THE TOXICOLOGY OF COMMERCIAL FORMULATION OF BACILLUS THURINGIENSIS BERLINER TO JAPANESE QUAIL AND HOUSE FLY LARVAE

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This is to certify that the

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THE TOXICOLOGY OF COMMERCIAL FORMULATIONS OF BACILLUS THURINGIENSIS BERLINER TO JAPANESE QUAIL AND HOUSE FLY LARVAE

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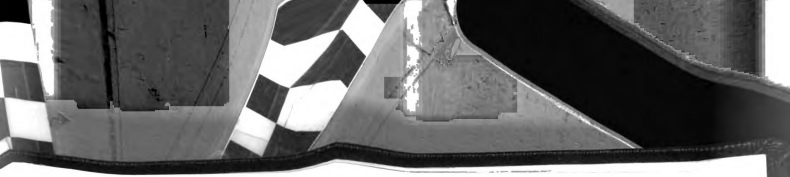

ABSTRACT

THE TOXICOLOGY OF COMMERCIAL FORMULATION OF BACILLUS THURINGIENSIS BERLINER TO JAPANESE QUAIL AND HOUSE FLY LARVAE

by Alfred L. Borgatti

The toxicology of commercial formulations of Bacillus thuringiensis Berliner was studied by feeding varied amounts of four formulations to Japanese quail (Coturnix coturnix japonica Tem. and Schl.) as a part of their normal diet. The effectiveness of the spore formulations which passed through the digestive tracts of the birds was evaluated by bioassay tests using four- to five-day old house fly larvae in the droppings. These data were correlated with the median lethal spore dose for the larvae.

Because of relatively high insecticidal contamination of two of the formulations tested and the resulting mortality to the quail, additional tests were undertaken to determine the amounts of insecticides present and their effect on the spores and on the control of house fly larvae.

A systematic treatment of the bacteria in each formulation confirmed the identity of the spores as Bacillus thuringiensis Berliner. Only slight variation was observed in the fermentation reactions. No bacterial contaminants were found which could be implicated as being responsible for mortality to the test animals.

The spores of B. thuringiensis were found to have no adverse

effect on the normal metabolic activity of quail or white mice. In quail feeding studies effective larval house fly control of 70% to 85% was achieved at feeding levels of 5.4×10^9 to 9.3×10^9 spores per bird per day. At spore concentrations in the formulations of 45 to 70 billion spores per gram, these levels were equivalent to feeding rates of 3.5 to 7 grams of formulation per pound of food. A comparison of these results with the median effective dose for the materials indicated that apparently relatively few spores failed to pass through the animals in a viable state, assuming that all the spores were originally viable.

Contamination of Agritrol with approximately 1000 ppm of DDT and 200 ppm of aldrin did not appear to affect the spore viability or influence the resulting mortality to house fly larvae. The median effective dose of Agritrol was found to be 1.6×10^9 spores per larva. Compared to the ED_{50} of Pure Spore of 2.3×10^9 spores per larva, there appeared to be no interaction between insecticidal contamination and spore activity to house fly larvae. The major effect of the insecticidal contamination appeared to be associated with the metabolic activity of the quail and mice, causing consistant mortality at the higher feeding levels.

At high feeding levels of Agritrol, a delay was encountered in the expression of control of adult fly emergence for two weeks after feeding was begun. The reason for this delay was not evident.

It was found that holding spores in droppings at -18° C. for one year did not seriously affect their viability when thawed and used as growth medium for house fly larvae.

Addition of at least 25% by weight of moisture to droppings

appeared to be effective in enhancing spore pathogenicity to house fly larvae. There was a consistant increase in effectiveness of the spores for control of house fly larvae with increases in moisture content of the droppings.

THE TOXICOLOGY OF COMMERCIAL FORMULATION OF *BACILLUS THURINGIENSIS* BERLINER TO JAPANESE QUAIL AND HOUSE FLY LARVAE

By

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INTRODUCTION

Bacillus thuringiensis Berliner was first isolated and described, in Germany, from sick and dying larvae of the Mediterranean flour moth, Ephestia (= Anagasta) kuhniella (Zeller), by Ernst Berliner (1915a). The isolated bacterium was described as a slightly motile, compact, straight, spore-forming bacillus with rounded corners and firm membrane, easily stained with all common aniline dyes, and Gram positive. Its length was reported as approximately 5 u, its thickness approximately 1 to 8 u, with chains of 3 to 4 cells and seldom of filament length. The spore stage was described by Berliner as being 2 u long and 1 u wide, with the spore occupying one end of the sporangium and a "Restkorper" or "residual body" at the opposite end. The "Restkorper" was seen to remain clinging to the spore upon its release from the sporangium.

This bacterium was not exploited as an agent for insect control until members of the International Corn Borer Institute reported it to be effective against the European corn borer, Pyrausta nubilalis (Hubner) (Husz 1928, 1929; Metalnikov and Chorine, 1929). Their reports prompted field study with laboratory preparations of spores but cultural practices for production of large quantities of spores were not available.

Interest declined, however, in the use of this and other bacterial pathogens for control of insects because of failure to be able to duplicate earlier studies and because of the disappointing

results in varied field trials using untested and uncontrolled samples of laboratory preparations. With the development of DDT and other effective insecticides, attempts to colonize bacterial populations or to treat foliage with bacterial preparations, became all but forgotten. However, with the appearance of insect resistance to DDT and the organo-phosphates as well as new reports of control of the white grub with a preparation of Bacillus popilliae (Dutky), research was again undertaken with B. thuringiensis and its close relatives with renewed vigor. Several diagnoses of Bacillaceae infections in a wide variety of insect species, especially the action of B. thuringiensis against the alfalfa caterpillar were reported by Steinhaus (1951a, 1951b). Others (Burgerjon and Klinger, 1959; De and Konar, 1955; Hall, 1954, 1955; Hall and Dunn, 1958; McConnell and Cutkomp, 1954; Rabb, et al., 1957; Steinhaus and Bell, 1953; Tanada, 1953; and Vasiljevic, 1957) reported good to poor results with the bacillus against the tobacco hornworm, cabbage looper, imported cabbage worm, Khapra beetle, Tortrix viridana (L.), and the salt-marsh caterpillar. The reader is referred to the excellent reports by Steinhaus (1956) and Tanada (1959) for a more detailed review.

The development of large quantities of dried spore preparations of B. thuringiensis from commercial pilot-plant operations in 1957 and 1958 prompted many investigators to attempt field studies with the bacteria in dust and wettable powder formulations. Due to a lack of knowledge as to the behavior of the bacillus under field conditions, and a lack of preliminary laboratory data on effective dosage levels, many investigators became discouraged with the results as compared with conventional chemical applications.

Preliminary tests with the bacillus at Michigan State University in 1958 gave evidence that B. thuringiensis might be effective in controlling larval house fly populations. It was postulated that it might be possible for these spores to be fed to animals, pass through their digestive tracts unchanged, and by their presence in the feces, be effective in reducing larval house fly populations.

Therefore this study was undertaken to investigate: (1) the effectiveness of B. thuringiensis against house fly larvae when passed through the digestive tracts of animals, (2) the effect of the bacterial spores on the metabolism of animals and the possibility of spore germination in the intestine of animals, (3) the relative stability of spores at varying moisture levels, and (4) the comparative effectiveness of the spores when incorporated in feces or when applied as a dust to the surface of droppings.

Due to the discovery of contamination of certain of the B. thuringiensis samples tested, and to the fear, expressed by many, that this bacterium, being so closely related to Bacillus anthracis Cohn, might prove pathogenic to vertebrates under certain conditions (Steinhaus, 1959), the reaction of a vertebrate species to a combination of a chlorinated hydrocarbon insecticide and spores was investigated. It was felt that this study might give further elucidation to the natural relationship between bacteria and insecticides in the field.

The birds used in this study were from a stock of Japanese quail (Coturnix coturnix japonica, Temenick and Schlegel) established at Michigan State University by David L. Cross (1960). These birds made an extremely valuable tool for growth and nutrition studies

because of their resistance to disease, ease of handling, small size, and rapid maturation. A test of three months covered all stages from a young rapidly-growing bird to a mature, reproductive adult. In a two and one-half year period of rearing and testing these birds, no outbreak of disease occurred even though no antibiotics or other prophylactic measures were administered, except for the normal antibiotics present in commercial feeds.

THE SYSTEMATIC TREATMENT OF THE COMMERCIAL PREPARATIONS USED IN THESE STUDIES

Berliner (1915b) described the position of the spore in the sporangium and other morphological variations of B. thuringiensis as the basis of giving this organism a separate species designation. This description was confirmed by Chorine (1929), Smith et al. (1952), and later by Heimpel and Angus (1958). The latter two papers reappraised the earlier works in an effort to establish a definite taxonomic position for this species as well as for other bacillus species pathogenic to insects.

Smith et al. (1952), on the basis of the oblique position of the spore in the sporangium, placed B. thuringiensis as a variety of Bacillus cereus Frankland and Frankland since all strains tested were otherwise identical with B. cereus.

Heimpel and Angus (1958) prefer to place all bacillus species conforming to the B. cereus pattern but being pathogenic to insects as a distinct species, B. thuringiensis. They further propose to designate as varieties of this species, those insect-pathogenic bacilli which do not contain a parasporal crystal. This crystal, or "Restkorper" was described by Berliner as a curiosity but Hannay (1953) reappraised its importance and attributed it to be the causal agent of pathogenicity of the bacillus. Earlier workers had completely ignored the inclusion bodies as a morphological tool for separation of this group of bacteria from the B. cereus group and this accounts for the confusion as to its

proper taxonomic position. For the purposes of this paper, the name B. thuringiensis will be used to designate the variety of B. cereus, as specified by Smith et al. (1952), which is pathogenic to insects.

The question remained of the possibility of this bacillus reverting to a pathogenic variety of B. cereus as is the problem, at least taxonomically, with Bacillus anthracis Cohn. Because of the number of deaths in initial tests on quail with certain of the commercial formulations it became a matter of prime concern to subject all of the formulations received in 1959 to a complete systematic treatment in order to confirm the identity of the organism, or possibly organisms, present in each sample. The suppliers of all formulations tested received their original culture of B. thuringiensis from the culture maintained by Dr. E.A. Steinhaus at the University of California.

Replicated tests were set up for each material using the media recommended by Smith et al. (1952). The wettable powder formulations (except one material which was a bran formulation) were suspended in sterile distilled water and pasteurized at 60° C. for 30 minutes to destroy any non-spore contaminants adhering to the materials, and these suspensions were then used to inoculate nutrient agar plates. All further inoculations and transfers were made from single colony isolates from these plates. All tests were incubated at 28° C. unless otherwise indicated.

The morphology of these cultures generally conformed to the following:

1. Vegetative cells: short, relatively thick, Gram positive rods.

2. Spores: subterminal, oval, lying obliquely in the sporangia.
3. Sporangia: oval, not swollen. Parasporal crystals were present but not all sporangia contained crystals.
4. Nutrient agar colonies: large, flat, dry, white, rhizoidal, opaque with irregular margins.
5. Glucose nutrient agar colonies: essentially the same appearance as on nutrient agar but the texture of the colonies was more moist, softer, and somewhat glistening. On light staining with methylene blue (1%), the cells appeared vacuolated.
6. Nutrient agar slant: abundant, echinulate, spreading, dull, butyrous growth.
7. Glucose nitrate agar: very scanty growth in 72 hours.
8. Nutrient broth: heavy pellicle formed which was easily disrupted and sank quickly. The broth was clear and only a scant sediment was present in the bottoms of the tubes.
9. Gelatin stab: stratiform liquifaction in 24 hours.
10. Indole production: negative(at 32° C.)
11. Methyl red test: negative
12. Voges-Proskauer (acetylmethylcarbinol) test: positive (negative for Agritrol) (at 32° C.).
13. Growth in sodium citrate: negative
14. Hydrolysis of starch: complete
15. Fermentation studies: A complete tabular presentation is given in Table 1. Acid but no gas production was

Table 1. Fermentation reactions with B. thuringiensis using $(NH_4)^+$ as a source of nitrogen.

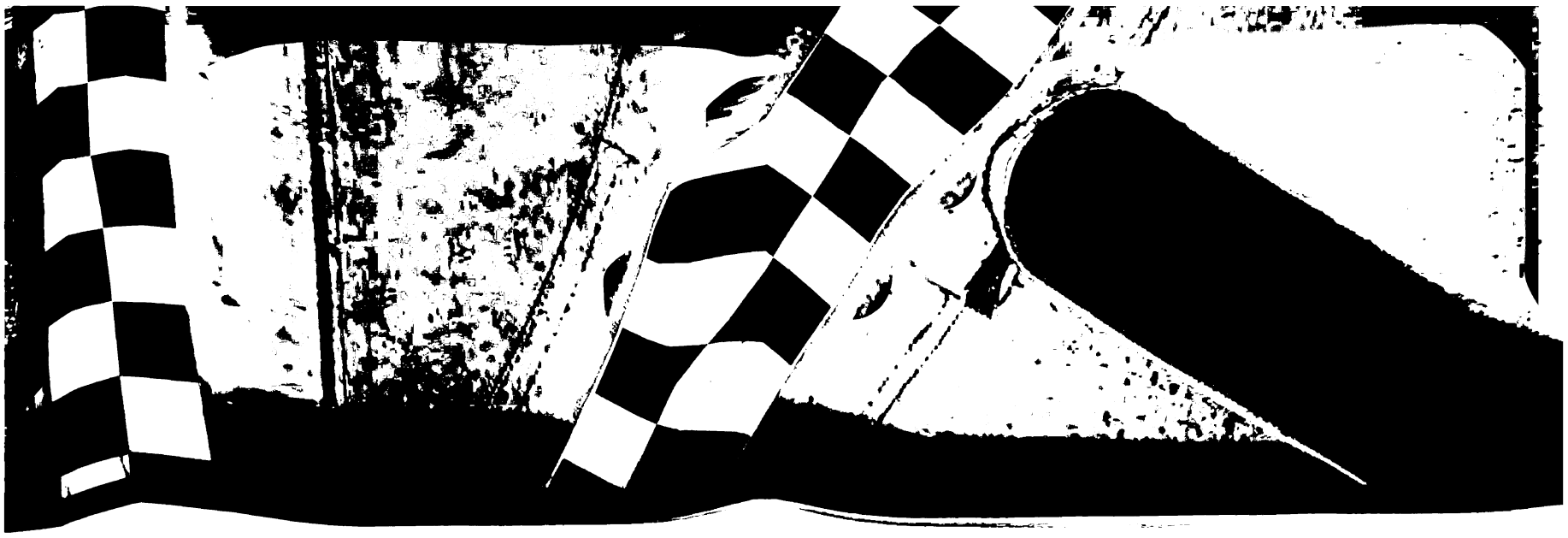
Source	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Lactose	Sucrose	Trehalose	Raffinose	Inulin	Glycerol	Adonitol	Mannitol	Sorbitol	Dulcitol	Inositol	Salicin
Pure Spore	N[a]	N	N	A	A	N	A	A	N	N	A	N	N	N	N	N	A
Thuricide (1)	N	N	N	A	A	N	A	A	N	N	A	N	N	N	N	N	A
Biotrol (2)	N	N	N	A	A	N	A	A	N	N	A	N	N	N	N	N	A
Agritrol (3)	N	N	N	A	A	N	A	A	N	N	A	N	N	N	N	N	A
Bakthane (4)	AG	N	N	AG	A	N	A	A	N	N	A	N	N	N	N	N	A

[a]) N = Negative; A = Acid; G = Gas
1) Stauffer Chemical Co.
2) Nutrilite Products, Inc.
3) Merck & Co.
4) Rohm and Haas Co.

present in glucose, fructose, sucrose, trehalose, glycerol, and salicin. Bakthane also produced acid plus gas in arabinose and gas in glucose.

The position of the spores in the sporangium and other morphological observations were made using a technique suggested by Angus (1959). The spores were placed on a coverslip, mixed with a background stain of 10% nigrosin, and allowed to dry. The coverslip was then inverted onto a paraffin frame affixed to a glass slide. This provided an air space between the slide and the suspension for better definition under the phase contrast microscope.

The reactions, of the organisms examined, to these tests indicate that the organism in every case was B. thuringiensis. The one exception might be the Agritrol material since the Voges-Proskauer test was negative, but all other reactions were identical to the reactions for B. thuringiensis. This reaction may, therefore, be a variable reaction with this test, since it is a rather delicate test and had to be repeated on all cultures in order to obtain definite results for all replications. One contaminant was found, a Sarcina sp., which was thought to have been an aerial entry into one of the original stock cultures.



GENERAL FEEDING STUDIES WITH QUAIL

Chronic tests

The test birds were reared and maintained from a stock group, in a quonset building on the Michigan State University Campus. Provisions for incubation and hatching of the eggs were furnished by the Poultry Department at the University.

Food for the quail consisted of two different formulations of commercially pre-mixed "crumbles" rations. Chicks, aged 1-14 days, were fed a starter ration consisting of 26% crude protein (min.), 3.5% crude fat (min.), and 6% crude fiber (max.). From age 14-20 days the chicks were fed a mixed ration of 50% starter and 50% grower mixes. Grower ration was fed to all birds after 20 days of age. The grower ration consisted of 20% protein, 3% fat, and 7% fiber. The reader is referred to Cross (1960) for further information concerning feed and rearing.

The first series of tests were chronic feeding studies of each commercially produced wettable powder formulation of B. thuringiensis. These tests were initiated to evaluate the reaction of the birds' metabolisms to low, moderate, and excessive dosages. Since each product had a different spore concentration due to considerably different spore-counting techniques, it seemed advisable to compare each product for its over-all effects. The materials were mixed with the food in 5 lb. lots and kept in covered 5 gallon metal containers.

Test birds were kept by mated pairs in pens consisting of five

[redacted]

compartments each one foot on a side and screened on two sides and top with one-half inch mesh hardware cloth. Each compartment was separated from the next by a one-half inch plywood partition. The tops of each 5-compartment section were covered by a removable plastic cover which tended to prevent restlessness and the passage of wastes from upper to lower cages. Bird wastes were collected in cafeteria-type trays placed beneath each compartment. Food and water were available to the birds at all times. The food was kept in individual plastic feeders within the cages, and the water in metal trays in front of the cages. Twenty-four hour lighting was provided for all birds. The average daily temperature was approximately 76° F. and the average relative humidity was approximately 25%, with considerable variation in the summer due to the influence of the sun on the metal roof of the building.

Chronic feeding studies were run for ten weeks starting with four replications of 20-day old birds to include all phases of rapidly metabolizing youth, reproductive stage, and full maturity. In this way it was possible to detect any abnormal reaction of the animals to the presence of spores. Data were taken each week on weight gain, amount of food consumed, and the general behavior of the birds. During the egg-laying period the eggs were collected, marked, weighed, rotated daily, and stored at a temperature of 20° C. in a ventilated refrigerator. Each week's egg collection was incubated at 99° F., with a relative humidity of 9?%, in Single Stage James (252B) incubators equipped with two-hour automatic rotators, and the percent hatch was recorded for each cage.

At each two-week interval, the weight of food spilled was

compartments each one foot on a side and screened on two sides and top with one-half inch mesh hardware cloth. Each compartment was separated from the next by a one-half inch plywood partition. The tops of each 5-compartment section were covered by a removable plastic cover which tended to prevent restlessness and the passage of wastes from upper to lower cages. Bird wastes were collected in cafeteria-type trays placed beneath each compartment. Food and water were available to the birds at all times. The food was kept in individual plastic feeders within the cages, and the water in metal trays in front of the cages. Twenty-four hour lighting was provided for all birds. The average daily temperature was approximately 76° F. and the average relative humidity was approximately 25%, with considerable variation in the summer due to the influence of the sun on the metal roof of the building.

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At each two-week interval, the weight of food spilled was

recorded by comparison with similar volumes of food previously weighed. This was the only practical method available since the food and droppings were of approximately the same size and could not be separated by sieves. A comparison of all tests gave comparable results and it was felt that little error was introduced by this method. As much waste food as possible was removed from the trays and the droppings were then collected, and stored in one pint ice cream containers for bioassay tests with house fly larvae.

The formulations in these tests were Thuricide Wettable Powder containing 42 x 10^9 spores/gram (Stauffer Chemical Co.); Biotrol Feed Additive (formerly Larvatrol) containing 10 x 10^9 spores/gram (Nutrilite Products, Inc.); Agritrol Wettable Powder containing 70 x 10^9 spores/gram (Merck and Co.); and Bakthane Wettable Powder containing 25 x 10^9 spores/gram (Rohm and Haas Co.).

A preliminary test with Agritrol, at dosages of 10, 15, 20 and 25 grams of wettable powder per pound of food (Table 2), produced mortality in the three highest dosage levels with death preceded by symptoms of extreme nervousness and convulsive attacks. This mortality was unexpected since an earlier trial feeding on a young white leghorn chicken gave no indication of such symptoms. A comparison test with Thuricide, using the highest rate of 25 grams/pound of food, was not significantly different from the control group.

One possible explanation of this difference in effect was thought to be the result of feeding high levels of bacteria to the birds. A comparison test, again with Thuricide, at an equivalent spore dosage rate amounting to 42 grams of Thuricide per pound of food (= 17.5 x 10^{11} spores/lb. of food) produced no significant differences in weight gain or food consumption from the control birds (Table 3).

Table 2. Mortality of quail fed high dosages of Agritrol wettable Powder for 56 days.

Treatment Level (Gms./lb. of food)	Mean Percent Mortality And Lethal Time			
	Males		Females	
	%	Days	%	Days
0	0	56	0	56
10	50	38	75	51
15	100	24	100	30
20	100	24	100	21
25	100	22	100	19

Table 3. Feeding test with quail using Thuricide Wettable Powder (42 grams/lb. of food) as a comparison with Agritrol (25 grams/lb. of food). Test duration = 49 days.

Weight Gain (gms.)	Grams of Food Consumed	No. of Eggs	Weight of Eggs (gms.)	% Hatch	Mortality
Male - 32 Female - 44	1844	34	376	41	0

Samples of dead and dying birds from the Agritrol diet were sent to the Avian Necropsy section of the Veterinary School at Michigan State University for examination. The reports stated that all birds examined showed the presence of spores in the blood, heart, liver, and intestine. Cause of death was attributed tentatively to bacteremia.

Since a check had been made as to the identification of the spores in each material, there was little thought of a contaminant, but to be certain, a sample of bacteria taken from the blood of sacrificed birds was sent to Dr. E. A. Steinhaus of the Insect Pathology Laboratory at the University of California for verification. Dr. Steinhaus reported the presence of a second organism but was unable to proceed to a specific identification due to sudden illness. Nevertheless the possibility of this unknown organism being the cause of death was discounted because of its low numbers in the specimens and its apparent absence from cultures of the original materials. It was thought to be a form normally present in the gut of the birds.

A check of all available commercial formulations by running simultaneous quail feeding tests with rates of 1, 3.5, 7, and 14 grams of material per pound of food resulted in the discovery that Agritrol and Bakthane were both toxic to the birds, at rates as low as 7 grams/lb. of food (Table 4). The formulations of Thuricide and Biotrol produced no such effect.

Figure 1 shows the mean weights of quail for each test with the mean weights of the controls superimposed on each series. These graphs show the extreme toxicity of Agritrol, at 7 and 14 grams/lb., and Bakthane at 3.5, 7 and 14 grams/lb. dosage levels. Samples of dead and dying birds were sent to the Avian Necropsy section again for

Table 4. Mean weight gain and percent mortality of quail in 70-day feeding studies.

Material	Treatment (gms./lb. of food)	Mean Weight Gain (grams)		Mortality			
				Males		Females	
		Males	Females	%	Days	%	Days
Thuricide	0	51	69	0	0	0	0
	1	48	69	0	0	0	0
	3.5	53	67	0	0	0	0
	7	43[a]	59[b]	25	37	50	41
	14	38	65	0	0	0	0
Biotrol	0	58	72	0	0	0	0
	4	50	77	0	0	0	0
	14	54	80	0	0	0	0
	28	52	63	0	0	0	0
	56	49	66	0	0	0	0
Agritrol	0	48[a]	61	25	49	25	66
	1	53	74	0	0	0	0
	3.5	41	53[c]	0	0	25	28
	7	27[c]	40[c]	25	61	50	35
	14	-21[c]	-25[c]	100	4	100	6
Bakthane	0	54	73	0	0	0	0
	1	41	63	0	0	0	0
	3.5	-16[c]	6[c]	75	7	50	33
	7	-25[c]	-21[c]	100	6	100	7
	14	-26[c]	-29[c]	100	5	100	5

(a) Average of three replications. Death due to excessive head picking.
(b) Average of two replications. Death due to excessive head picking.
(c) Data taken from all birds at time of death or at end of the test.

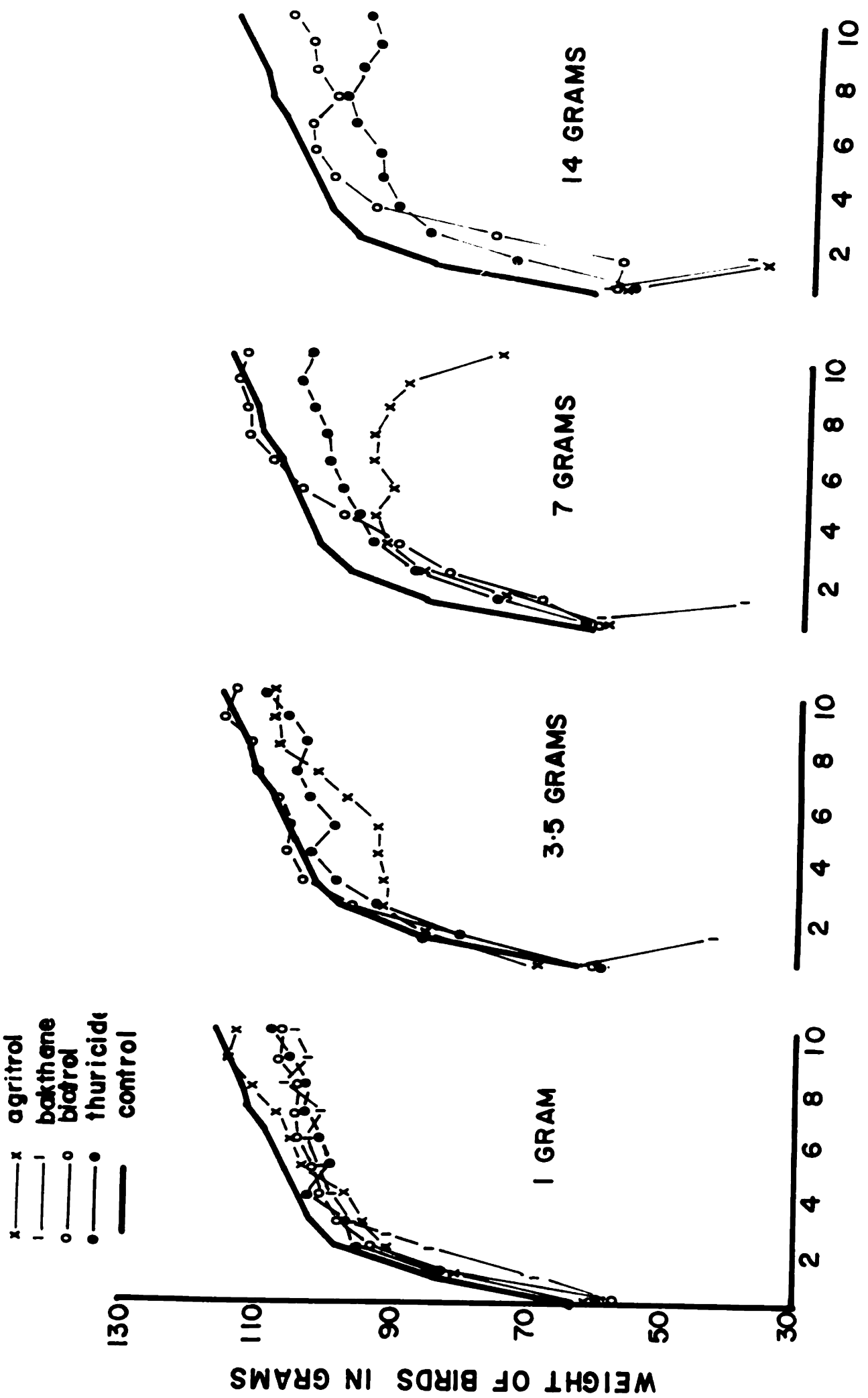


Figure 1. Mean weights of quail fed various levels and formulations of B. thuringiensis

bacterial and histological examination. The reports stated that spores were found in abundance in the heart, liver, kidney, spleen, and intestine (a bacteremia). No lesions were found in the tissues.

Two distinct types of symptoms were also noticed in the birds fed the toxic materials. After several days of feeding with high rates of Agritrol, the birds became extremely nervous and were seen to have convulsive attacks after which they would lie stiff and prone for a minute or two. Feeding with Bakthane produced listlessness and eventually an inability to balance properly. These latter birds also tended to "ball up" or "round out" and their feathers appeared ruffled. The droppings from these two sets of birds were also different from the control birds. The Agritrol-fed birds had considerable bile in their droppings as evidenced by the green coloration present, while the Bakthane-fed birds excreted extremely dry and hard droppings.

Other reactions checked in this series of tests were food consumption, number and weight of eggs and percent hatchability (of the total egg production) for the four materials under consideration (Tables 5 to 8).

Food consumption in these tests varied as did the weight gain, depending on the material used. This appeared due to a combination of repellency coupled with a decreased appetite due to the onset of sickness. Repellency was especially noticeable in the Biotrol material at the two highest rates. In order to compare all materials at equivalent spore dosages it was necessary to feed Biotrol at rates approximately four times those of formulations with higher spore counts. At rates of 28 and 56 grams/lb. of food, some of the birds had so much difficulty discerning the difference between the food and the bran

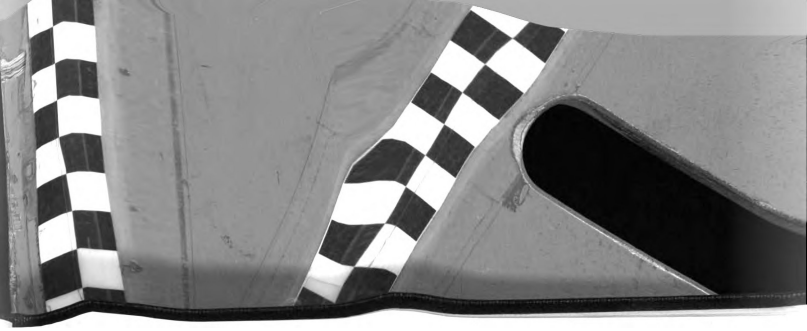


Table 5. Chronic feeding studies on quail for 70 days using Thuricide Wettable Powder (42 x 10^9 spores/gram).

Treatment (gms./lb. of food)	Food Consumption (gms.)	No. of Eggs	Weight of Eggs (gms.)	% Hatch
0	2574	36	354	54
1	2528	24	212	44
3.5	2707	40	368	31
7	2327[b]	16	141	34
14	2493	30	270	36
Maximum 5% Range (a)	472	33.5	265	30

(a) Maximum range at 95% confidence level from a multiple range test (Duncan 1955).
(b) Mean of three replications.

Table 6. Chronic feeding studies on quail for 70 days using Biotrol Feed Additive (10 x 10^9 spores/gram).

Treatment (gms./lb. of food)	Food Consumption (gms.)	No. of Eggs	Weight of Eggs (gms.)	% Hatch
0	2449	39	359	44
4	2455	42	398	43
14	2208	24	205	28
28	2212	32	279	37
56	1997	36	305	31
Maximum 5% Range (a)	508	31	265	25

(a) Maximum range at 95% confidence level from a multiple range test (Duncan 1955).

Table 7. Chronic feeding studies on quail for 70 days using Agritrol Wettable Powder (70 x 10^9 spores/gram).

Treatment (gms./lb. of food)	Food Consumption (gms.)	No. of Eggs	Weight of Eggs (gms.)	% Hatch
0	2178	36	334	40
1	2360	38	362	33
3.5	2036	15	155	21*
7[a]	2416	18	177	46
14[a]	----	--	---	--
Maximum 5% Range (b)	371	25	236	17

(a) Not treated statistically because of missing data due to mortality of the test birds.
(b) Maximum range at 95% confidence level from a multiple range test (Duncan 1955).
* Indicates significance from the control at the 5% level.

Table 8. Chronic feeding studies on quail for 70 days using Bakthane Wettable Powder (25 x 10^9 spores/gram).

Treatment (gms./lb. of food)	Food Consumption (gms.)	No. of Eggs	Weight of Eggs (gms.)	% Hatch
0	2565	38	340	56
1	2092	25	200	14
3.5	1420*	4*	23*	0*
7[a]	----	--	---	--
14[a]	----	--	---	--
Maximum 5% Range (b)	516	21.5	198.7	47.7

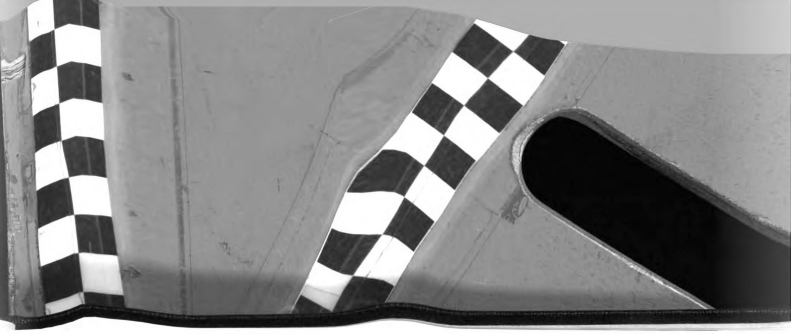
(a) Not treated statistically because of missing data due to mortality of the test birds.
(b) Maximum range at 95% confidence level from a multiple range test (Duncan 1955).
* Indicates significance from the control at the 5% level.

carrier that they either became frustrated and refused to eat or ate the bran only and died or lost considerable weight due to this poor diet. However, once the birds discovered the food in the dishes, they immediately began eating well (although much food was spilled because of their searching pecks for the food without the bran) and quickly put on weight.

Except for the two materials which caused death at the higher rates, no significant differences were obtained between the control birds and test birds for the weight gain, food consumption, number and weight of eggs, and percent hatchability.

These tests presented three questions concerning the effect of this bacillus in warm-blooded animals: 1) Since no bacterial contaminants were found in any of the samples which were responsible for the death of the birds, was the sporangia disrupted by the action of the bird's enzyme and digestive systems thus releasing a potential toxin (the parasporal crystal) into the gut causing a protein reaction which resulted in mortality; 2) Was it possible for the spores to germinate in the digestive tract, where conditions of temperature and moisture might be suitable, causing liberation of the parasporal crystal or some bacterial metabolic waste products which were toxic to the birds; 3) Could there be a carry-over of sufficient culture material, from which the spores were harvested, which might disrupt normal metabolism?

In attempting to find answers to these questions it was decided to institute a series of acute oral toxicity studies by which the amount of formulated material consumed per day per bird could be controlled as well as avoiding any repellent action of the materials by the birds.



Acute oral studies

The following materials were used in acute oral toxicity tests: Agritrol; Agritrol dry-heat treated at 112° C. for 24 hours (hereafter designated as Dead Agritrol); a pure culture of dried Bakthane spores without diluent or wetting agent (hereafter designated as Pure Spore); and the pure culture of dried spores dry-heat treated at 112° C. for 24 hours (hereafter designated as Dead Pure Spore).

The heat-treated samples were tested for the presence of viable spores by placing a subsample in sterile distilled water, heating at 65° C. for 30 minutes, and inoculating the pasteurized samples on nutrient agar plates. The plates were incubated at 30° C. and examined for growth in 24 hours. None of the heat-treated samples gave growth on the agar plates and thus the spores were considered to have been rendered non-viable.

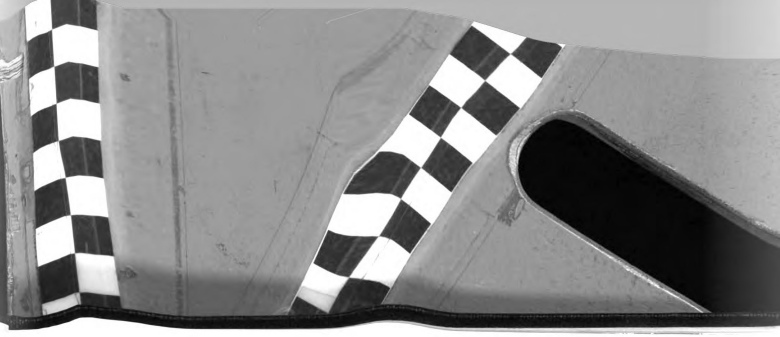
These materials were placed in number 5 gelatin capsules and force-fed to four mated bird-pairs at the rate of one capsule per day per bird for 21 days. Data were recorded for weight gain, food consumption, and mortality (Table 9).

It was evident that the normal Agritrol formulation was not causing any upset in the birds and that the rate of feeding was too low to result in mortality. However, the interesting observation was that the Bakthane at only 0.03 grams/day, or approximately 0.12% of the diet, had enough effect on the quail to significantly lower the amount of weight gained during the test period. It was also significant to note that this material, at the rate applied, contained only approximately one-fourth the spore concentration of the other materials tested, indicating that the spore itself did not appear to be responsible for the weight decline.

Table 9. Acute oral toxicity studies in quail receiving one number five gelatin capsule of material/day/bird for 21 days. Four 25-day old bird-pairs were used per test.

Treatment	Mn. Gms. of Form./day	Mn. No. of spores/day	Mean Weight Gain (grams) Males	Females	Mean Food Consumption (grams)	% Mortality
Control	----	----	17[b] 1/	45	663[ac]	0
Pure Spore	0.0803	5.7×10^9	32[b]	43	652[ac]	0
Dead Pure Spore	0.0853	6.1×10^9	29[b]	42	702[cd]	0
Agritrol	0.0417	2.9×10^9	38[b]	46	706[cd]	0
Dead Agritrol	0.0455	3.2×10^9	31[b]	43	686[cd]	0
Bakthane	0.0338	0.9×10^9	18[a]	35	568[bd]	0

1/ Means with the same letter are not significantly different.



Subsamples of birds from each test were sent to Drs. E. J. Bicknell and C. C. Ellis of the Department of Veterinary Pathology at Michigan State University for examination of bird livers. The histopathological study found a diffuse fatty metamorphosis - mild, with passive hyperemia in the pure spore -, and Bakthane-fed birds, and a marked change in the Agritrol-fed birds. Mild peripheral lobular fatty metamorphosis with passive hyperemia was found in the livers of Dead Pure Spore-fed birds, while dead Agritrol-fed birds showed marked peripheral lobular fatty metamorphosis.

In a further search for the cause of mortality in these samples, dosages were increased to approximately three times the amounts in Table 9, and these amounts were fed daily to thirty-day old males for 21 days. As with the other tests, these materials were placed in number 5 gelatin capsules. All birds were allowed food and water ad. lib. and kept under 24 hour lighting conditions.

It can be seen in Table 10 that Agritrol and Bakthane, at approximately equal rates of formulation, produced significantly reduced weight gains and fairly rapid mortality in the test birds.

A most significant result was the effect of Dead Agritrol at high rates of feeding similar to Agritrol. This material produced no mortality and the weight gain of the birds was no different than the other birds' weights. There were also no significant differences obtained between the weight gains and food consumption of the birds fed Dead Agritrol and those fed Dead Pure Spores.

The histopathology of the livers of these groups also verified this change in effect of the Dead Agritrol since the livers of these birds appeared to be essentially normal with no appearance of the

Table 10. Acute oral toxicity studies in quail receiving two number five gelatin capsules of material/day/bird for 21 days. Figures are means of four 20-day old males/test.

Treatment	Mn. Gms. of Form./day	Mn. No. of Spores/day	Mean Weight Gain (grams) Males	Mean Weight Gain (grams) Females	Mean Food Consumption (grams)	% Mortality
Control	----	----	17[b] 1/	45[b]	663[b]	0
Dead Pure Spore	0.1560	10.9×10^9	18[b]	44[b]	682[b]	0
Dead Agritrol	0.1552	10.9×10^9	27[b]	46[b]	691[b]	0
Agritrol	0.1168	8.2×10^9	-11[a]	-13[a]	11[a]	75 (3-4d)
Bakthane	0.1454	3.6×10^9	-20[a]	-18[a]	3[a]	100 (3d)

1/ Means with the same letter are not significantly different.

fatty changes. Therefore, it appears that there is no possibility for the hypothesis that germination of the spores in the intestinal tract of the birds with subsequent release of some toxic waste product or the release of the potentially toxic parasporal crystal was the cause of mortality in the quail. Also, since spores were recovered intact from the gut of the birds, it would appear that there is no release of the parasporal crystals into the intestine and thus no problem with any protein reactions with the crystals. Instead, from the data reported, it appeared that there was some material external to the spore which was harmful to the birds and which was also heat labile.

In order to further study this idea of an external source of toxicity, tests were devised to determine the effects of the diluents in the toxic formulations. In the formulation of the Agritrol wettable powder, two inert ingredients were added -- Bentonite (a clay) as a carrier or diluent at 10 percent of the material by weight, and Igepon, a wetting agent, constituting 5 percent of the formulation. The Bakthane contained 33 1/3 percent by weight of "supercell filter aid", a diatomaceous earth, as a diluent plus 1 percent of a wetting agent. These materials were mixed with the food at the equivalent rate of 25 grams of normal formulation per pound of food and given ad. lib. No significant differences were obtained between control and test birds with respect to weight gain, food consumption, egg production, or hatchability (Table 11). Thus the diluents in Agritrol and Bakthane would appear to have no relationship with the cause of mortality in the quail.

Table 11. Chronic feeding studies on quail using the diluents only from the formulations of Agritrol and Bakthane. Figures below are means of three replications of 20-day old bird-pairs tested for 37 days.

Treatment	Weight Gain (gms) Males - Females	Food Consumption (in grams)	No. of Eggs	Weight of Eggs	Mortality
Control	59 - 62	1344	15	132	0
Agritrol Diluent	46 - 58	1240	15	142	0
Bakthane Diluent	49 - 68	1382	10	92	0

COMPARATIVE MOUSE STUDIES WITH COMMERCIAL FORMULATIONS

Studies were conducted with mice to discover whether the toxicity accompanying the feeding of certain commercial formulations of B. thuringiensis might be a species specific phenomenon. According to Padgett and Ivy (1959), the Japanese quail is more resistant to disease than the bob-white quail and thus it was postulated that a laboratory mouse might show more demonstrable symptoms which could be analyzed with more authority since most pathologists are more certain of tissue and organ reactions in these animals than in such a relatively obscure exotic bird as the Japanese quail.

The animals used in these tests were 6- to 8-week old white Swiss mice purchased from a commercial supplier. The mice were housed, three to a cage, in standard laboratory mouse cages in the "rat room", Giltner Hall, on the Michigan State University campus. The room was air conditioned with a mean daily temperature of 68° F., and light was controlled to give an eighteen hour day. The mice were fed Hoppart Mouse Ration with the following formula (prepared by Merlyn Swab, Veterinary College Technician):

Formula: 140 parts corn
100 parts wheat
24 parts alfalfa meal
80 parts whole milk
12 parts yeast
40 parts linseed meal

4 parts iodized table salt

This ration would supply the following nutritional requirements:

18% protein

8% fat

3.7% fiber

94% T.D.N.

56% N.F.E.

Food was placed in small specimen jars with covers cut to permit insertion of a small piece of one-half inch mesh hardware cloth. This screen allowed the mice to feed but reduced waste of food and kept fecal deposition in the food to a minimum. Food and water were available to the mice at all times, the water being supplied by standard mouse watering bottles on each cage.

Agritrol, Bakthane, Thuricide, and the Agritrol diluent were used in this series of tests. The formulations were mixed with the mouse ration at the following rates: Agritrol at 10, 15, 20, and 25 grams/pound of food; Bakthane at 42 grams/pound; Thuricide at 43.75 grams/pound; and the Agritrol diluent at 5 grams/pound (equivalent to 25 grams/pound of the normal formulation). Observations were made every two days and data were recorded for weight gain, food consumption, appearance, and general behavior.

Due to the amount of time involved and lack of space in the rat room, only the Agritrol-fed mice could be tested with any real degree of reliability. Four replications of three mice per cage were arranged in this test while the other formulations were applied to only one or two replications of three mice per cage. However, as is evident in Table 12, these tests resulted in comparable reactions to those

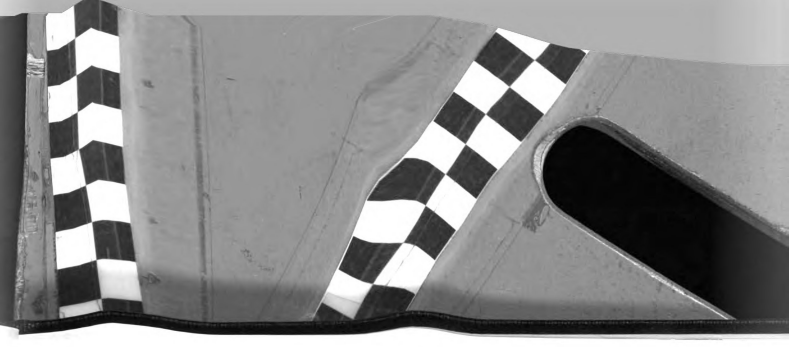
with the quail. Agritrol and Bakthane continued to cause weight loss, decreased food consumption, and eventual mortality in the mice while excessively high rates of Thuricide and Agritrol diluent did not appear to upset the normal functioning of these animals.

Table 12. Mean weight gain, food consumption, and percent mortality in 6- to 8-week old mice fed various formulations of B. thuringiensis.

Source	Feeding Time (days)	Weight Gain (grams)	Food Consumption (grams)	Mortality (%)
Control	28	5	339	0
Agritrol:				
10 gms./lb.	14	-6	134	67
15 gms./lb.	14	-8	171	50
20 gms./lb.	9	-10	79	75
25 gms./lb.	7	-12	48	100
Bakthane (42 gms./lb.)	14	-12[a]	73	33
Thuricide (43.75 gms./lb.)	35	7[b]	311	0
Agritrol diluent (5 gms./lb.)	28	0[a]	311	0

(a) Based on 2 replications
(b) Based on 1 replication

The toxic symptoms in the mice were somewhat different from those in the quail and were probably due to the difference in the behavior, structure, and action of the digestive systems of the different species. The Agritrol-fed mice became extremely nervous and active



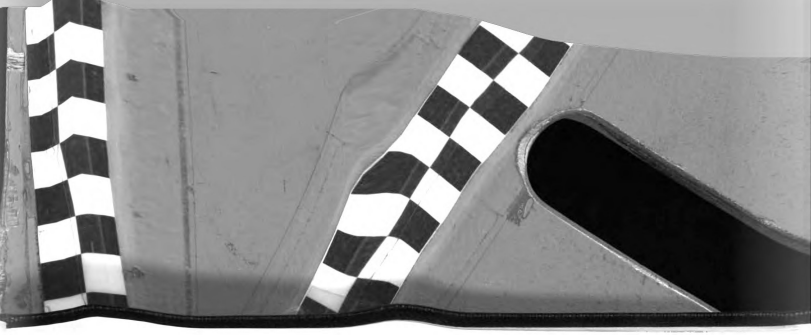
until close to death they retreated to a back corner of the cage where they remained while being observed. The Bakthane-fed mice, on the other hand, became reluctant to move even when prodded and eventually their abdomens became swollen and edematous. The droppings of these latter mice were lighter colored than normal and quite hard and dry.

Samples of sick and dying mice were sent to the Diagnostic Bacteriology Laboratory of the Department of Microbiology and Public Health at Michigan State University for examination. Spores were found in the heart, liver, and intestine of all the animals examined. The Bakthane-fed mice were also found to have a collapsed intestine which accounted for the enlarged abdomen. It was also noted that the intestines of both the Agritrol-fed and Bakthane-fed mice were practically devoid of their normal flora.

The problem still remained, however, as to what was present in these two formulations which resulted in animal mortality. Table 13 shows the results of two presumptive tests on two groups of three mice per cage with two new samples of Agritrol produced for 1960 experimental field work. Both of these formulations gave no sign of interference with normal digestion. Therefore it appeared that some contaminant was introduced into the formulations either during the drying process in which the spores were removed from the culture vats and dried in the open air, or during the formulating process when the diluent and wetting agents were added.

Table 13. Results of 35-day trial feedings of 1960-produced Agritrol with 3 mice/cage in each trial at 25 grams of formulation per pound of food.

Formulation Sample Number	Weight Gain/Cage (grams)	Food consumed/Cage (grams)	Mortality (%)
# 0656	14	427	0
# 9334	10	348	0



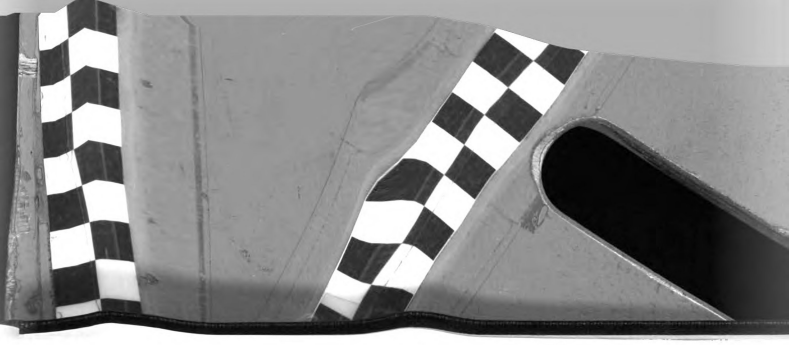
STUDIES ON THE CONTAMINATED FORMULATIONS
OF B. THURINGIENSIS

Determination of the amounts and kinds of contaminants present

The two materials which caused mortality in the quail (Agritrol and Bakthane) were reported by Stuart (1960) to contain chemical contaminants of the chlorinated hydrocarbon insecticide groups. Agritrol was reported to contain DDT [1, 1, 1-trichloro-2, 2-bis (p-chlorophenyl) ethane] and aldrin (1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 8, 8a-hexahydro-1, 4, 5, 8-endo, exo-dimethano-naphthalene). Bakthane was found to contain a substance related to Kelthane [1, 1-bis (p-chlorophenyl) 2, 2, 2-trichloro ethanol] or Perthane [1, 1-dichloro 2, 2-bis (p-ethylphenyl) ethane].

To determine the amounts of chemical contaminants, the Agritrol and Bakthane formulations were subjected to benzene extraction in a Soxlet condenser for 24 hours. The benzene extract was slowly evaporated to dryness by introducing air down the side of the flask with a capillary pipette. The dried residue was then redissolved in acetone and filtered twice to remove the insoluble saponification products which came through the condensation process with the benzene.

The formulation residue, remaining after the condensation process, was dried and fed to quail to test for further toxic factors. As reported in the quail feeding studies, the dried residue of Agritrol was no longer toxic to the birds but the Bakthane continued to produce toxicity. Further extraction of Bakthane by Soxlet condensation with



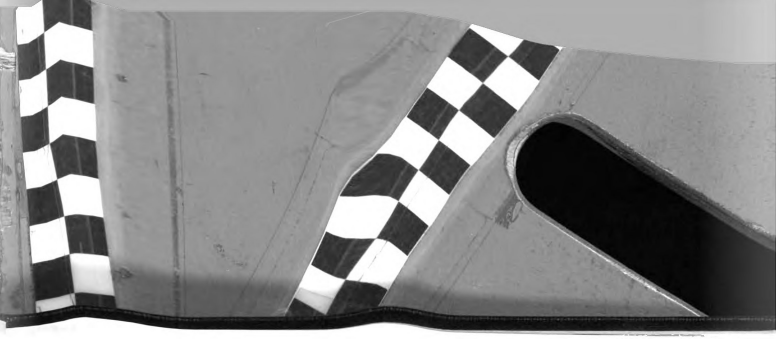
ether for 24 hours also failed to stop the toxic reactions in the birds and the Bakthane was removed from any further consideration.

Two methods were used to test the filtered acetone solution of the Agritrol extract for the DDT and aldrin present. The solution was divided into two equal portions, one of which was placed in a glass-stoppered Erlenmeyer flask for use in determination of the combined effects of DDT and aldrin, and the other portion was treated with 10% alcoholic potassium hydroxide in a reflux condenser for one hour to destroy the DDT present. This saponified material was evaporated to dryness, redissolved in acetone, and filtered twice to remove the undissolved sediment for use in bioassay tests.

The amount of DDT present in Agritrol was determined by Mr. Richard Bernard of the Zoology Department at Michigan State University. For this determination Mr. Bernard employed the Schecter-Haller technique as described by the Association of Official Agricultural Chemists (1955). The results of the several trials are shown in Table 14.

Table 14. Results of chemical analysis of formulations of B. thuringiensis

Material	Weight (grams)	ugrams of DDT/gm. of sample
Agritrol	1.128	1000+ (= 0.011%)
Agritrol	1.356	1000+ (= 0.001%)
Agritrol	1.563	1000+ (= 0.001%)
Thuricide	1.435	0
Pure Spore	1.892	0
Pure Spore	1.783	0
Distilled Agritrol	1.576	119
Distilled Agritrol	1.279	106



The organisms used in the bioassay tests for the determination of the chemical contaminants were wild-type pomace flies, Drosophila melanogaster Meigen. The rearing media for larvae and food for adults was a basic formula consisting of the following:

800 ml. distilled water

100 grams sucrose

50 grams brewers' yeast

15 grams agar

1 gram K_2HPO_4

These ingredients were mixed in an Osterizer blender and then autoclaved at 121° C. for ten to fifteen minutes at 15 lbs. pressure. After autoclaving, the following items were added:

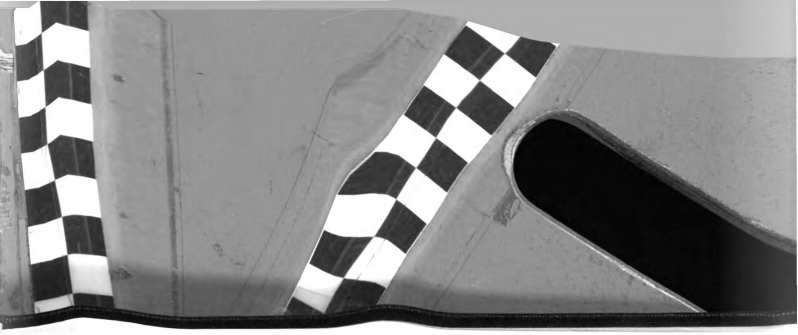
200 ml. distilled water

10 grams Wesson salt mixture "W"

Note: For stock cultures, 5 ml. of propionic acid were added/liter of media to prevent fungus growth.

Stock cultures of Drosophila were maintained at 30° C. in one-half pint glass milk bottles containing approximately 2 to 2-1/2 inches of media and plugged with non-absorbent cotton. Sexually mature adult flies were introduced into the bottles and allowed to mate and oviposit for two to three days after which time they were removed and sacrificed. Generation time, with the media and temperature used, was approximately ten to eleven days.

The same media as described above, with the exception of propionic acid, was used in the bioassay tests for DDT and aldrin. The flies were first tested for normal susceptibility to known amounts of technical samples of the chemicals. Predetermined quantities of



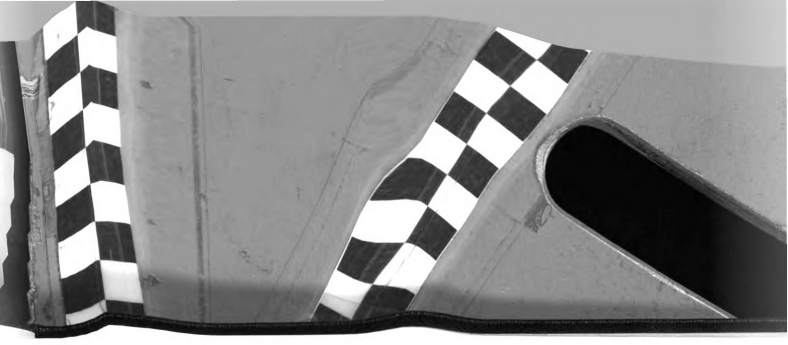
acetone solutions of DDT and aldrin were mixed with twenty grams of warm media and poured into 4 dram shell vials to a depth of 1/4 inch and allowed to set. Five replications of each dosage were made, and ten, one to two-day old pomace flies were added to each vial. The vials were plugged with non-absorbent cotton and placed in a 30° C. incubator for 24 hours. Data were recorded for the number of dead and moribund flies (Table 15).

Table 15. Comparison of determinations of the ED_{50} to Drosophila (in media) of technical grade aldrin and the aldrin contamination in Agritrol.

Source	Replicates	ED_{50}*	$F_{ED_{50}}$ (95% Confid.)	Slope**	F_{slope} (95% Confid.)
Tech. grade aldrin	1	0.195	1.70	1.31	1.06
	2	0.198	1.11	1.52	1.20
Aldrin contam. in Agritrol	1	0.160	1.11	1.32	1.08
	2	0.190	1.05	1.31	1.08

*$F_{P.R.}$ P.R. (Potency Ratio) within experimental error.
**$F_{S.R.}$ S.R. (Slope Function Ratio) within experimental error.

After an indication was obtained for the normal susceptibility of the Drosophila, similar tests were run with the saponified extract recovered from the Agritrol condensation and redissolved in 100 ml. of acetone. These data are also recorded in Table 15. The results of these tests showed that the ED_{50} and slope were quite similar to those of the tests of technical grade aldrin indicating that the aldrin recovered from the extraction and purification processes was at a level



of approximately 200 parts per million. Sun and Tung Sun (1952) reported that this extraction recovered approximately 85% of the total aldrin present in the formulation and therefore the level of 200 ppm reported, was considered to be very close to the true level of aldrin present initially.

The ED_{50} and slope were calculated by the method of Litchfield and Wilcoxon (1949). The DDT was found to have been detoxified in the media so that no mortality data were available for this chemical.

In an attempt to get a reliable ED_{50} and slope for DDT, the flies were treated by the method reported by Sun and Pankaskie (1954). Known amounts of DDT, aldrin and saponified Agritrol extract in acetone solutions, were each carefully hand-mixed with canned pumpkin. Sufficient treated pumpkin was used to cover a piece of paper hand towel 3/4 x 1/2 inch. This piece of towel was placed in a 4 dram shell vial and ten adult flies were placed in the vial. Five replicates of each dosage were prepared and each vial was placed in a 30° C. incubator in such a way that the pumpkin-covered towel acted as a roof over the flies. After 24 hours the number of dead and moribund flies/vial was recorded and an analysis of the ED_{50} was calculated (Table 16).

Table 16. Comparison of determinations of the ED_{50} to Drosophila (on pumpkin) of technical grade aldrin, aldrin contamination in Agritrol (extract), and the complete formulation of Agritrol.

Source	ED_{50}	$F_{ED_{50}}$ (95% Confid.)	Slope	F_{slope} (95% Confid.)
Tech. grade aldrin	0.255	1.16	2.04	1.22
Extract of aldrin contaminant in Agritrol	0.200	1.13	2.02	1.20
Agritrol formulation	0.036	1.28	2.45	1.34

As with the media tests, such great variation was obtained in the DDT tests that no reliable estimate of the ED_{50} or slope could be calculated. Thus it was necessary to resort to the use of the LD_{50} of DDT for Drosophila, on pumpkin, of 10 parts per million (ppm) as reported by Sun and Pankaskie (1954).

Further tests were employed to determine the effects of a combination of DDT and aldrin and the effect of spores on Drosophila using pumpkin as a food source for the flies. Thuricide and Agritrol were used in their normal formulations and treated as in the other pumpkin tests. No effects were observed on the flies with the Thuricide treatments indicating that the bacillus was non-pathogenic to the adults or the larvae. The results of the Agritrol test are presented in Table 16. In the presence of DDT and aldrin an ED_{50} of 0.036 ppm was obtained which, compared to the ED_{50} of aldrin (0.25 ppm) and the assumed ED_{50} of DDT (10.0 ppm), appeared to be a greater than additive effect between the two insecticides. However, since a reliable test was not obtained with DDT in these studies, the possibility of interaction with the crystals contained in the spores also cannot be completely discounted. Figure 2 gives the comparative curves for the aldrin, and Agritrol tests and the assumed DDT value in pumpkin to give a better perspective of the slopes of the three curves.

Determination of the resistance to DDT in the house flies used in the adult fly emergence from droppings

One set of tests was conducted with house flies, using the topical application method and equipment described by Elmosa (1960), to compare the level of resistance of the barn strain of flies used in the emergence tests in bird droppings with a non-resistant strain (CSMA-48)

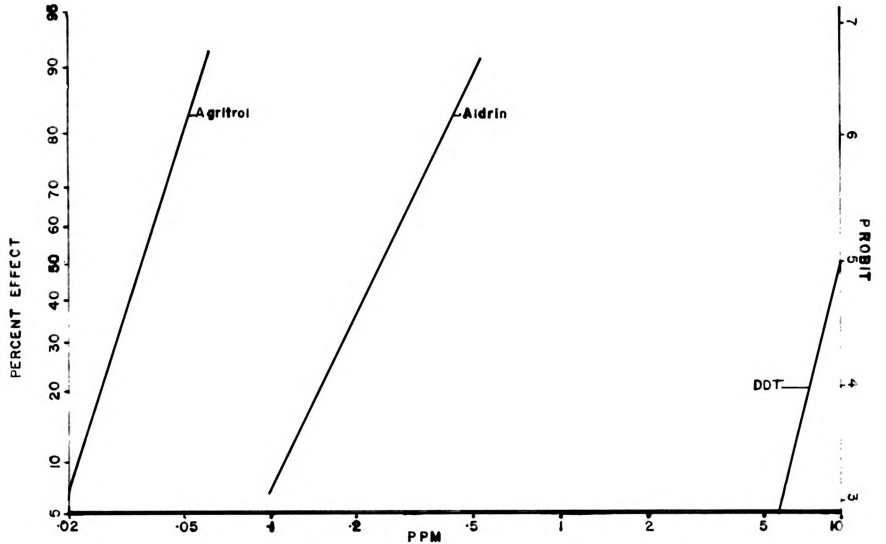


Figure 2. Comparative slope and ED_{50} in Drosophila for technical DDT, technical aldrin, and Agritrol in pumpkin.

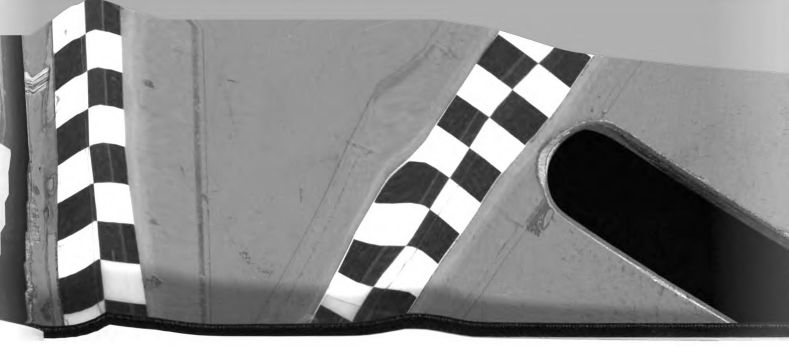
of house flies supplied by John F. Tighe of the United States Food and Drug Administration.

The ED_{50} of the non-resistant flies, under the conditions of temperature, rearing media, and techniques used in these studies, was 0.035 ugms. and the ED_{50} of the barn strain was found to be 0.35 ugms. of DDT/fly. Unfortunately, these tests were quite variable due to circumstances over which there was little control, especially in the excessive use of the room in which the tests were run. This resulted in the death of all flies in one test because a window was left open next to the test boxes during a cold night. Therefore, these results were compared with the results of a class project in the course Insecticides and Their Use (Entomology 423) conducted by Dr. R. A. Hoopingarner. The results of this project closely paralleled the ED_{50} values reported above. The observations were then thought to be valid that the strain of house flies used in the emergence tests were approximately ten times more resistant to DDT and its analogues than were the non-resistant CSMA-48 flies. With this information, a more reliable observation could be drawn as to the effect, if any, of the DDT and aldrin contaminants in Agritrol, on the developing house fly larvae.

Acute oral toxicity studies in quail with the contaminants found in Agritrol

Feeding studies were conducted in quail using the dried residue remaining from Soxlet condensation of the Agritrol and Bakthane formulations. Comparative studies were also undertaken with known amounts of the contaminants fed singly or in combination with pure spores.

All tests in this group were run with 30-day old male quail with five replications per test. Each bird was fed a measured amount



of material daily in number 5 gelatin capsules and given food and water ad lib. Data were recorded for weight gain, food consumption, general behavior, and mortality for a period of 21 days.

The materials used were: 1) Benzened Agritrol (the dried residue from benzene washing of Agritrol); 2) Benzened Bakthane (dried residue from benzene washing of Bakthane); and 3) Ethered Bakthane (dried residue from ether washing of Bakthane).

Table 17 reports the results of feeding the three treatments and the percent mortality in each treatment. It appeared that the contamination was completely removed from the Agritrol formulation by the benzene washing, or at least reduced to a minimal amount, since there was no mortality in the five birds and weight gain and food consumption did not differ appreciably from the controls. A chemical analysis showed that only 106-119 ugms./gm. of sample remained (Table 14).

Benzene washing of Bakthane, however, failed to remove enough of the contaminant in that formulation since mortality was still complete and rapid. Ether washing of Bakthane also failed to remove enough of the contamination from the formulation, although more appeared to have been removed by ether than benzene as shown by the extended life of the birds fed the dried residue from the ether extraction. No further consideration was given to the contamination present in Bakthane.

Comparison feeding studies were undertaken, in quail, using known amounts of DDT and aldrin, singly and in combination, and mixtures of these insecticides with spores. DDT was fed at the rate of 1000 parts per million (ppm) and mixed with Bentonite (the Agritrol

Table 17. Comparison of chemically purified formulations of Agritrol and Bakthane fed to 30-day old male quail by capsule for 21 days.

Observations	Treatment				
	Control	Benzened Agritrol	Benzened Bakthane	Ethered Bakthane	Maximum 5% Range[a]
Mean weight of formulation consumed/day (grams)	---	0.1660	0.1454	0.1476	--
Mean number of spores consumed/day	---	11.6×10^9	3.6×10^9	3.7×10^9	--
Mean weight gain (grams)	18	19	-18*	-12*	12
Mean food consumption (grams)	285	316	9*	13*	19
Percent mortality	0	0	100	100	--
Mean lethal time (days)	0	0	8	14	--

(a) Maximum range at 95% confidence level from a multiple range test (Duncan 1955).
* Indicates significance from the control at the 5% level.

clay diluent) and with Pure Spore. Aldrin was fed at a rate of 200 ppm and mixed with Bentonite and with Pure Spore. These rates were determined as approximate levels of DDT and aldrin, in the Agritrol formulation, by chemical and bioassay determinations. Combination trials were also made with DDT plus aldrin mixed with Bentonite and with Pure Spore in two different proportions. The results of these trials are reported in Table 18.

Tests with 1000 ppm of DDT mixed with Bentonite and with Pure Spore showed that this amount in the normal formulation was sufficient to upset the normal metabolic balance of the quail but not at a high enough level to cause mortality. As a comparison, Cross (1960) found that a level of 300 ppm was needed to effect mortality in this species of quail with the male more susceptible than the female.

Histopathological examination of the livers in these test birds revealed that the birds fed DDT + Bentonite displayed diffuse fatty infiltration in the liver while birds fed DDT + Pure Spore had essentially normal livers. It was postulated that this difference in effect to the liver may have been due to the dissolution of the fatty layer on the surface of the spores by the action of the acetone in the DDT solution, either allowing the DDT to penetrate the wall of the spore or attach to the spore and be carried out of the birds before any damage could be effected.

Similar tests with 200 ppm of aldrin did not appear to affect the quail. No significant differences were obtained between the control and aldrin tests for weight gain and food consumption.

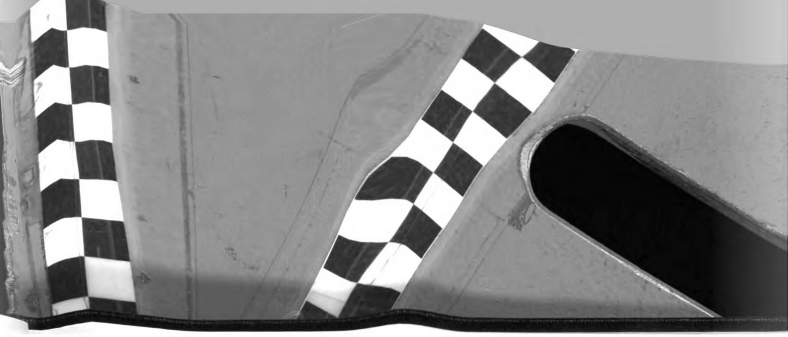
A combination test of 15 ppm of DDT plus 7.5 ppm of aldrin mixed with Pure Spore showed no significant deviation from control

Table 18. A comparison of the effects of DDT and aldrin in combination with each other and with Pure Spore and Bentonite when fed to 30-day old male quail by capsule for 21 days.

Measurements	Treatment							
	Control	DDT + Bentonite	DDT + Spore	Aldrin + Bentonite	Aldrin + Spore	DDT (15 ppm) + Aldrin (7.5 ppm) + Spore	DDT (100 ppm) + Aldrin(200 ppm) + Bentonite	DDT (100 ppm) + Aldrin(200 ppm) + Spore
Mean weight of formulation consumed/day (grams)	---	0.1518	0.1241	0.1490	0.1207	0.2326	0.1526	0.1256
Mean numbers of spores consumed/day (x 10^9)	---	---	8.69	---	8.45	16.6	---	10.8
Mean weight gain (grams)	24	14[a]	27	15	17	17	17	16
Mean food consumption (grams)	281	283	302	283	297	267	302	310
Percent mortality	0	0	0	0	0	0	0	0

(a) $t_{.95}$ = 8.8 for the least significant difference between DDT + Bentonite and control.

birds with respect to weight gain and food consumption. Subsequent tests using 1000 ppm of DDT plus 200 ppm of aldrin mixed with Bentonite and with Pure Spore also gave no significant differences in weight gain and food consumption from male control birds. These latter tests, however, were run concurrently with bioassay tests on Drosophila and, in the bioassay tests, great variation was observed with the DDT solution used. Therefore it was felt that the technical DDT used in these studies might have become reduced in effectiveness due to age or to some impurity in the crystals or the acetone solvent.



EFFECTIVENESS OF B. THURINGIENSIS ON HOUSE FLIES

The house flies (Musca domestica L.) used in all tests of fly emergence and dosage mortality studies were reared from a population collected in the dairy barn on the Michigan State University campus in 1959.

The adult flies were housed in wood-framed cages, 16 x 16 x 12 inches, with three sides and top covered with 1/16 inch mesh galvanized screen. The floors of the cages consisted of 5/8 inch plywood boards. The doors of the cages were covered with muslin sleeves sufficiently long to allow them to be knotted.

Adult food consisted of fresh whole milk with approximately 0.2% formaldehyde added as a preservative. The milk was placed in small ice cream sundae cups with a small piece of synthetic sponge (1 x 1-1/2 inches) to give the flies a solid footing from which to feed.

Oviposition and larval rearing media consisted of one part by volume of oat hulls, two parts by volume of CSMA (Chemical Specialties Manufacturers Association) house fly medium, and 500 ml. of hot water. The dry medium was thoroughly mixed by hand, the water added and carefully mixed again. The wet media was then transferred to seven inch glass specimen dishes (fingerbowls) which were placed in the cages with the adult flies. Two days of exposure to gravid females was sufficient to produce several thousand individuals in the next generation from each cage. After two days of egg deposition, the dishes were removed

from the cages, dated, and placed in clean empty cages at room temperature. No further attention was given to the rearing dishes or larvae since there was sufficient water to allow the larvae to reach the pupal stage and by the time a pupation site was needed, the top one-half to one inch of media was dry enough for this purpose. Fungi developed rapidly over the surface of the media during the first two or three days after preparation but the larval movements constantly stirred the media, destroying the mycelia and preventing further formation of a fungus mat on the surface.

Larval age was dated from the time of oviposition which occurred generally two days after the adults had emerged. Hatching of the eggs usually occurred within 24 hours after the egg masses were observed in the dishes. The length of larval life ranged from 5 to 7 days. At room temperature, and depending on the time of year, the flies were observed to complete a full cycle (egg to adult) in 10 to 14 days.

Tests of adult fly emergence from treated quail droppings

The droppings from each cage of the test birds in the 70-day quail feeding studies were collected at two-week intervals and placed in one pint ice cream cartons to a depth of one-half the height of the box. Twenty-five, 4- to 5-day old house fly larvae were placed in each container together with 25 ml. of water. The boxes were covered with cheesecloth and kept at room temperature (68° - 70° F.) for twenty days to insure complete adult fly emergence. The contents of each box were then carefully examined for adult flies, unopened pupae, and dead larvae in an attempt to account for the original deposition of 25 larvae.

Two comparison tests were also made using equivalent rates of pure spores hand-mixed with untreated droppings and CSMA media to compare the effectiveness of dusting or hand-mixing the spore materials in the droppings, and spores pre-mixed with the droppings in the intestines of animals.

Table 19 shows the results of fly control in droppings from birds fed rates of 0 to 25 grams of formulation/pound of food in the preliminary Agritrol tests. It was interesting to note that the first test of droppings collected during the first two weeks of the study gave only 40% control and only at an exceedingly high rate of feeding. Subsequent tests showed a marked decline of adult fly emergence to a point where all treatments gave significantly lower emergence than the control. The first emergence test was also significantly different from the other tests. The reason for this delay in expression of larval pathogenesis is not understood. There was no delay in appearance of pathogenicity to the house fly larvae in any of the other materials tested as well as with the lower rates of Agritrol. The results of droppings tests for each material used in the quail feedings studies are presented in Tables 20 to 23.

Table 24 gives the comparative results of adult fly emergence in droppings from the birds used in the comparative feeding studies. As a result of the mortality of birds in the Bakthane tests, no valid analysis was possible for comparison of the effectiveness of this material with the other materials used. A separate two-way analysis of variance (Table 23) on Bakthane alone, however, showed that there were no significant differences between tests but that treatments of 1 and 3.5 grams of formulation/pound of food fed to the quail produced

Table 19. Percent adult house fly emergence from 25 larvae placed in droppings from quail fed high rates of Agritrol.

Test Interval	Grams of Agritrol/lb. of Food					
	0	10	15	20	25	Mean[1/]
1	100	100	100	100	60	92.0[a]
2	100	0	4	4	16	24.8[b]
3	100	36	8	24	0	33.6[b]
4	100	4	--	--	--	[2/]
Mean[1/]	100[a]	45.33[b]	37.33[b]	42.66[b]	25.33[b]	

[1/] Means with the same letters are not significantly different.
[2/] Not treated statistically because of missing data due to bird mortality.

Table 20. Percent emergence of adult house flies from droppings of quail fed Thuricide Wettable Powder.

Test Interval	Grams of Thuricide/lb. of Food				
	0	1	3.5	7	14
1	100	9	27	8	4
2	98	32	7	6	8
3	78	65	25	28	19
4	44	43	21	33	16
5	56	32	29	16	11
Mean[1/]	75.2[a]	36.2[b]	21.8[c]	18.2[c]	11.6[c]

[1/] Means with the same letters are not significantly different.

Table 21. Percent emergence of adult house flies from droppings of quail fed Biotrol Feed Additive.

Test Interval	Grams of Biotrol/lb. of Food				
	0	4	14	28	56
1	37	28	11	11	8
2	74	11	18	7	2
3	75	32	6	15	0
4	62	13	3	4	2
5	56	20	15	3	7
Mean[1]	60.8[a]	20.8[b]	10.6[b]	8.0[b]	3.8[b]

[1] Means with the same letters are not significantly different.

Table 22. Percent emergence of adult house flies from droppings of quail fed Agritrol Wettable Powder.

Test Interval	Grams of Agritrol/lb. of Food				
	0	1	3.5	7	14
1	59	32	8	17	---[2]
2	85	0	0	6	---
3	75	20	7	18	---
4	82	58	12	35	---
5	71	69	26	15	---
Mean[1]	74.4[a]	35.8[b]	10.6[b]	18.2[b]	---

[1] Means with the same letters are not significantly different.
[2] No data due to mortality of the birds.

Table 23. Percent emergence of adult house flies from droppings of quail fed Bakthane Wettable Powder.

Test Interval	Grams of Bakthane/lt. of Food				
	0	1	3.5	7	14
1	61	1	0	--2/	--2/
2	71	8	0	--	--
3	76	10	0	--	--
4	72	1	0	--	--
5	47	3	0	--	--
Mean1/	65.4a	4.6b	0b	--	--

1/ Means with the same letters are not significantly different.
2/ No data due to mortality of the birds.

Table 24. Comparison of mean percent emergence of adult house flies from quail droppings and CSMA media treated with various formulations of B. thuringiensis.[1]

Grams of form./ lb. of food	Formulation: Pure Spore Hand-mixed w/CSMA media	Pure Spore Hand-mixed w/droppings	Agritrol	Thuricide	Biotrol	Mean[2]
0	65.6	56.8	74.4	75.2	60.8	66.6[a]
1	11.2	50.4	35.8	36.2	20.8	30.9[b]
3.5	3.2	1.6	10.6	21.8	10.6	9.6[c]
7	0	41.6	18.2	18.2	8.0	17.2[c]
14	0	1.6	4.5[3]	11.6	3.8	4.3[c]

[1] Bakthane results were omitted due to missing data caused by excessive mortality of the test birds.
[2] Means with the same letter are not significantly different.
[3] Correction for missing data (Snedecor 1956).

significantly less adult emergence than the control group yet were not different from each other. Two-way analyses of each material also showed no significant differences between test periods. All treatments showed significantly less adult fly emergence from control droppings and in most cases significant differences were obtained between feeding levels of 1 gram of formulation/pound of food and the higher rates. A comparison of the percent effectiveness in Table 25 also shows this trend.

Table 25. Mean percent effectiveness* of B. thuringiensis against house fly larvae in droppings of treated quail and in CSMA media.

		Percent Effectiveness				
Gms./lb. of Food	Mn. Spores Cnsmd/d/pr	Spores Hand-mixed w/media	Spores Hand-mixed w/droppings	Thur-icide	Agri-trol	Bio-trol
1	33.4×10^8	83	11	52	52	66
3.5	10.8×10^9	95	97	71	86	83
7	18.6×10^9	100	27	76	76	87
14	4.6×10^{10}	100	97	85	94	94

*Percent effectiveness = $\frac{\% \text{ emergence in control} - \% \text{ emergence in test}}{\% \text{ emergence in control}} \times 100$

Tests of the effectiveness of hand-mixing known amounts of spores with normal CSMA house fly rearing media and with untreated quail droppings are presented in Tables 26 and 27. Treatments were used which corresponded to the rates at which the quail were fed assuming almost complete passage of the spores through the digestive tract of the birds. Complete absence of adult fly emergence was noted

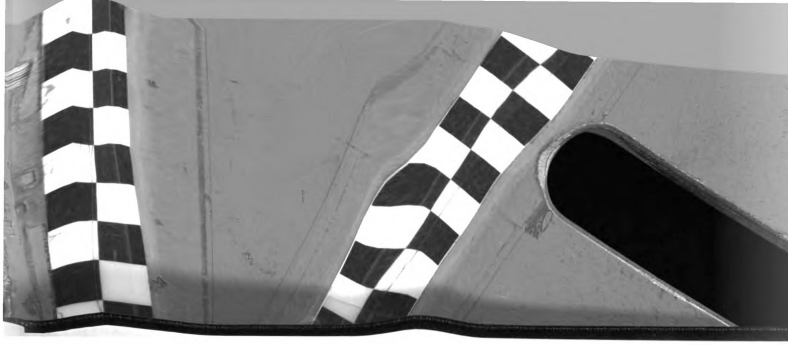


Table 26. Percent emergence of adult house flies from untreated quail droppings hand-mixed with known amounts of Pure Spore.

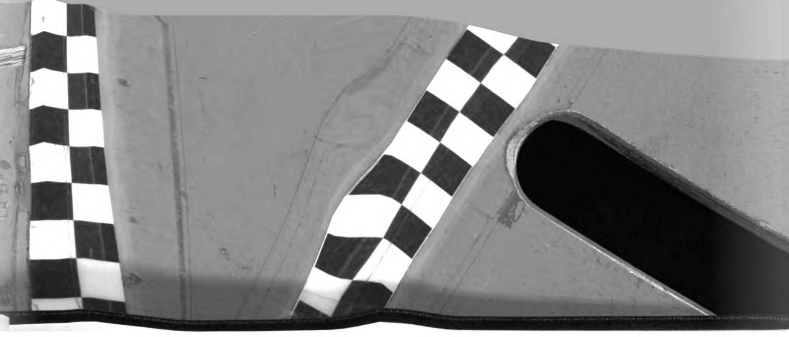
	Treatment (grams of spore/lb. of food)				
Test	0	1	3.5	7	14
1	64	36	0	32	0
2	56	48	0	20	0
3	84	64	0	40	0
4	44	48	0	48	0
5	36	56	8	68	8
Mean[1/]	56.8[a]	50.4[a]	1.6[b]	41.6[a]	1.6[b]

1/ Means with the same letter are not significantly different.

Table 27. Percent emergence of adult house flies from CSMA media hand-mixed with known amounts of Pure Spores.

	Treatment (grams of spore/lb. of food)				
Test	0	1	3.5	7	14
1	52	8	4	0	0
2	76	20	8	0	0
3	60	0	0	0	0
4	80	8	0	0	0
5	60	20	4	0	0
Mean[1/]	65.6[a]	11.2[b]	3.2[b]	0[b]	0[b]

1/ Means with the same letter are not significantly different.



in the media treatments corresponding to 7 and 14 grams of formulation per pound of food with a marked decline noticeable in the 1 gram/lb. level. The droppings test showed a marked variation in emergence at the three highest levels but no difference in emergence between the control, 1 gram/ lb., and 7 gram/lb. levels.

All emergence tests were analysed for interaction with the treatment levels. Bakthane was omitted from this comparison for reason of insufficient data due to the heavy mortality of the test birds. Table 28 shows that there was an interaction between the droppings and media tests. This was also evident in a combined test for interaction involving all formulations (Table 29). From these analyses it appeared that a more uniform mixing of spores and droppings occurred when the spores were fed to the birds and allowed to pass through their digestive tracts than when spores were hand-mixed with the droppings. This more even distribution of spores was reflected in the more uniform reduction in adult fly emergence in all tests except the hand-mixed droppings test. Uniform dispersal was also evident in the media test.

A more graphic illustration is presented in Figure 3, with a corrected value of the hand-mixed droppings test included to show the percent mortality which should have occurred. With the use of this corrected value, a strong interaction was still obtained between the two hand-mixed tests but only a mild interaction was obtained in the total analysis (just significant at the 5% level). No interaction was found to occur between the hand-mixed media, Thuricide, Biotrol, and Agritrol tests.

The effect of moisture on spore viability

The effect of moisture on spore viability was examined by two

Table 28. Analysis of interaction between treatments and tests in studies of adult fly emergence from CSMA media and untreated quail droppings hand-mixed with Pure Spore.

Treatment (gms./lb. of food)	Total Percent Emergence in Tests: Spores Hand-mixed With Media	Spores Hand-Mixed With Droppings
0	328	284
1	56	252
3.5	16	8
7	0	208
14	0	8

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	"F" Ratio
Treatments	4	24419.2	6104.8	59.06**
Tests	1	2592.0	2592.0	25.08**
Interaction	4	5782.4	1445.6	13.99**
Deviation	40	4134.4	103.36	
Total	49	36928.0		

** Significant at the 1% level.

Table 29. Analysis of interaction between treatments and tests in studies of adult fly emergence from treated CSMA media and quail droppings.

Treatment (gms./lb. of food)	Total Percent Emergence in Tests				
	Spores Hand-mixed w/media	Spores Hand-mixed w/droppings	Agritrol	Thuricide	Biotrol
0	328	284	372	376	304
1	56	252	179	181	104
3.5	16	8	53	109	53
7	0	208	91	91	40
14	0	8	35[a]	58	19

(a) Replacement of missing data (Snedecor 1956)

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	"F" Ratio
Treatments	4	62957.8	15739.5	103.72**
Tests	4	4822.4	1205.6	7.89**
Interaction	16	7726.4	482.9	3.16**
Deviation	100	15274.8	152.7	
Total	124	90781.4		

** Significant at the 1% level.

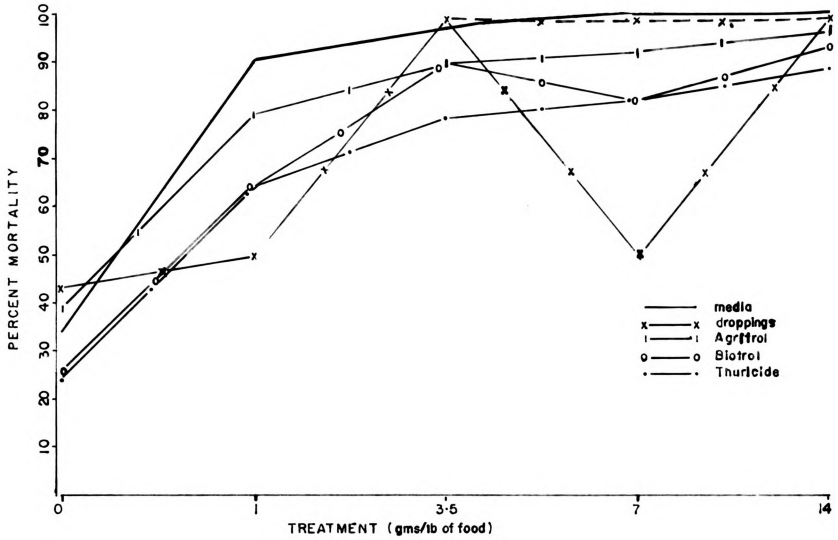
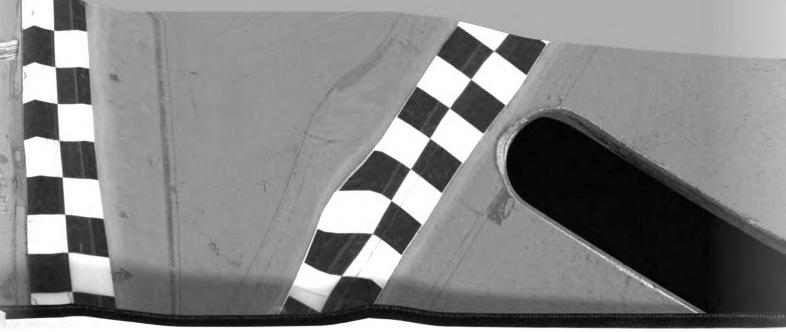


Figure 3. Percent mortality to house fly larvae in quail droppings and CSMA media treated with various formulations of B. thuringiensis.

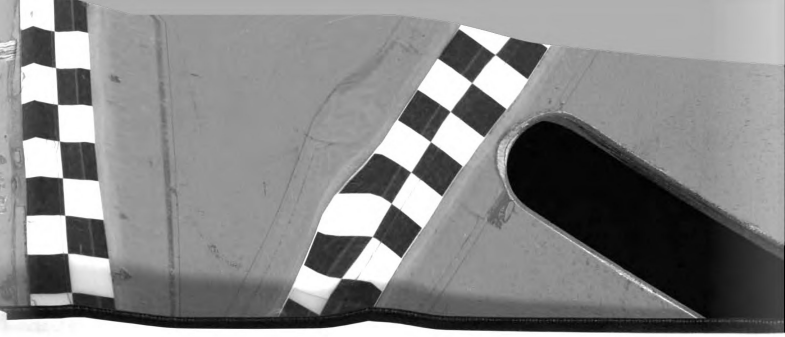


methods: (1) observation of the results of larval tests in droppings collected from birds on feeding studies, assuming the spores were held in an environment of 100% moisture in the digestive tracts of the animals, and (2) by addition of given amounts of water to droppings containing a known amount of spores.

Samples of droppings were collected from quail fed Thuricide at a rate of 7 grams of Thuricide/pound of food, and 50 grams subsamples were placed in one pint ice cream cartons. A 150 gram subsample was placed in a dry-heat oven at 112° C. for 24 hours to remove all water. Upon reweighing, the droppings were found to have originally contained 15% by weight of water.

Six moisture levels were then established, with five replications for each level, using the normal droppings with 15% by weight of moisture as control. Water was added to each box to increase the levels by 25, 50, 75, 100, and 150% by weight of moisture. These moisture levels were held constant for 10 days, after which 25 four- to five-day old house fly larvae were added to each box. Twenty milliliters of water were added to each box to keep the water addition constant and still allow enough moisture for larval development in the controls. The boxes were covered with gauze and kept at room temperature (68° to 70° F.) for 20 days, to allow sufficient time for complete emergence to occur. The boxes were then opened and data recorded for number of adults, unopened pupae, and larvae. The adult emergence results are shown in Table 30.

Analysis of these data indicated that mean adult emergence in all moisture levels was significantly lower than the control. The mean adult fly emergence in the control also was not significantly



different from the mean emergence of adult flies from the 7 gram/lb. treatment level of the 70-day Thuricide test (Table 20).

Table 30. Analysis of the effects of addition of water to droppings from quail fed 7 grams of Thuricide/pound of food as shown by adult fly emergence.

	Number of adults emerged/% moisture level					
Replication	0	25	50	75	100	150
1	8	0	0	3	0	0
2	14	2	1	0	0	0
3	10	0	3	0	0	0
4	11	2	0	0	0	0
5	21	6	3	0	0	0
Mean	12.8	2	1.4	0.6	0	0

Maximum 5%
Range (a) = 4.32

(a) Maximum range at 95% confidence level from a multiple range test (Duncan 1955).

A comparison of these moisture level studies with the normal fly emergence data of Table 24, indicated that additional moisture appeared to enhance pathogenicity of the spores. Addition of 25% by weight of moisture to treated droppings gave an increased percent effectiveness of 84% over the normal dry droppings. Addition of 100% by weight of moisture produced 100% increase in effect.

The effect of extreme temperatures on the viability of spores

Berliner (1915a) reported, as optinum for viability, a temperature range of 30° to 40° C. for spores of B. thuringiensis. He also

found that the spores could withstand temperatures up to 100° C. and were only killed when held at this temperature for at least two minutes. Steinhaus (1951a) reported that Mattes found that spores dried at 60° C. remained viable after six years. Husz (1929) reported that better pathogenicity was obtained with growth of the organisms at 30° C. and that spores would remain alive, as demonstrated by successful culture transfer, when subjected in test tubes to outdoor temperatures from 0° C. to -26° C.

These observations were made on laboratory cultures in test tubes under varying conditions of temperature and nutritional base and gave no indication as to the behavior of the bacteria in commercially prepared materials after exposure to extremely low temperatures. Therefore, a test was arranged to give an indication of what changes might be expected in pathogenicity of spores in droppings after an extended exposure to a temperature of -18° C.

Droppings were collected in one pint ice cream cartons from birds fed spores at the rate of 2.8×10^{11} spores/pound of food and from control birds and arranged in four replications. Control and treated droppings were placed in a walk-in freezer at -18° C. and left for one year. At the end of this time the droppings were slowly warmed to room temperature and 25 four- to five-day old house fly larvae were placed in each box to which had also been added 25 ml. of water. After 20 days the number of emerged adults, unopened pupae and larvae were counted in all boxes. The results are shown in Table 31.

These figures show that the spores are quite resistant to low temperatures and therefore should exhibit some carry-over effect in droppings used as compost or plant cover in the winter in northern regions.

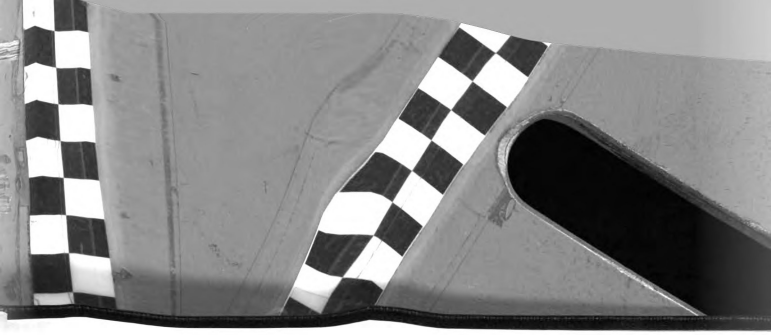


Table 31. Mean percent of all stadia of house flies recovered after development in thawed treated droppings held at -18° C. for one year.1/

Source	Adults	Pupae	Larvae
Control	40	44	8
Treated	0	20	36

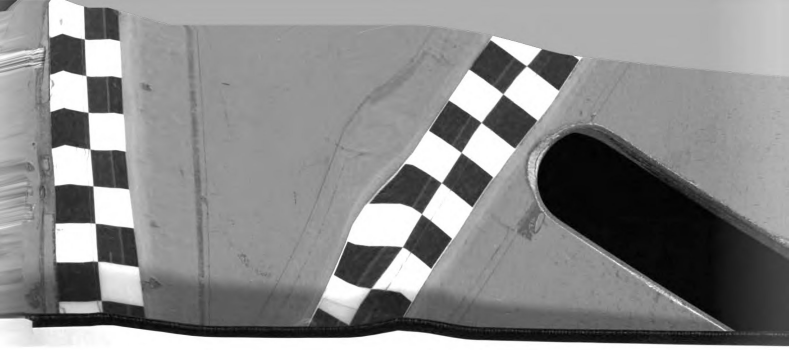
1/ Data not analyzed statistically.

Studies on the median effective dose of B. thuringiensis to house fly larvae

Known amounts of Pure Spore were added to CSMA media in one pint ice cream cartons to give dosage levels of 0.36 grams of spores per box to 2.0 grams of spores per box. The media level in each box was kept at approximately two-thirds of the height of the box. After carefully mixing the spores and media, 25 four- to five-day old house fly larvae were placed in each box. Five replications of each dosage level were made and all boxes were covered with gauze and kept at room temperature (68° to 70° F.) for 20 days. At the end of this time all boxes were opened and the media carefully examined. Data were recorded for the number of emerged adults.

A comparison test was conducted with Agritrol using exactly the same procedure.

Table 32 gives the results of these tests on Pure Spore and Agritrol. All calculations presented were determined by the method of Litchfield and Wilcoxon (1949). The ED_{50}'s for Pure Spore and Agritrol were found to be statistically quite similar and no significant difference in potency was obtained. The slopes of the two curves were

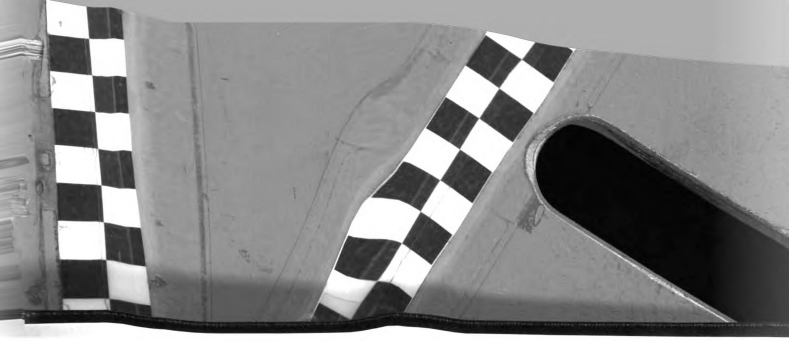


also found to be similar and parallel. Therefore it was postulated that the insecticidal contaminants in Agritrol had no effect on the larval house fly development, and subsequent adult emergence, in the tests of adult fly emergence from quail droppings.

Table 32. Nomograph calculations of the ED_{50}, slope, and potency, to house fly larvae, of Pure Spore and Agritrol mixed with CSMA media.

Observation	Treatment	
	Pure Spore	Agritrol
ED_{50}	2.3×10^9 spores/larva	1.6×10^9 spores/larva
95% Error Factor for the ED_{50}	2.4	1.2
Slope	2.2	2.5
95% Error Factor for the Slope	1.12	1.17
Potency Ratio	0.70[1/]	
95% Error Factor for the P.R.	2.45	
Slope Ratio	1.15[2/]	
95% Error Factor for the S.R.	1.21	

1/ No significant difference in potency.
2/ No significant difference in slope. The curves are parallel.



SUMMARY

The toxicology of commercial formulations of Bacillus thuringiensis Berliner was studied by feeding varied amounts of four formulations to Japanese quail (Coturnix coturnix japonica Tem. and Schl.) as a part of their normal diet. The effectiveness of the spore formulations which passed through the digestive tract of the birds was evaluated by bioassay tests using four- to five-day old house fly larvae in the droppings. These data were correlated with the median lethal spore dose for the larvae.

Because of relatively high insecticidal contamination of two of the formulations tested and the resulting mortality to the quail, additional tests were undertaken to determine the amounts of insecticides present and their effect on the spores and on the control of house fly larvae.

A systematic treatment of the bacteria in each formulation confirmed the identity of the spores as Bacillus thuringiensis Berliner. Only slight variation was observed in the fermentation reactions. No bacterial contaminants were found which could be implicated as being responsible for mortality to the test animals.

The spores of B. thuringiensis were found to have no adverse effect on the normal metabolic activity of quail or white mice. In quail feeding studies effective larval house fly control of 70% to 85% was achieved at feeding levels of 5.4×10^9 to 9.3×10^9 spores per bird per day. At spore concentrations in the formulations of 45

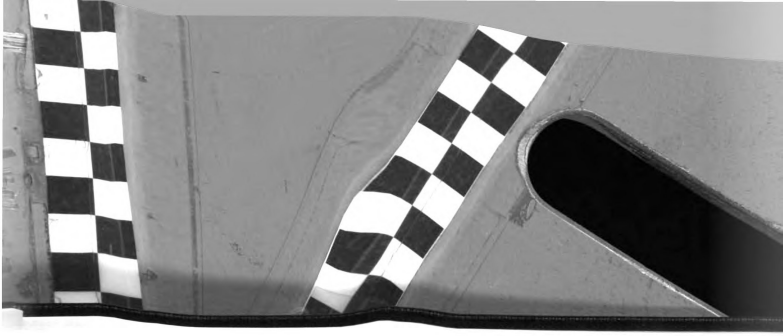
to 70 billion spores per gram, these levels were equivalent to feeding rates of 3.5 to 7 grams of formulation per pound of food. A comparison of these results with the median effective dose for the materials indicated that apparently relatively few spores failed to pass through the animals in a viable state, assuming that all the spores were originally viable.

Contamination of Agritrol with approximately 1000 ppm of DDT and 200 ppm of aldrin did not appear to affect the spore viability or influence the resulting mortality to house fly larvae. The median effective dose of Agritrol was found to be 1.6×10^9 spores per larva. Compared to the ED_{50} of pure spores of 2.3×10^9 spores per larva, there appeared to be no interaction between insecticidal contamination and spore activity to house fly larvae.

At high feeding levels of Agritrol, a delay was encountered in the expression of control of adult fly emergence for two weeks after feeding was begun. The reason for this delay was not evident. The major effect of the insecticidal contamination appeared to be associated with the metabolic activity of the quail and mice, causing consistant mortality at the higher feeding levels.

It was found that holding spores in droppings -18° C. for one year did not seriously affect their viability when thawed and used as growth medium for house fly larvae.

Addition of at least 25% by weight of moisture to droppings appeared to be effective in enhancing spore pathogenicity to house fly larvae. There was a consistant increase in effectiveness of the spores for control of house fly larvae with increases in moisture content of the droppings.



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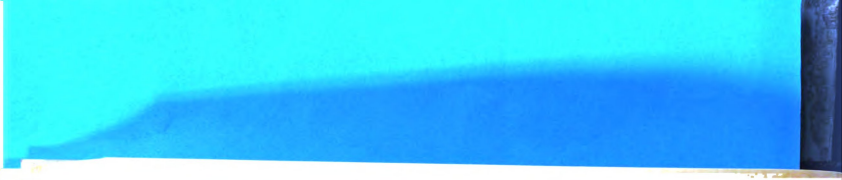

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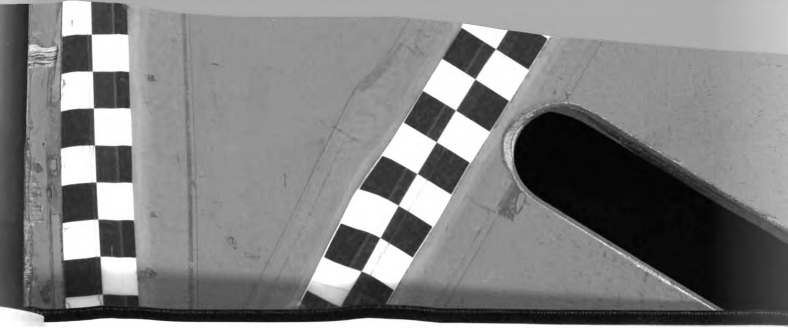

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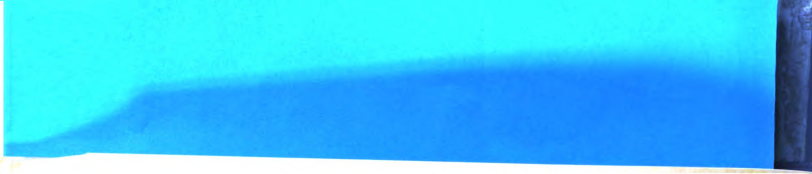

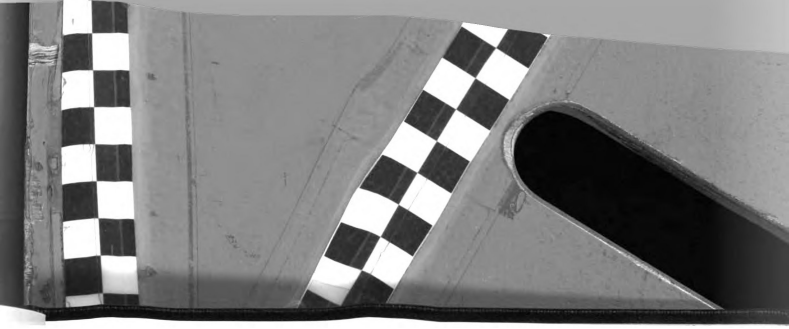

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