca. 1000	ca. 300	112	**0**	0
No. emerged at 16 weeks	N/A	N/A	N/A	N/A	**0**	0

N/A = not assessed. Columns with boldface numbers denote observed times to extinction for each strain. Test insects: *Sitophilus* spp. Experiment started on 4 August 1992. PH_3 concentration: 0.015 mg L^{-1}. Temperature: 25°C.

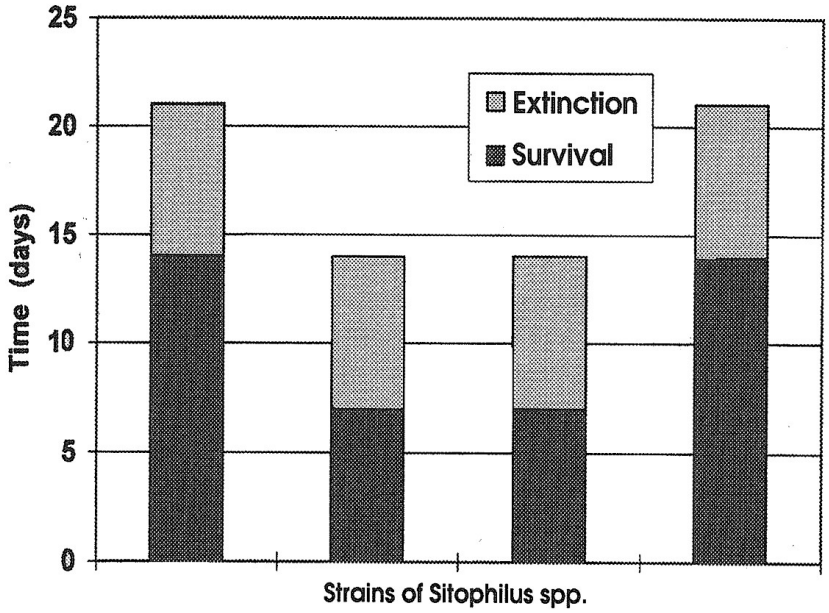

Fig. 2. Graphical presentation of data from mixed-age culture experiments showing the time at which extinction was recorded (top of the upper portion) and the previous sample time from which survival was observed (top of the bottom portion).

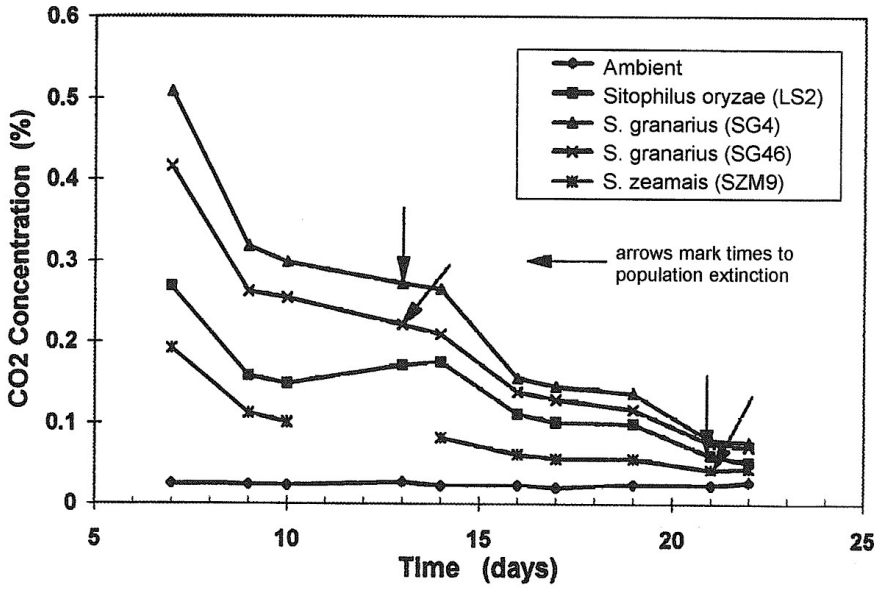

Fig. 3. Decay of CO_2 concentrations in effluent air from mixed-age cultures of *Sitophilus* spp. exposed to 15 g/L PH_3 at 25°C, 60% r.h.

incubated samples, possibly due to metabolic activity of fungi in the cultures. It was also difficult to discriminate between low levels of CO_2 in the effluent produced by one or two insects and the normal variation in atmospheric levels. The CO_2 level was thus rejected and incubated samples adopted as the basis for determining times to population extinction.

EVALUATION OF THE METHOD

The data in Table 2, as well as showing the relative abundance of the different stages of *Sitophilus granarius* in a series of different experiments, also provide a measure of the repeatability of the mixed-age culture technique. The data span a period of 8 years and are the result of the work of two to three laboratory technicians. The variation evident in the number of pupae was not considered excessive, and it may be seen that for each of the tests in question a sufficient number of pupae was present. Normally no more than six sub-samples were drawn from each culture (i.e. six dosage times). Therefore, from these data, approximately 80 to 1,000 pupae were expected in each sub-sample. This should have been sufficient to provide a reasonable indication of the time to extinction, assuming pupae to be the most tolerant stage. The same argument would hold if eggs were the most tolerant stage. A key aspect of the technique is the presence of a sufficient number of the most tolerant stage at the beginning or during the test to ensure a reasonable expectation that the result obtained is derived from the most tolerant age or stage. Clearly, the dosage times assumed and allowed for development during exposure to PH_3. This is exactly analogous to practical fumigations. Examples of the use of this technique are described by Winks and Hyne (1994, 1997). Winks and Hyne (1994) describe the use of this mixed-age culture technique to determine resistance factors in strains of *R. dominica* that were clearly more closely aligned with the difficulties of controlling such strains in the field than other factors derived from tests on adults.

A key question with this technique is the level of repeatability that can be achieved in estimating the time to population extinction. Figure 4 provides observed times to population extinction, together with the previous dosage time from which survival was recorded, for a resistant strain of *R. dominica* based on a number of experiments at different concentrations. The systematic decrease in times to population extinction with increasing concentration, together with the comparison of times at each of 0.015, 0.030 and 0.050 mg L^{-1} provide evidence that a reasonable level of repeatability can be achieved with this technique. It is significant that the experiments from which these data were extracted were conducted over a period of 4 years.

It is logistically difficult to obtain a large number of sub-samples at different treatment times. Thus the time between the previous sample from which survival was obtained and that from a later one from which no survival was obtained is frequently longer than is desirable. It follows that a precise estimate of the time to population extinction would be earlier than that recorded. This may be seen in the data for 0.03 mg L^{-1} in Fig. 4. Experiments have been repeated with different sample intervals to obtain closer estimates,

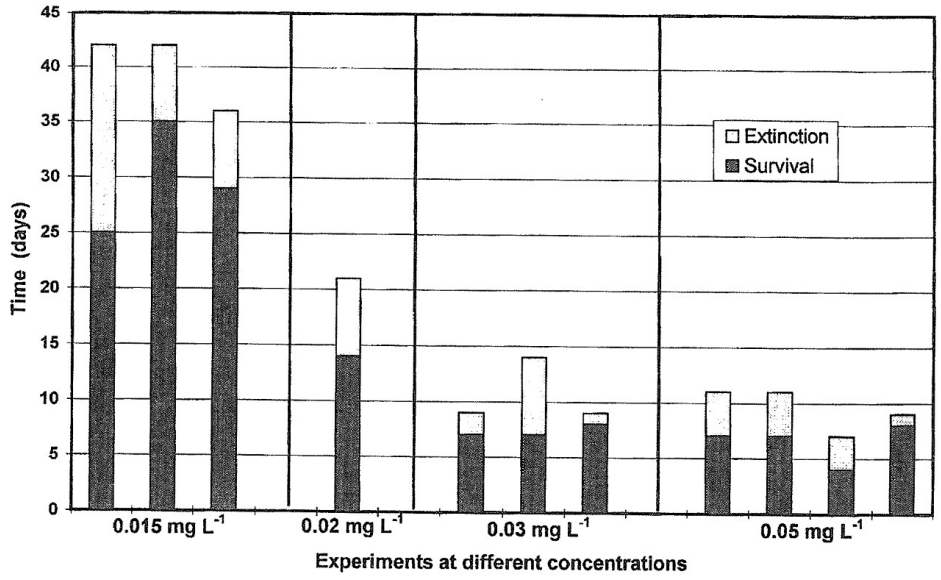

Fig. 4. Repeatability of times to population extinction of a Group 1 strain of *Rhyzopertha dominica* (RD316) exposed to constant concentrations of PH_3 at 25°C, 60% r.h.

but since these experiments can take up to 6 months to complete, obtaining such estimates is a protracted process.

This method has been used as the basis for determining minimum concentrations required for different exposure periods to control the range of pests likely to be found in Australian grain. These minimum concentrations (Table 4) have been submitted for incorporation in the revised guidelines for registration of PH_3-generating products issued by the Australian National Registration Authority.

CONCLUSION

Although experiments using the mixed-age culture technique require a longer period of time before results are available than do other methods based on selected stages with stable tolerance distributions, the data obtained from mixed-age cultures for PH_3 have enabled recommendations to be made for fumigations in the field and have thus been far more useful. Moreover, as described by Winks and Hyne (1994), this method also provides a far more meaningful measure of resistance to PH_3 than do those methods based on adults.

The most effective validation of the technique has been in the use of data derived in this way in practical control situations. These data have formed the basis of the recommendations for the use of PH_3 in general and SIROFLO® in particular in Australia for at

TABLE 4
Alternative minimum PH_3 concentrations in g m^{-3} with ppm in brackets at various dosage times to achieve complete control of infestations of a range of insect species

Group	Species	Temperature (°C)	Time (d) 5	7	10	14	21
1	*Sitophilus* spp., *Lasioderma serricorne*, *Trogoderma variabile*, *Dermestes* spp., Acarina (mites) and strains of Group 2 insects with low level of PH_3 resistance	>20	NA[1]	1.0 (700)	0.3 (200)	0.05 (35)	0.03 (20)
		15–20	NA	NA	NA	0.15 (100)	0.10 (70)
2	Susceptible *R. dominica* and *Tribolium* spp., *Oryzaephilus* spp., *Cryptolestes* spp., *Callosobruchus* spp., *Acanthoscelides obtectus*, *Palorus subdepressus*, *Carpophilus* spp., *Typhaea stercorea*, *Gnathocerus* spp., Ptinidae, Lepidoptera, Psocids	>20	0.15 (100)	0.04 (30)	0.035 (25)	0.03 (20)	0.015 (10)
		15–20	NA	0.15 (100)	0.07 (50)	0.05 (35)	0.02 (15)
3	*Bruchus pisorum* (pea weevil)	>20	NA	NA	NA	NA	0.04 (30)
		15–20	NA	NA	NA	NA	0.1 (70)

[1]NA means not applicable.

least the last 8 years. SIROFLO® has been widely adopted by most of Australia's Grain Handling Authorities and is currently used to treat approximately 8 million t of grain annually. It is the predominant fumigation method used in vertical silos in Australia and is now being extended into control in sheds. SIROFLO® has enabled Australia's grain industry to continue to meet the requirement for nil tolerance of insects in both domestic and export grain. Throughout this period PH_3 concentrations for use in SIROFLO® have been derived entirely from laboratory experiments using the mixed-age culture technique.

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