LABORATORY STUDIES OF THE EFFECTIVENESS OF *BACILLUS THURINGIENSIS* SEROTYPE H-14 AGAINST *CULEX PIPIENS* LARVAE

BY

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DEDICATION

This thesis is dedicated to my

Father, Alhaji Shehu Mohammed;

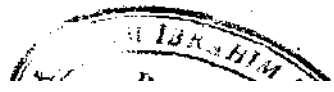
Mother, Hajiya Y. Mohammed

and

To those people all over the world

who have fallen victims of mosquito

transmitted diseases.



DECLARATION

I hereby decalre that this thesis has been composed by myself and that it is a record of my own research work. It has not been accepted in any previous application for a higher degree. All sources of information are specifically acknowledged by means of references.

HAJARA SHEHU

OCTOBER 1996.

CERTIFICATION

This thesis entitled 'Laboratory Studies on the effectiveness of Bacillus thuringiensis H-14 against Culex pipiens larvae' by Hajara Shehu meets the regulations governing the award of the degree of Master of Sciences of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The effectiveness of Bacillus thuringiensis H-14 as a biological control agent was tested in the laboratory against culex pipiens larvae by bic>assay methods. The second, third and fourth instar larvae were exposed to different bacterial concentrations. The percentage mortality of mosquito larvae increased with increase in concentration, exposure time and water volume but decreased with the increase in larval age from second to fourth instars.

The LC50 values after 6 and 24 hours in 150 ml of bacterial suspension were 0.020 mg/1 and 0.0036 mg/3 *for* second instars; 0.04 mg/1 and 0.009 mg/1 for third instars; 0.063 mg/1 and 0.013 mg/1 for fourth instar larvae. In 30 ml of bacterial suspensions, these values were 0.038 mg/1 and 0.008 mg/1; 0.05 mg/1 and 0.015 ng/1; 0.158 mg/1 and 0.017 rag/I for second, third and fourth instar larvae after 6 and 24 hours exposures respectively.

The LC90 values after 6 - 24 hours in 150 ml of bacterial suspensions for second, third and fourth larval stages were 0.'i58 mg/1 - 0.022 mg/1, 0.199 mg/1 •• 0.050 mg/1 and 0.251 mg/1 - 0.071 mg/1 while in 30 ml bacterial suspensions, these values were 0.211 mg/1 - 0.093 mg/1, 0.251 mg/1 - 0.079 mg/1 and 0.791 mg/1 - 0.081 mg/1 respectively.

Statistical tests to determine differences in mortality due to exposure time, beakers and larval instars were all found to be significant ($P>0.05$).

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CHAPTER ONE

1. INTRODUCTION

Mosquitoes are still considered one of the most important vectors of human and domestic animal diseases and a nuisance pest even after massive control and eradication. They have a world wide distribution and are found at elevations of 5,500m and in mines at depths of 1,250m below sea level (Service, 1980).

All mosquitoes belong to the family Culicidae. This family consists of 34 genera arranged in 3 subfamilies, Toxorhynchitinae, Anopheles and Culicinae. There are some 3,100 species of mosquitoes and the most important man-biting species belong to the genera Anopheles, Culex, Aedes, Mansonia, Haemagogus, Sabethes and Psorophora (Knight and Stone, 1977). They are vectors of human diseases like Malaria, Filariasis, Yellow fever, Dengue fever and encephalitides which continues to limit progress both in developed and developing nations (Anonymous, 1973).

Malaria is possibly the most prevalent disease of mankind today. It affects between 200 - 400 million people in 107 countries. It is estimated that at least 100 million cases of malaria occur annually resulting in about 2 million deaths (Newmark, 1983). In Africa, this disease is responsible for the deaths of about 1 million people alone.

In Nigeria, about 1 million cases of this disease is reported annually accounting for 100,000 deaths. This makes malaria the fourth principal cause of death in the country (Adedayoh and Fayiga, 1978). Malaria disables more people and exert a higher material cost than any other parasitic disease (Alvarado and Bruce-Chwatt, 1962).

Although filariasis has more limited distribution than malaria, it is still a common disease in tropical areas. The incidence of this disease has increased in recent years due to urbanization which has led to an increase in breeding areas for the mosquitoes vector. Today, more than 250 million people are affected by this disease (Anonymous, 1973).

Yellow fever is also one of the most virulent diseases of man. Today, this viral disease has been controlled by the use of an effective vaccine. Yellow fever is now limited to Central and South America and some parts of Africa.

Dengue fever, another viral disease, occur throughout the tropical and subtropical regions of the world. Unlike yellow fever, Dengue fever is a prostrating and not a killer disease. However, a more virulent form of this disease called Dengue haemorrhagic fever has resulted in deaths among children in South East Asia (Anonymous, 1973).

Anopheles spp to DDT was reported in 1951 and by 1960, resistance had been observed in about 29 species (Alvarado and Bruce-Chwatt, 1962). Today, over 93 species of mosquitoes have developed resistance to chemicals (WHO, 1982). It appears that effective insecticides are vanishing and their replacement are scarce. The development of new pesticide products is now much reduced as compared to the recent past (Hertlein, et al., 1980).

One of the most promising alternative to chemicals is biological control. It is the influence of predators, parasites and pathogens introduced by man to control target pests (Burges and Hussey, 1971). To date, the most promising biological control agent is the bacterium called Bacillus thuringiensis (Goldberg and Margalit 1977; WHO, 1981; Klowden et al., 1983 and Rishikesh and Quelennec, 1983). Its potential application as a microbial agent has been studied by many scientists. It has been found to be specific for target insects, does not produce toxic residual effects and may persist in field population thus lowering the resistance of insect pests (Heimpel, 1967). Laboratory and field trials with B.t H-14 have been carried out in many parts of the world (WHO, 1982). However, little work has been done in Africa which form one of the mainstreams of mosquito-borne diseases of the world (WHO, 1982).

This present study is undertaken to determine the effectiveness of Bacillus thuringiensis H-14 on the different instars of Culex pipiens larvae in 250 ml beakers containing 150 ml of bacterial suspension and 50 ml beakers with 30 ml of B.t suspensions under laboratory conditions.

CHAPTER TWO

2. LITERATURE REVIEW

The entomopathogen, Bacillus thuringiensis var thuringiensis was originally thought to affect only lepidepterous larvae (Heimpel, 1967; WHO, 1982 and Klowden et al., 1983). However, several early isolates of B.t when used at very high concentration were found to show some activity in mosquitoes (WHO, 1982). In 1977, an isolate called B. thuringiensis israelensis serotype H-14 (B.t H-14) was discovered (WHO, 1980 and 1982). This isolate has been shown to be highly effective (Toxic) against the larvae of medically important dipterans, such as mosquitoes and Blackflies (Goldberg and Margalit, 1977, and Undeen et al., 1978). However, this subspecies of B.t appears to be non-toxic to lepidepterans (Goldberg and Margalit, 1977). The most susceptible mosquito species reported are Aedes aegypti; A. triseriatus and Culex tarsalis (WHO, 1982).

2.1 ORIGIN AND NATURAL DISTRIBUTION

Bacillus thuringiensis was first isolated under this name by Berliner in 1915 (Angus, 1971). However, the mosquito factor, B.t H-14 was first isolated in Negev, Israel in 1977 (WHO, 1982). Since then, twenty-five isolates of B.t H-14 have been isolated in different parts of the world. These areas included the Mediterranean regions, Africa, South east Asia, Europe and North America (WHO, 1982).

2.2 TAXONOMY

One of the most important aspect of any biological investigation is the precise identification of the organism under study. Toumanoff and Le Controller (1957) classified crystalliferous bacteria as varieties of Bacillus cereus Fran and Fran. However, Heimpel (1967) grouped these bacteria as varieties of B. thuringiensis. Early classifications were based on morphological and metabolic characteristics and toxicity on Bombyx mori. Modern classification is based on some biochemical characteristics like specificity of flagellar antigens of vegetative cells, estarase types, antigenic composition of delta-endotoxin and production of toxins (de Barjac, 1981 and Norris, 1964). Crystal serology which was introduced by de Barjac and Bonnefoi, (1963) is also used as a criterium of classification (Krywienczyk and Fast, 1980). To date, the species B. thuringiensis has been subdivided on the basis of its flagellar antigens (H-antigens) into 19 serotypes (WHO, 1982).

2.3 BIOLOGICAL CHARACTERISTICS

Bacillus thuringiensis is a spore-forming bacterium. It produces one or more crystals of toxic proteins (Delta-endotoxin) during the phase of spore-formation (Heimpel , 1967). This property of B.t distinguishes it from B. cereus to which it is similar in morphology, physiology, fatty acid composition, flagellar antigens and have high degree of DNA homology (Yoshimura et al., 1982).

Bacillus thuringiensis are aerobic, gram-positive rods. They measure between 2 - 6 micron long. Their endospores measure 1-1.8 by 0.9 micron while their crystals are 0.4-0.8 by 0.8-1.4 microns. These crystal bodies stain like other material. They are liberated by the lysis of the sporangium at the end of sporulation. It is now known that the crystals production is not linked with spore formation except in a single serotype called the H-2 serotype (WHO, 1982).

2.4 BACILLUS THURINGIENSIS TOXINS

Toxins produced by B.t are the useful metabolites which are harmful to sensitive hosts (Lysenko and Kucera, 1971). They are considered as one of the possible mechanisms of pathogenicity. According to their locations B.t toxins can be grouped into endotoxins and exotoxins. Endotoxins are bound to bacterial cells from which they are extracted while exotoxins are the soluble compounds extracted out during the bacterial growth. This bacterium has the ability to produce several toxins which enables it to control a wide spectrum of insects. Heimpel (1967) reported that more than 137 insects species belonging to the order Lepidoptera, Hymenoptera, Diptera and Coleoptera are affected by this bacterium.

During the last decade, a lot of work has been done on B.t toxins. Heimpel (1967) reviewed that four different toxins have been isolated in cultures of B.t. These are:

alpha-endotoxin or phospholipose C,

beta-exotoxin or Thermostable exotoxin;

gamma-exotoxin (the unidentified enzyme), and

delta-endotoxin or crystal parasporal body.

Today, seven different toxic components in B.t strains have been postulated (WHO, 1982). The two most important toxins are the Beta-exotoxin and the Delta-endotoxin (Quarashi, 1977). These are the only toxins being reviewed here.

2.4.1 BETA EXITIXUB

MCconnell and Richard (1959) demonstrated that the supernatant from liquid cultures of B.t was toxic to insects belonging to several orders. This toxic material was later on classified as Beta-exotoxin (Burgerjon and Galichet, 1965). Beta-exotoxin is a small, water soluble, heat stable dialyzable molecule which is formed only by some strain of B.t (Falcon, 1971). This toxin is an adenine nucleotide that appears to be an ATP analog. It is toxic to invertebrates, birds and mammals (WHO, 1982). In North America and Western Europe, commercial formulation of B.t are exclusively based on strains which do not produce Beta-exotoxin (WHO, 1982).

2.4.2 DELTA-ENDOTOXIN

B.t delta-endotoxin was first discovered by Hannay (1953). Later, Angus (1956) showed the proteinous nature of the crystal and its high toxicity on lepidopterous larvae.

Today, crystals of some B.t strains are found to be toxic to some medically important dipterans (WHO, 1982). Delta-endotoxins are contained within the crystals which are found during sporulation. These crystals are typically diamond-shaped except crystals of B.t H-14 which are of all sizes and shapes (WHO, 1982). Moreover, its crystalline lattice is much smaller than that of the rest. The crystals of B.t H-14 are serologically distinct because their antigenic material is different from those toxins active for other group of insects. They also differ in amino acid composition and contain 3 times more lysine and 15½ times less arginine than the crystals of var Kurstaki (Tyrell et al., 1979; Undeen and Bert, 1979).

2.5 MODE OF ACTION OF EXOTOXIN AND ENDOTOXIN

EXOTOXIN

The present knowledge on the mode of action of B-exotoxin is exclusively based on the work done by Sebesta and Horska (1968). They found that its mode of action is possibly due to the competitive inhibition of enzymes that catalyze the splitting of ATP and pyrophosphate. Earlier, Burgerjon and Galichet (1965) showed that B-exotoxin affect insects only during moulting or metamorphisis. This implies that the toxin interfers with hormones responsible for these physiological changes. Toxicity of B-exotoxin in mammals is mostly the necrosis of the liver which is the principal organ

affected. Other organs that show signs of damage are the kidneys, spleen and adrenal glands (Bond et al., 1971).

ENDOTOXIN

The protenous crystals of B.t H-14 contains protoxins. Each protein is composed of a single subunit of approximately 134,000 dalton molecular weight. However, most subspecies of B.t produce crystals containing at least one protein whose molecular weight is about 130,000 dalton units. When a crystal is ingested by a susceptable host, the protoxin is quickly converted to smaller toxic subunits in the presence of high PH and suitable enzymes in its gut (Tyrell et al. 1979). The specificity of B.t H-14 is probably due to the absence in most invertebrates of the enzymic system that transform its protoxin to the actual toxin (WHO, 1982).

The gross pathology associated with Delta-endotoxin is characterised by a rapid paralysis of the mouthparts and gut (Anderson and Rogoff, 1965 and WHO, 1982). Effect of toxin is seen some 10 minutes after ingestion which is reflected in the increasing alkalinity of the haemolymph (Anderson and Rogoff, 1965). This rapid PH change brings about the total paralysis within 1-7 hours which eventually results into the death of insects within 24 hours. Paralysis consist of the loss of integrity of the gut epithelium as a result of the swelling, distortion and final bursting of cells (WHO, 1982).

Histopathological studies showed that the cells of the midgut epithelium were mostly affected. The gastric caeca and the posterior stomach showed the greatest signs of damage (WHO, 1992). These cells become free from one another and also from the basement membrane of the gut (Anderson and Rogoff, 1965). Death results probably, from disruption of the ionic regulation capacity of the midgut and the subsequent leakage of gut content into the haemocoel (Couch, 1980). Earlier, Ramakrishnan (1968) attributed the rise in hemolymph level of K^+ as responsible for the paralysis.

Some species of Bacillus thuringiensis, a spore-forming bacteria, produce endospores which allow them to persist in a dormant state outside the body of the intended host. Upon ingestion by a susceptible host, the spores germinate in the gut to produce the vegetative cells. These cells enter the hemocoel where they multiply rapidly, destroy certain tissues and soon fill the cavity. This stage of infection is called septicemia (Falcon, 1971). Prior to death of host, thick walled spores are formed which are released after death of the host. However, B.t H-14 does not kill mosquito larvae by the development of spores but through the direct action of the Delta-endotoxin (Rishikesh and Quelennec, 1983).

2.6 STANDARDIZATION

Bacillus thuringiensis produce several toxic materials which has created problems with its production and

standardization (Heimpel, 1967). Standardization, according to Burges and Thomson, (1971) is the adoptation of the most appropriate units for measurement of insecticidal potency of a pathogenic preparation. It enables a manufacturer to reproduce a product of constant potency comparable to the potency of other products. In the past, spores were the only units available for standardization (Fisher, 1963). However, the spore-counts do not give an accurate estimate of crystal contents of a preparation. Similarly, the number of spores or crystals contents bear no relationship to the activity of the delta-endotoxin. Also, the proportion of the spore and endotoxin containing crystals of preparation of B.t varies with the Bacillus and its mode of production. Due to these short-comings of the spore-counts, other better methods like bioassay has been adopted (Rishikesh and Quelennec, 1983).

As a result of this, the International Organisation of Biological Control (IOBC) has agreed that the standard reference material should be the 'Institut Pasteur Standard' 1978 (IPS, 78) (WHO, 1980). The first standard was developed by the International Reference Centre on B. thuringiensis H-14 in 1978. It was assigned the arbitrary fixed potency of 1000 toxic units per milligram (WHO, 1980 and 1982). The re recommended target insect was the late third instar or early fourth instar larvae of Aedes aegypti. This standard powder contains spore-crystal complex of serotype H-14. Because of

the failure in stability after exposure at 50°C (Larget, 1982) and significant variation in data of several scientists, a second reference material, the IPS-80 was prepared (Rishikesh and Quelennec, 1983). This has been avaluated at 15,000 toxic unit per milligram on Aedes aegypti (WHO, 1982). Until the final standard IPS-82 becomes available, the IPS-80 remains the reference material (Rishikesh and Quelennec, 1983). An alternative to bioassay is currently being developed using immunochemical technology (WHO, 1982). Here, the amount of crystal in a spore-crystal preparation of B. thuringiensis is determined by its reaction with specific antiserum in an immunological test (Burges and Thomson, 1971).

2.7 PRODUCTION AND FORMULATION

All Bacillus thuringiensis toxins are commercially produced both in semi-solid and submerged fermentations (Dulmage and Rhodes, 1971). The semi-solid process yield wettable powders and dust formulation containing spores, crystals and exotoxin. The submerged process, on the other hand, produce flowable formulation containing spore-crystal complex. Bacillus thuringiensis, especially serotype H-14 can be grown in a variety of protenaceous material such as commercial powder of soya bean products, fish meal, dried milk products, blood and serum from a variety of animal as well as human faecal material (Hertlein et al., 1980). Out of all these media, soya broth substrate had been shown

to produce the highest larvicidal activity, beef and egg albumen substrate provided for the formation of few spores with high toxin production per spore while dried milk medium produced many spores with little toxin per spore (WHO, 1982).

Bacillus thuringiensis have been formulated into dust, wettable powders, granules, aqueous and emulsified concentrates (Angus and Luthy, 1971). For developing countries, cottage-industry type of production, which is simple and does not require industrial formulations have been suggested (Hertlein et al., 1980).

2.8 STABILITY

It has been shown that the delta-endotoxin of B.t H-14 can withstand a temperature of up to 80°C during 24 hours without losing its activity but exposure to 120°C for 15 minutes result in its inactivation (WHO, 1982). Ultra-violet or gamma radiations do not harm the crystals but spores are killed by UV light (WHO, 1982). Primary powder of B.t H-14 was found to rapidly loss its biological activity when stored in ethanol while it is preserved in neutral distilled water (WHO, 1982). When the stability of toxicity of the bacterium toward Culex quinquefasciatus larvae was tested in polluted water, the percentage mortality of the larvae decreased. This is because mud and other particulate matter affect bacterial activity (WHO, 1982).

A laboratory test carried out in Thailand by Pantuwatana (1980) using B.t H-14 in distilled water at room temperature, showed that the bacterium was still active for about 165 days. A similar study on the longivity of B.t H-14 was carried out in Nigeria. The powder was stored for 22 months both at room temperature and in refrigerator. No significant reduction in the potency of the powder was observed when it was later tested against 2nd instar larvae of Aedes aegypti (Prasertphon and Rishikesh, 1979). Similarly, the stability of the toxin under tropical conditions was also studied in Barkina Faso and French Guianae. No loss in activity was observed when primary powder was kept in sealed dry containers for up to 6 months. Apparent loss of activity was however observed in powder exposed to air and humidity (WHO, 1982).

Laboratory studies have shown the stability of the crystal endotoxin on dead larvae. This may provide additional control when mosquito larvae feed on B.t H-14 killed larvae that contain vegetative cells with crystals and spores of the bacterium.

2.9 BACILLUS THURINGIENSIS AS A BIOLOGICAL CONTROL AGENT

It is about over a hundred years since man first learnt that bacteria can cause diseases in insects. Today, over 90 species and varieties of pathogenic bacteria have been isolated and described from insects (Anonymous, 1969). Of

these, Bacillus thuringiensis which have shown a wide host range in various orders of insects have been developed on commercial basis for the possible control of pests. This bacterium has been produced in many countries under various trade names such as Biotrol, Dipel, Microtrol and Thuricide (Guarashi, 1977). These preparations have been tested successfully against more than 137 insect species mostly belonging to the order lepidoptera (Heimpel, 1967). Most of these pathogenicity tests have been carried under laboratory conditions while others have been tried in the fields (WHO, 1962). Large scale tests have been conducted in Russia, France, Holland, United States and Canada. Some of the developing nations that have shown interest in the use of this microbial agent for the pest control are Nigeria, Philippines, Republic of Korea and Thailand (WHO, 1981).

In recent years, commercial preparation of B.t. have been produced by at least 12 manufacturers in five countries. In the United States, hundreds of tons of this material are manufactured each year with continous increase in production annually. One firm, for example, increased production more than 200 folds over the past eight years (Falcon, 1971). A very conservative estimate indicated that over 5,000 tons of B.t have been used for the control of insects (WHO, 1982). In the United States alone, B.t products are currently registered for use on more than 20 crops (Falcon, 1971). These included alfalfa, bananas, cabbages, cauliflower, lettuce, melons,

potatoes, tomatoes and forest trees (Heimpel, 1967).

In the past, products of B.t have yielded a generally good to excellent results in the control of cabbage worm, Pieris brassicae (Herf and Krieg, 1963). Angus (1968) reported the protection of over 50% of the cole-crop and lettuce from the cabbage looper, Trichoplusia ni in Southern California during the period between 1965 and 1966. Preparation of B.t have also been used against the European cornborer, Ostrinia nubilalis (Raun, 1963). This biocide was formulated into a new encapsulated form to achieve control of the pest. Allen (1961) reported the use of B.t as cornmeal bait to kill the tobacco budworm Heliothis virescens. The control of the cornworm Heliothis zea have been achieved using this bacterium (Tanada and Reiner, 1962). The tobacco hornworm, Manduca sexta, is also controlled by commercial preparations of B.t, especially in the production of chemical free tobacco in the U.S.A. (Beggs, 1964).

During the past decade, B.t have been tested extensively for cotton pest control (Aragon, 1964). Leaf damage by T. ni was reduced significantly in large scale field tests in the United States (Falcon et al., 1968). Also significant reduction in larval population of cotton leaf perforator Bacculatrix thruberiella was obtained (Aragon, 1964).

Since the 1950s, B. thuringiensis has been tested for control over 20 species of lepidopterous pests on more than 10 kinds of fruit trees. The codling moth, Cydia pomonella, because of its great economic importance had received considerable attention. In most tests, worm damage to apple was reduced considerably but insufficient by western standard because the larvae feed only slightly on the surface before tunneling into apple (Falcon, 1971).

In peaches, oriental fruit moth, Grapholitha molesta could not be controlled even with several concentration of Thuricide and Biotrol (Falcon, 1971). However, an important lepidopoteran pest of peaches, the peach twig boarer, Anarsia lineatella was susceptible to B. thuringiensis (Krieg, 1963).

Bacillus thuringiensis has also been used to control the grape leaf folder on grapes in California (Jenson, 1966). The bacterium is now an important part of the integrated pest control programme for grape pests (Falcon, 1971).

2.10 EFFECTIVENESS OF B.T H-14 ON INSECTS

CULICIDAE

Laboratory tests have shown that Aedes and Culex larvae are more sensitive to this bacterium than Anopheles larvae (WHO, 1982). This is probably due to the surface feeding habit

of Anopheline mosquitoes while the other two species are bottom feeders. Nicolescu (1982) reported that Culex pipiens larvae have higher susceptibility to B.t H-14 than Anopheles atroparus, while Aedes aegypti have the highest susceptibility. There is also some variation in sensitivity to this bacterium between strains of the same species of mosquitoes (WHO, 1982). For example Culex molestus has been observed to display high variation in susceptibility between field collected larvae and those reared in the laboratory (Nicolescu, 1982).

The results of both field and laboratory evaluations have shown that water temperature, residual chlorine in tapwater, depth of water, sunlight, organic and inorganic matter in water may affect the activity of the bacterium against mosquito larvae (WHO, 1882). For instance, B. thuringiensis H-14 was found to be about 85 times more active against 4-day old larvae of Aedes aegypti in distilled water than in pond water (WHO, 1982).

Bacillus thuringiensis have been shown to mix well with other chemical insecticides to produce better effects (Heimpel, 1967). A study carried out on Aedes aegypti showed a synergic effect of about 1.8 to 1.9 times with chlorophoxim and temephos (WHO, 1982). Pyrethroid compounds decrease the effectiveness of B.t H-14 because they act quickly and disturb the feeding of larvae (WHO, 1982).

The possibility of cross resistance of this bacterium to mosquito larvae which are resistant to conventional insecticides has been examined. The larvae of Culex quinquefasciatus and Anopheles albimanus were used for the tests. It has been observed that the existing mechanisms of resistance toward carbamates, organophosphate and pyrothroids do not appear to confer a significant advantage to mosquito larvae in presence of B.t H-14 toxin (WHO, 1982). Also cross resistance to DDT does not appear to extend to B.t (Van Essen and Hembree, 1980).

B.t H-14 is very active and has rapid and specific action against mosquito larvae (WHO, 1982). This is true even in harsh environment such as salt marshes or at relatively low temperature in ponds containing large quantities of vegetal material (Luthy et al., 1980). There is no evidence of recycling of B.t H-14 in any of the habitats where evaluations were carried out even though survival of the bacterium has been observed in dead larvae in the laboratory (WHO, 1982).

SIMULIIDAE

The potential of B.t H-14 as a blackfly control agent has been investiguted under laboratory conditions since 1978 and later in the field since 1979.

Tests carried out in the laboratory demonstrated the high potential of the bacterium in the control of black flies. Different species of simuliid vary in their response. The younger instars are more susceptible than older blackfly larvae (Molloy et al., 1981). Preparations of B.t H-14 differ in their effectiveness. In a test conducted, 84% mortality was recorded after 1 minute exposure to $5x10^6$ spores/ml (IPS-78 powder) and 99% after 1 minute exposure to $1x10^6$ spores/ml (Undeen and Berl, 1979). A direct correlation was found to exist between efficiency and particle size (WHO, 1982). Tests carried out on S. damnosum complex showed that the larvae select particles in relation to their size. These particle sizes are related to the capacity of their ingestion and toxicity, larger particles have greater lethal effect (Elsen, 1982).

Field evaluations conducted with B.t H-14 have generally confirmed laboratory exposures. They showed high toxicity to a wide range of blackfly species under diverse conditions in different parts of the world. Temperature of the water body plays a significant role in the activity of this bacteria. For example, the warm water temperature ($26.8^{\circ}C$) of Marahoué river in West Africa facilitated the efficacy of the bacteria during a large scale field trial (Lacey et al. 1982). However, high levels of simuliid mortality were reported at temperature as low as $3^{\circ}C$ (Undeen, and Colbo, 1980).

2.11 SAFETY OF NON TARGET ORGANISMS

Microbial insecticides used for the control of insects must be safe enough before they are accepted for control purposes. In countries like the United States, West Germany, Holland, New-Zealand, Canada, Britain, Japan and France, bodies were set up to look into the safety of these biological control agents before they are registered (Heimpel, 1971).

Safety studies including human volunteer tests, were necessary. These tests carried out between 1956 to 1958 allowed the safe use of this bacterium and its varieties on vegetables and forage crops. By 1960, commercial applications of B. thuringiensis based preparations to food and forage crops were allowed (Heimpel, 1971). Extensive laboratory and field safety trials are being regularly conducted (WHO, 1982).

In aquatic environments, the only non-target fauna susceptible to B.t H-14 at dosages used for the control of mosquitoes were dixids and chironomids (Culicidae) (WHO, 1982). Chironomids are moderately susceptible at these dosages in the laboratory and the field except for Chironomus thummi which was found to be very susceptible (WHO, 1982). The predatory mosquito, Toxorynchites splendens was reported unharmed by B.t H-14 intoxicated mosquito larvae (WHO, 1982). Similarly, B.t H-14 do not harm aquatic lepidopterans (Pyralidae) but terrestial Lepidopterans are

moderately susceptible. Toxicity studies carried out on honey bees (Apis indica) showed no adverse effect on the organisms or on honey production and quality (Bailey, 1971).

Toxicological studies were carried out with B.t H-14 on various laboratory animals like mice, rats, guinea pigs and rabbits. Different routes of administration of bacteria, like subcutaneous, intra-peritoneal, oral, percutaneous, inhilation, occular and scarification were used. No acute or chronic toxicity could be demonstrated in these animals (Heimpel, 1971). Eye irritation safety tests carried out in rabbits showed only slight irritation (Hertlein et al., 1980). However, the bacteria can persist in the eye for at least one month (WHO, 1982). Another safety test carried out with mice receiving B. thuringiensis H-14 in their food for over two generations showed no effect on weight changes, mortality or fertility (WHO, 1982). Intracranial injection of B.t H-14 into rats at less than $1x10^6$ variable organism was not harmful (Hertlein et al., 1980). Anaphylactic shock could not be induced in guinea pigs, while successive serial passage in mice did not lead to the appearance of virulence (WHO, 1982). Acute and chronic feeding tests showed no apparent toxicity on ducks, laying hens and fish (Heimpel, 1971).

Eighteen human volunteers commenced a 5-day course of injestion of 1gm each of the bacterium. In addition five of the volunteers inhaled 100 mg of the powder daily for 5 days.

All subjects when thoroughly examined were found to be healthy and all the extensive laboratory tests were negative (Heimpel, 1971). Multiplication of B. thuringiensis does not occur in mammalian tissue in vivo (Hertlein et al., 1980). The bacteria persist for three to four hours at the site of injection and later collects in the spleen where they may be destroyed (WHO, 1982). The above results confirmed the safety of Bacillus thuringiensis and its varieties for mammals.

2.12 ECONOMICS

There is no sufficient knowledge on the economics of microbial insect control (Dulmage, 1971). Except for few private and confidential studies by industries, the cost of production and application of microbial organisms have not been thoroughly investigated. Essential basic research is being carried by universities and government agencies in countries like Russia, Canada, England, Germany, The Netherland and the U.S.A. since the financial risks would be too high for private companies to undertake such studies.

In 1971, the U.S. government spent about two million dollars for research and assigned 40 - 60 senior scientists to these projects (Dulmage, 1971). This money was spent primarily in searching for new pathogens, producing and utilizing them. This contrasts to the amounts spent by the

governments for research on chemical pesticides. The money was largely devoted to evaluate compound previously synthesized and screened by industry. Even though the overall expenditures for research were higher for chemical insecticides, the expenditures for the development of new products were probably greater for the microbial agents (Dulmage, 1971). The world-wide total for industrial investigation of insect control with B.t probably did not cost more than 600,000 dollars per annum in 1971. This contrast with the 100 million dollars per year spent by industries in investigating chemical pesticides (Dulmage, 1971).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 MAINTENANCE OF MOSQUITO COLONY

Culex pipiens, a medium sized culicine mosquito with uniformly brown thorax and stripped abdomen with rounded tip were reared in the laboratory. Male and female mosquitoes were collected by means of sweep net (Plate I) and confined to two separate cages marked as A and B (Plate II). Cage A measured 41.0 x 37.5 x 70.0 cm while cage B measured 45.2 x 45.0 x 79.5 cm.

3.1.1 EGG LAYING

Female mosquitoes in the cages were provided with blood meals from pigeons for maturation of eggs. This is because greater number of eggs are laid by females that have fed on birds rather than on mammals (Krishnamati and Pal, 1958). For this purpose, the thoracic and abdominal feathers of the pigeons used were plucked off to make their skin clear and accessible for feeding by the mosquitoes. The wings and legs of these birds were tied and placed inside the cages in the evenings and left overnight. The birds were duly fed with some grains and water. Thick layers of cotton wool soaked in 4% sugar (sucrose) solutions covered with filter paper in petri dishes were also placed in the cages for oviposition by the gravid female mosquitoes. After the first batches of eggs

were laid, the female mosquities regularly fed on blood at an interval of 12 hours (Subra, 1980).

Male mosquitoes fed on 7% sugar solutions poured into small glass bottles containing cotton wool. About 2cm of the cotton wool was left sticking out of each bottle for the mosquitoes to feed on, 4% sugar solutions were provided in the cages for the females to feed on in the absence of blood meals.

3.1.2 REARING OF LARVAE

Egg rafts laid on moist blotting paper were collected and transferred into rearing pans (Plate III). The pans used were enamel containers of about 21.0 cm in diameter and 8.20 cm deep. The lids of these pans have their centres covered with net measuring 13.5 cm in diameter. These pans were half filled with deionized water and dried grass of the species Pennistum pedicellatum. The contents of these pans were left for 24 hours to allow for the infusion of the grass and the solution to attain room temperature. The infused grass solution provided the necessary organic and inorganic food needed by the larvae.

Eggs which were whitish at the time of laying turned dark grey at the onset of hatching. Larval development from one instar to another was roughly taken as 48 hours as recommended by Subra (1980). The first set of larvae which hatched from

the eggs were regarded as the first instars. Forty eight hours after their emergence, some of these larvae were used for bioassays as second instars. The remaining second instar larvae were kept for another 48 hours after which they were labelled as third instars which later developed into the fourth instars after another 48 hours. New adult mosquitoes for the colony emerged from the pupae that had developed from the fourth instar larvae.

The temperature of the cages was maintained between 28 $\pm$ 1°C. Light bulbs were used to increase the temperature during cold days and also to induce mating. Relative humidity was maintained at about 60 - 85% by placing damp cloths over the cages when necessary. Aerators were connected to the rearing pans to prevent scum formation and provide adequate aeration. Water was added after every 4 - 5 days to replace the amount lost from the rearing pans. Mosquito colony was maintained until the end of the experiment.

3.2 PREPARATION OF SERIAL DILUTIONS

Thoroughly homogenized dilutions of the bacterial powder was made from a stock solution. This stock solution was prepared by weighing 50 mg (0.05g) of Bacillus thuringiensis H-14 powder and mixing it with 10 ml of deionized water in 1.5 x 1.7 cm test tube. The contents were thoroughly mixed with a stirring rod for 5 minutes. The stock solution had

a concentration of 5,000 mg of bacterial powder per litre. From this homogenate, another stock solution was made in another test tube by adding 0.1 ml of the homogenate to 9.9 ml deionized water. This was then mixed thoroughly with a stirring rod for a few minutes. The concentration in this stock solution was 50 mg per litre (UNDP/World Bank/WHO, 1985).

3.3 EXPOSURE OF LARVAE TO B.t SOLUTIONS

Bioassays were carried out in 250 ml plastic beakers with 150 ml of B.t H-14 suspension and 50 ml beakers containing 30 ml of bacterial suspensions.

150 ml of deionized water was poured into each of the six 250 ml beakers used in a bioassay. Twenty (20) live larvae of Culex pipiens were transferred to each beaker with the help of a brush. By means of micropipette, 120, 90, 60, 30, 24 and 15 microlitre suspensions from the stock solution of B.t H-14 were added to beakers marked 1, 2, 3, 4, 5 and 6 respectively giving a final solution of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg per litre in each of the series of beakers respectively.

Similarly, 30 ml of deionized water was placed into each of the six 50 ml beakers. Twenty (20) live larvae and 24, 18, 12, 6, 4.8 and 3 microlitre of the stock solution containing bacterial suspension were added into each of the beakers which

also gave final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg per litre in containers marked 1, 2, 3, 4, 5, and 6 respectively. Second, third and fourth instar larvae were exposed separately to various concentrations of bacterial suspension in both 250 ml beaker containing 150 ml of bacterial suspensions and 50 ml beakers with 30 ml of bacterial suspensions. First instar larvae were not included in these tests because of the difficulty involved in handling them.

Small amount of brewer's yeast and bread crumbs was added to each container to avoid excessive mortality. Mosquito larvae during these trials were exposed to bacterial suspension for 24 hours and mortality recorded after every 6 and 24 hours. During the course of this work, a total of 10,080 larvae were used. Each bioassay test was repeated 6 times (Appendix, 1 - 6).

Control beakers contained deionized water and food material. Pupae were not included in mortality counts. The temperature of the laboratory where bioassays were carried out was maintained at approximately 30 $\pm$ 1^{O}C.

3.4 DATA ANALYSIS

Concentrations and their corresponding mean percentage mortalities were plotted on Probit-log scale graph sheets. Logarithms to the base of 10 were used for transformation of

bacterial concentrations. The equation for the lines of best fit was used as

$$Y = a + bx \quad (1)$$

where Y is the mean percentage mortality in probit; a = intercept on Y-axis, b = the slope and x = log concentration. Both the intercept (a) and slope (b) were calculated using the equations:

$$6a + bEx = Ey$$

$$Exa + bEx^2 = Exy \quad (2)$$

standard statistical methods like correlation and analysis of variance were used to analyse the data. All the values were tested at 5% significant level.

Standard error of LC50 values was determined by

$$\frac{1}{b}\sqrt{\frac{1}{Snw}} \quad (3)$$

where b = slope, n = number of live larvae used in each beaker and w = the weighting coefficients, while the S.E of LC90 was calculated using

$$\frac{1}{b}\sqrt{\frac{1}{Snw} + \frac{m-\bar{x}}{Snw}\,(x-\bar{x})^2} \quad (4)$$

where m= LC90 value in log and $\bar{x}$ = mean concentration in log (Finney, 1964).

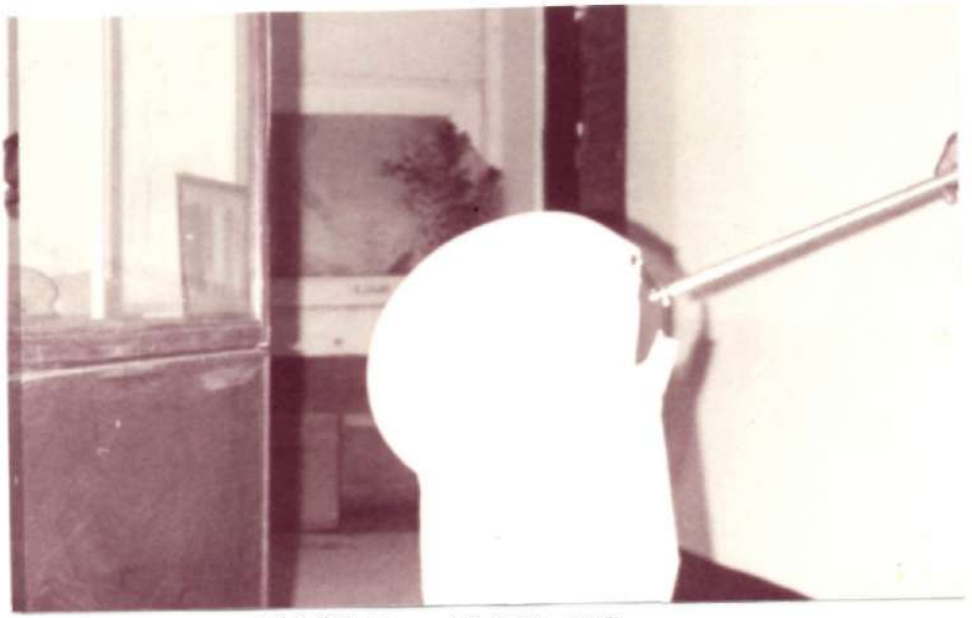


PLATE I: SWEEP NET.



PLATE IIA. CAGE A.



PLATE IIB. CAGE B.



PLATE III. REARING PAN.

CHAPTER FOUR

4. RESULTS

The efficacy of Bacillus thuringiensis H-14 against second, third and fourth instar larvae of Culex pipiens was tested in the laboratory in two types of containers. These were 250 ml beakers containing 150 ml of bacterial suspensions and 50 ml beakers with 30 ml of bacterial suspension. Six (6) beakers of each type were used during bioassay. Twenty (20) live larvae form the initial larval pupulation in each container were exposed to bacterial suspensions. Each bioassay test was repeated six (6) times (Appendix 1 - 6). Observations on the mortality and the number of fourth instars larvae which have developed into pupae were recorded against their corresponding dosage concentrations after every 6 and 24 hours exposures (Appendix 1 - 6).

It has been observed that the dying larvae at first became sluggish and lerthagic as paralysis sets in after ingestion of B.t H-14 and spreads to all parts of the body. After 24 hours exposure when death have occurred, the colour of the dead larvae changed from the usual dark grey to pale grey. The body surfaces and parts started to disintegrate. However, no dead larvae were found in any of the containers marked as controls throughout the experiment.

Food particles (Yeast and Bread crumbs) were added to all B.t H-14 suspensions used for bioassays. This was because during the early part of this experiment, when food materials were not added, the rates of mortality were very high which also occurred in control beakers.

4.1 EFFECT OF B.t H-14 ON DIFFERENT LARVAL INSTARS

Three different larval stages, second, third and fourth instars were reared for the purpose of bioassays. They were exposed to B.t H-14 suspension in 250 ml beakers for 6 and 24 hours durations.

4.1.1 SECOND INSTAR LARVAE

Mortality of these mosquito larvae increased with increase in concentration of bacterial suspension. The highest mortality occurred in solutions of 0.04 mg/l concentrations (Figures 1 and 2). The highest number of deaths observed in 250 ml beakers after 6 and 24 hours ranged between 63.3 and 97.5 percent. In the 30 ml series of bacterial suspensions contained in 50 ml beakers, the highest mortality rates were 54.16 and 80.0 percent.

The mean percentage mortalities of this group of larvae when plotted against concentrations on Probit-log graph give a linear relationship (Figures 1 and 2). The mean LC50 and LC90 which are the concentrations at which 50 and 90 percent

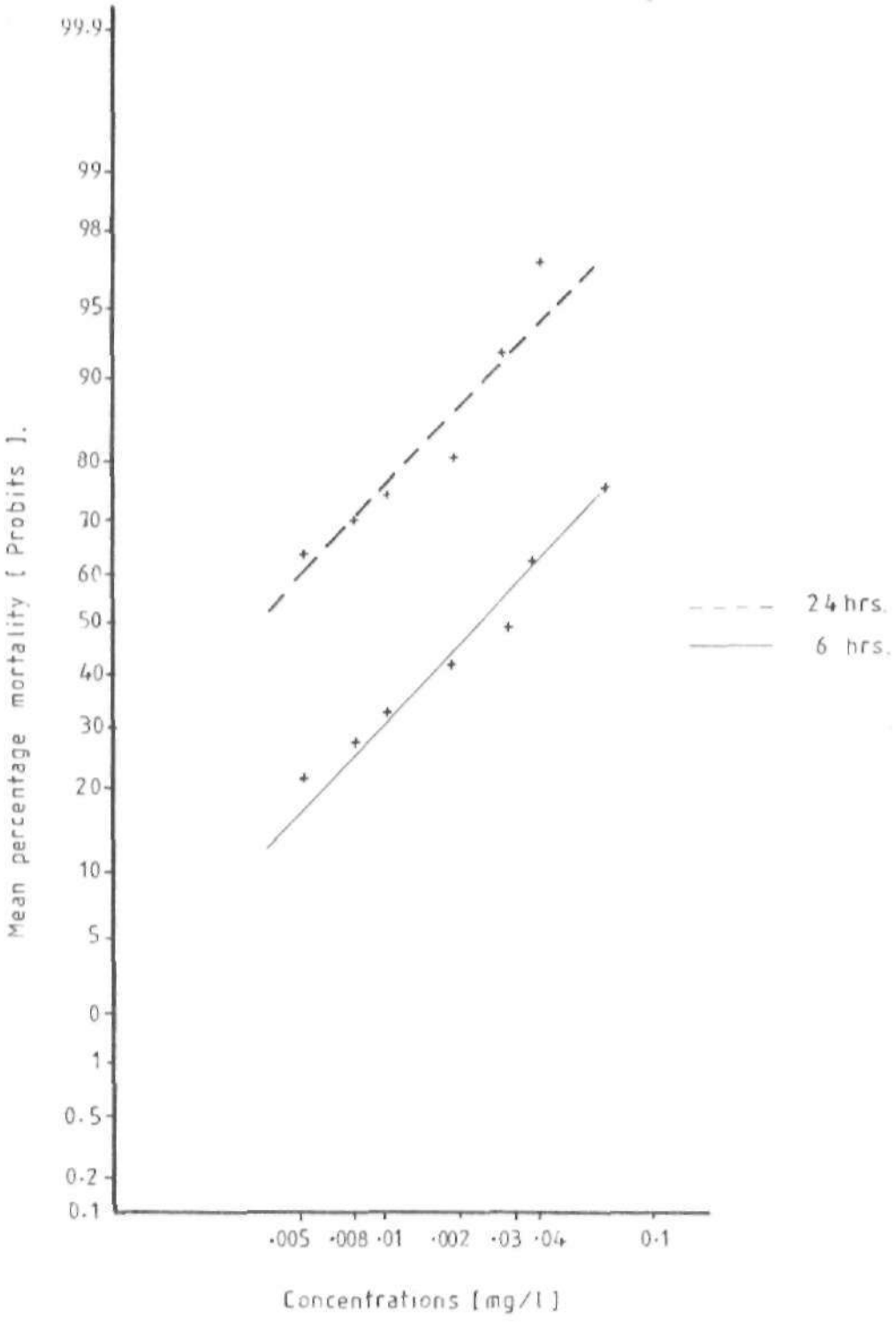


Fig. 1 Dosage / mortality regression lines for second instar larvae in 150 ml of bacterial suspensions.

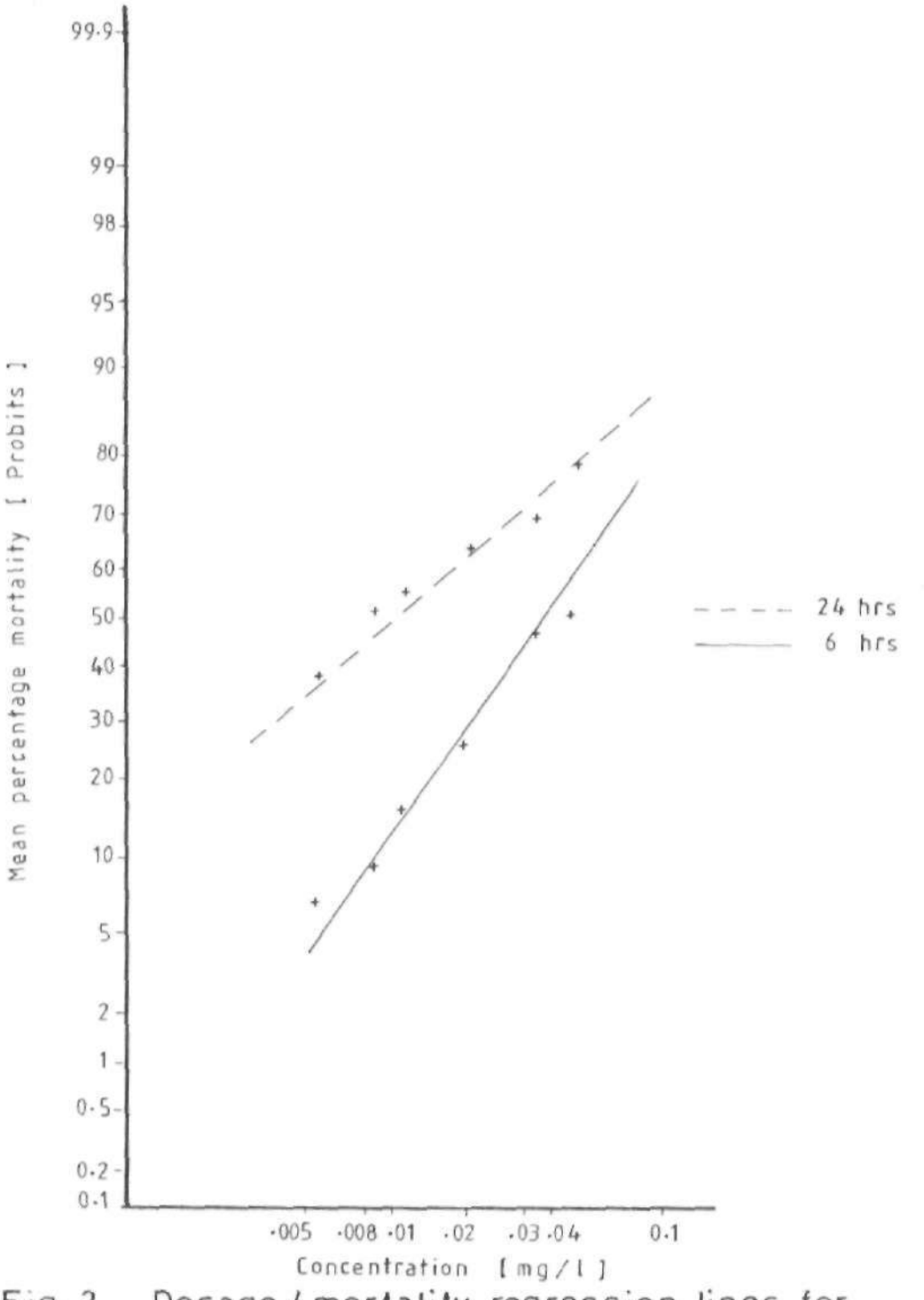


Fig. 2. Dosage / mortality regression lines for second instar larvae in 30 ml of bacterial suspensions.

respectively of the mosquito larvae would die were graphically determined. The LC50 values after 6 and 24 hours were 0.02 mg/l and 0.0036 mg/l in 250 ml beakers while 0.035 mg/l and 0.009 mg/l have been determined for bioassays in 50 ml beakers after 6 and 24 hours exposures. Similarly, the LC90 values after 6 and 24 hours in 250 ml cups were 0.158 mg/l and 0.022 mg/l, and 0.211 mg/l and 0.093 mg/l in 50 ml containers respectively.

4.1.2 THIRD INSTAR LARVAE

The mortality of this larvae also increased with concentration with the highest mortality occurring in solutions of concentration of 0.04 mg/l. In the bigger containers (250 ml beakers) containing 150 ml of bacterial suspension, the highest mortality for the third larval instars recorded during 6 and 24 hours exposures were 40.8 and 90 percent. Smaller 50 ml beakers, on the other hand had their mortilities ranging from 34.2 percent after 6 hours to 75.8 percent after 24 hours (Figures 3 and 4).

Estimated 50 percent lethal concentrations of 0.04 mg/l and 0.009 mg/l were graphically determined for 6 and 24 hours exposures in 150 ml of B.t solutions; the 30ml-LC50 values were estimated at 0.056 mg/l and 0.015 mg/l after 6 and 24 hours respectively. While the 150ml-LC90 values for third instars were 0.190 mg/l and 0.050 mg/l after 6 and 24 hours,

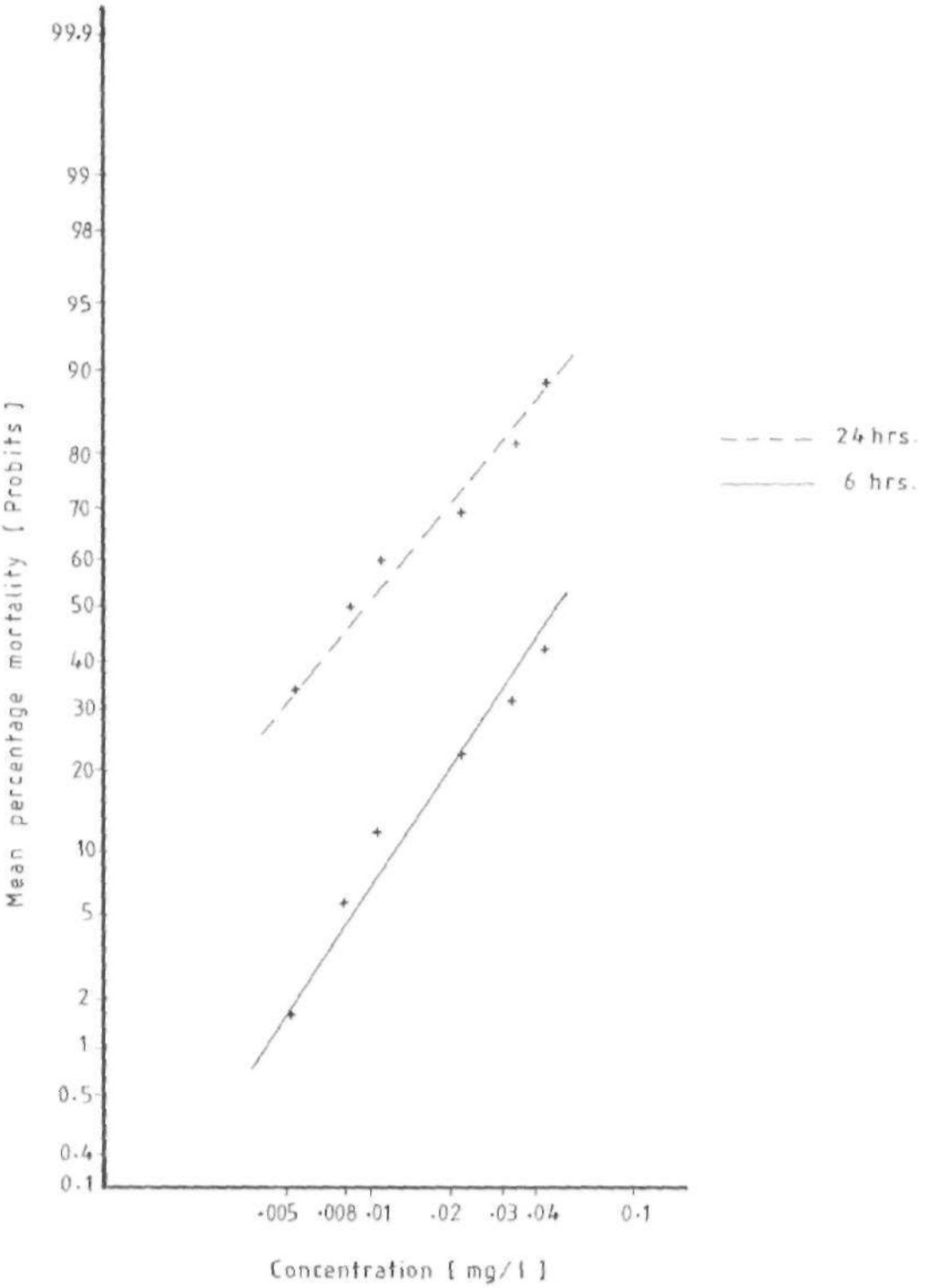


Fig. 3. Dosage / mortality regression lines for third instar larvae in 150 ml of bacterial suspensions.

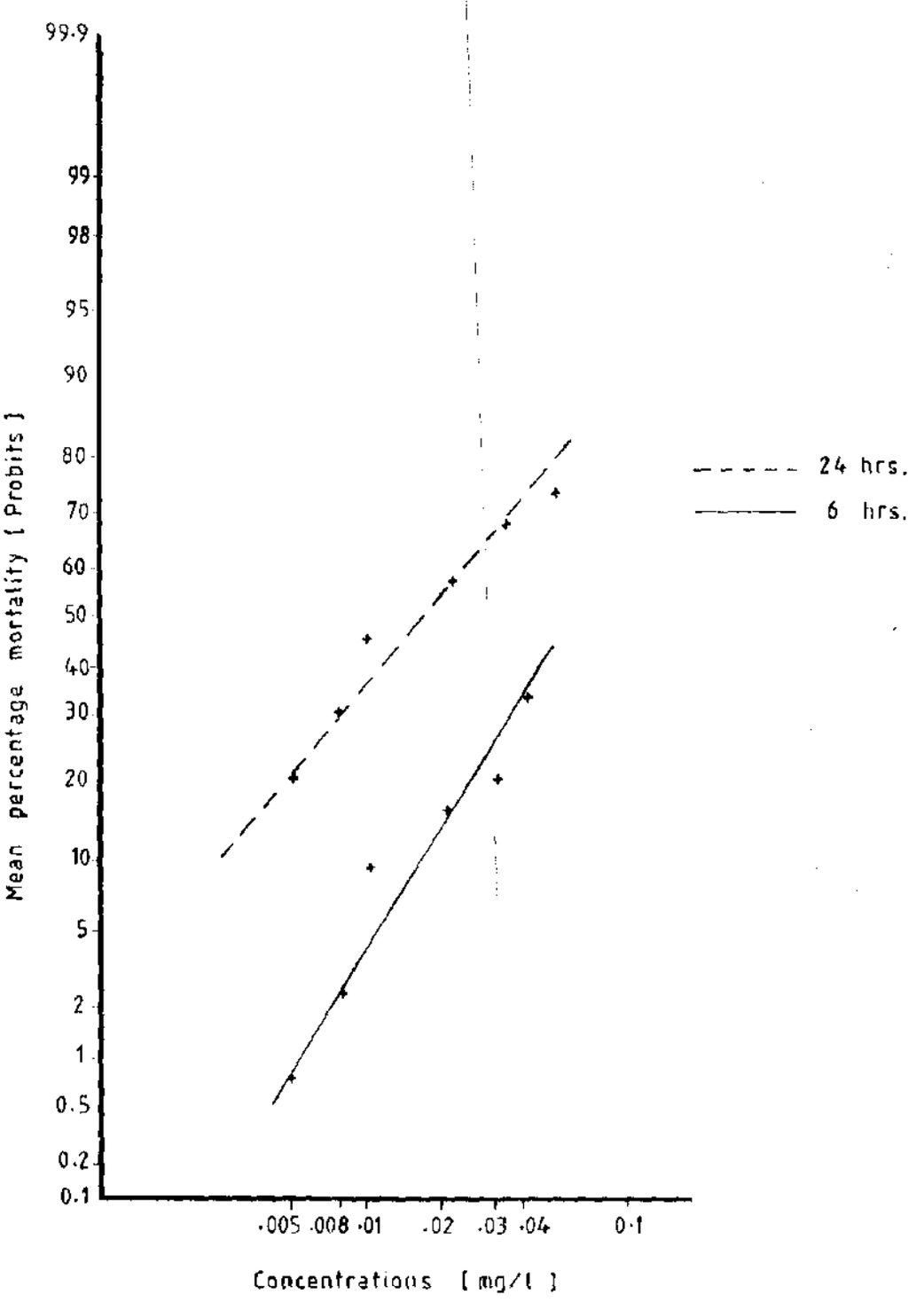


Fig. 4 Dosage / mortality regression lines for third instar larvae in 30 ml of bacterial suspensions.

the 30ml-LC90 were determined at 0.251 mg/l after 6 hours and 0.079 mg/l after 24 hours exposure to B.t suspensions.

4.1.3 FOURTH INSTAR LARVAE

Highest percentage mortalities recorded for fourth instar larvae in 150 ml of bacterial suspensions were 31.67 and 79.2 percent; 28.3 and 71.20 percent in 30 ml suspensions after 6 and 24 hours respectively (Figures 5 and 6).

The graphically determined LC50 for this instar larvae in 150 ml suspensions after 6 and 24 hours were 0.063 mg/l and 0.013 mg/l whereas in 30 ml B.t suspensions, the LC50 were 0.158 mg/l and 0.017 mg/l. Similarly, the LC90 values for 150 ml bacterial suspension was 0.251 mg/l after 6 hours and 0.071 mg/l after 24 hours, while 0.79 mg/l and 0.089 mg/l were determined after 6 and 24 hours respectively for bioassays carried out in 30 ml of B.t H-14 suspension.

4.1.4 TOXICITY OF B.t H-14 ON THE THREE LARVAL INSTARS

It has been observed that for a given exposure time and water volume, the second instars larvae responded as the most sensitive developmental stage of this mosquito species to bacterial powder. This is followed by the third and then the fourth instar larvae which were comparatively the least sensitive. The rates of mortality, which are represented by

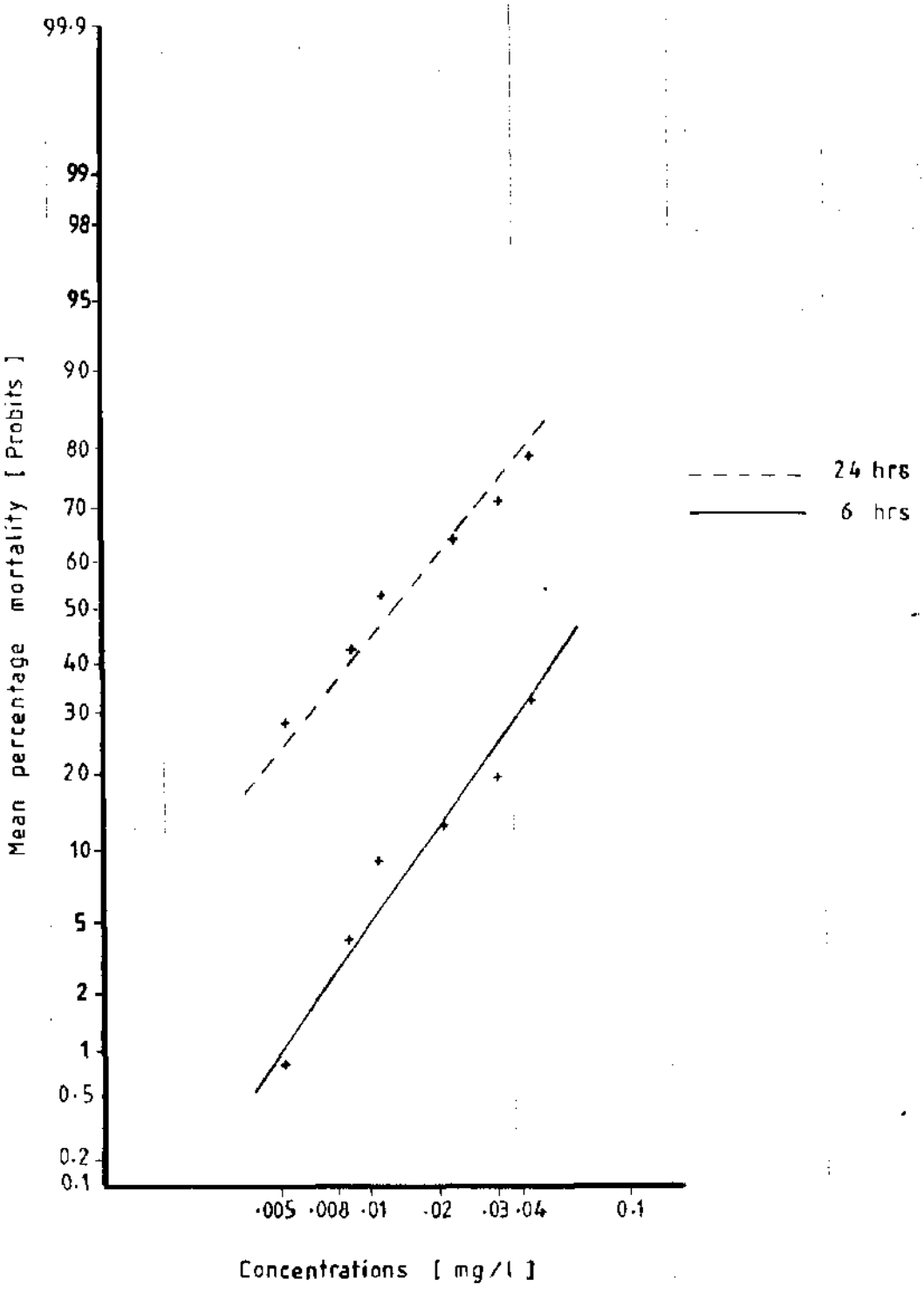


Fig. 5 Dosage/mortality regression lines for fourth instar larvae in 150 ml bacterial suspensions.

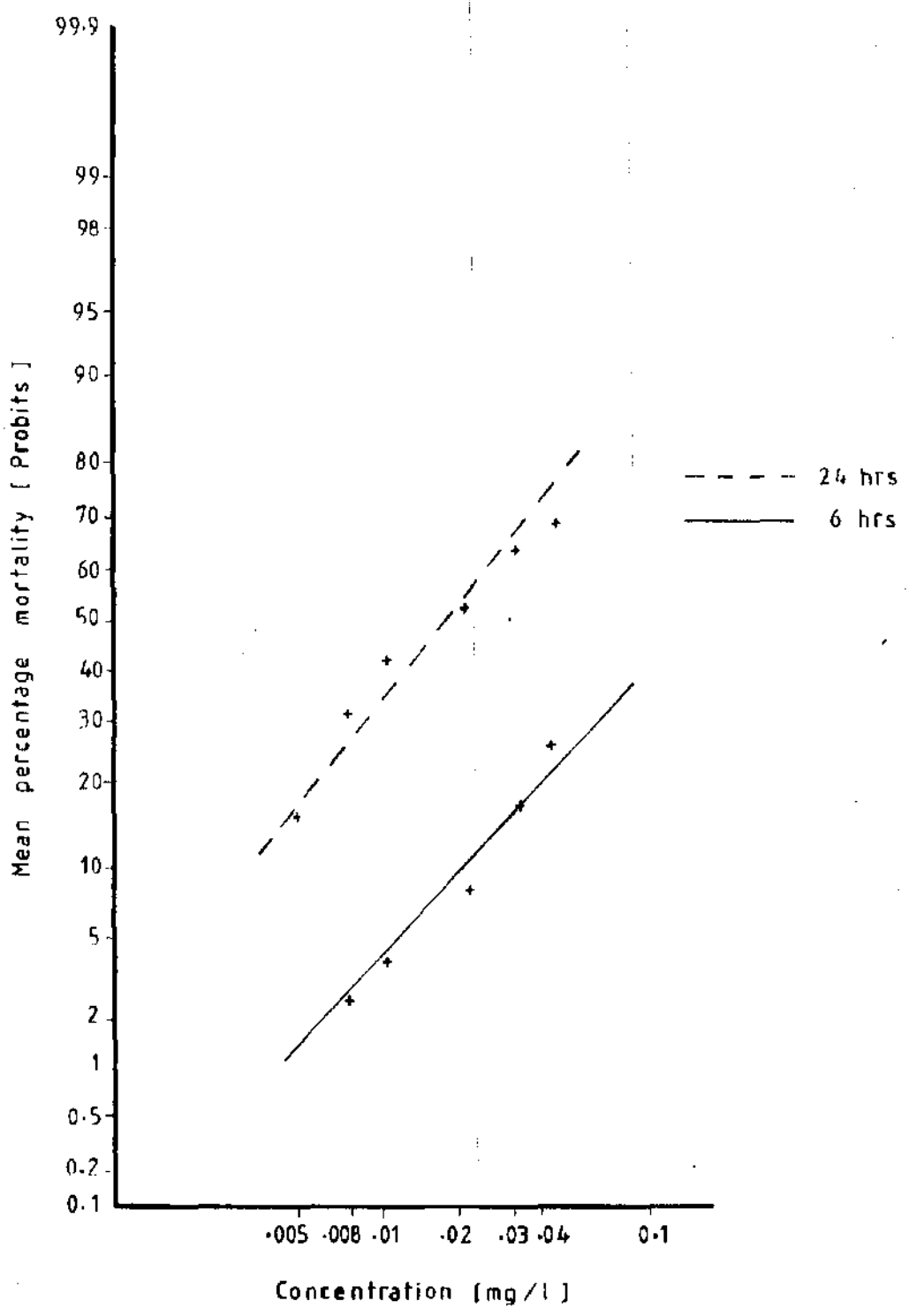


Fig. 6 Dosage / mortality regression lines for fourth instar larvae in 30 ml of bacterial suspensions.

slopes of graphs, were highest for second instar larvae when compared with that for third and fourth larval groups (Figs. 7 and 8).

The LC50 values for 150 ml bacterial suspension after 6 and 24 hours exposures at 0.02 mg/l and 0.0036 mg/l for second instars were the lowest as compared to 0.04 and 0.009 mg/l for third instar larvae response and 0.063 mg/l and 0.013 mg/l for fourth instar larvae (Table 1). In 30 ml suspensions the least LC50 values of 0.035 mg/l and 0.008 mg/l were recorded for second instars than 0.056 mg/l and 0.015 mg/l for third and 0.158 mg/l and 0.017 mg/l recorded for the fourth instars after 6 and 24 hours exposure periods respectively (Table 2).

Similarly, the 150 ml suspensions - LC90 values after 6 and 24 hours exposure increased from 0.158 mg/l and 0.022 mg/l for second instar larvae, 0.199 mg/l and 0.050 mg/l for third, to 0.251 mg/l and 0.071 mg/l for fourth instar larvae (Table 3). In 30 ml of bacterial suspension these values increased from that of second instars, 0.211 mg/l and 0.093 mg/l through that of the third 0.251 mg/l and 0.079 mg/l; to that of the fourth, 0.79 mg/l and 0.089 mg/l after 6 and 24 hours exposure time (Table 4).

There was a steady increase in lethal concentrations (LC50 and LC90) with increase in larval age. The following

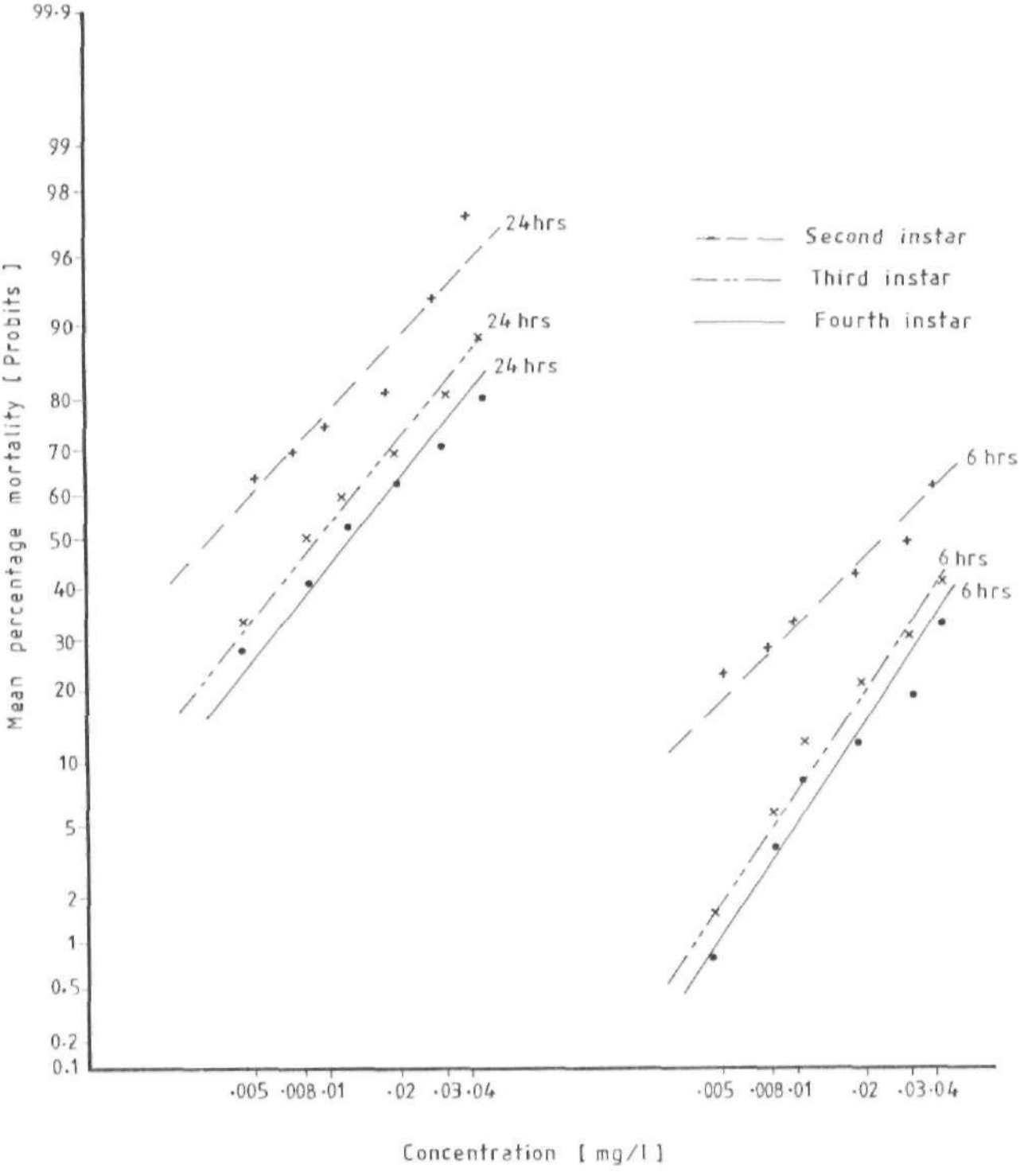


Fig. 7 Influence of larval stages on the activity of Bacillus thuringiensis H-14 on Culex pipiens in 150 ml of bacterial suspensions.

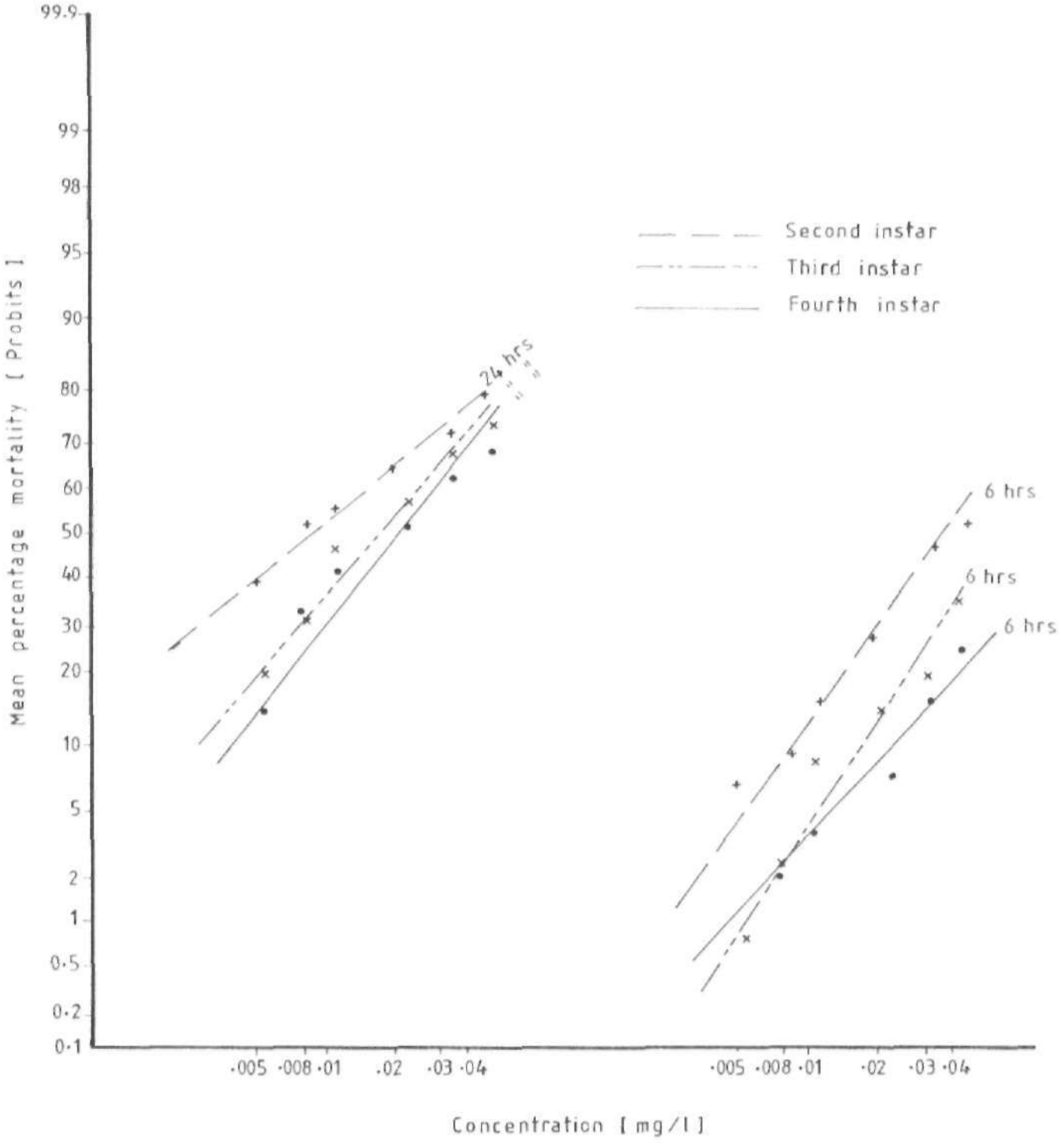


Fig. 8 Influence of larval stage on the activity of B.t. H-14 on Culex pipiens in 30 ml of bacterial suspensions.

Table 1: LC50 (mg/l) of B.t H-14 on second, third and fourth instar larvae of Culex pipiens (Means of 6 experiments in 150 ml of bacterial suspension).

Larval instar	6 hours	24 hours
Second	0.0200 ± 0.0048 mg/l	0.0036 ± 0.0007 mg/l
Third	0.0400 ± 0.0070 mg/l	0.0090 ± 0.0015 mg/l
Fourth	0.0630 ± 0.0126 mg/l	0.0130 ± 0.0025 mg/l

Table 2: LC50 (mg/l) of B.t H-14 on second, third and fourth instar larvae of Culex pipiens (Means of 6 experiments in 30 ml of bacterial suspension).

Larval instar	6 hours	24 hours
Second	0.0355 ± 0.0058 mg/l	0.0090 ± 0.0020 mg/l
Third	0.0560 ± 0.0103 mg/l	0.0150 ± 0.0025 mg/l
Fourth	0.1580 ± 0.0171 mg/l	0.0170 ± 0.0029 mg/l

Table 3: LC90 (mg/l) of B.t H-14 on second, third and fourth instar larvae of C. pipiens (Means of 6 experiments in 150 ml of bacterial suspension).

Larval instar	6 hours	24 hours
Second	0.1580 ± 0.1264 mg/l	0.0220 ± 0.006 mg/l
Third	0.1090 ± 0.0452 mg/l	0.0500 ± 0.0179 mg/l
Fourth	0.2510 ± 0.1190 mg/l	0.0710 ± 0.0310 mg/l

Table 4: LC90 (mg/l) of B.t H-14 on second, third and fourth instar larvae of C. pipiens (Means of 6 experiments in 30 ml of bacterial suspension).

Larval instar	6 hours	24 hours
Second	0.2110 ± 0.1270 mg/l	0.0930 ± 0.0649 mg/l
Third	0.2510 ± 0.1870 mg/l	0.0790 ± 0.0337 mg/l
Fourth	0.7900 ± 0.4233 mg/l	0.0800 ± 0.0509 mg/l

series was obtained for the 3 instars tested, second, third and fourth in the order of decreasing susceptibility (Figs. 9 and 10).

During the experiment on larval age, the second instar larvae were found to be 2.00 folds more susceptible to B.t H-14 after 6 hours exposure and 2.57 folds more sensitive after 24 hours than third instar larvae. This group of larvae were also 3.15 and 3.75 times more sensitive than fourth instars larvae after 6 and 24 hours in 150 ml of bacterial suspension.

In 30 ml solutions, the second instars were observed to be 1.5 folds more sensitive after 6 hours and 1.9 folds after 24 hours exposures than third instars. They were also 3.20 and 2.10 times more susceptible than the fourth larval instars.

The third instars were about 1.5 times more sensitive than the fourth instars in 150 ml bacterial suspensions. In 30 ml suspensions, this instar larvae was 2.03 times more sensitive after 6 hours and 1.3 times more susceptible than fourth instars after 24 hours exposures.

Statistical analysis was done to see any significant difference between these larval stages. The susceptibility of these different instar larvae to B.t H-14 was significant

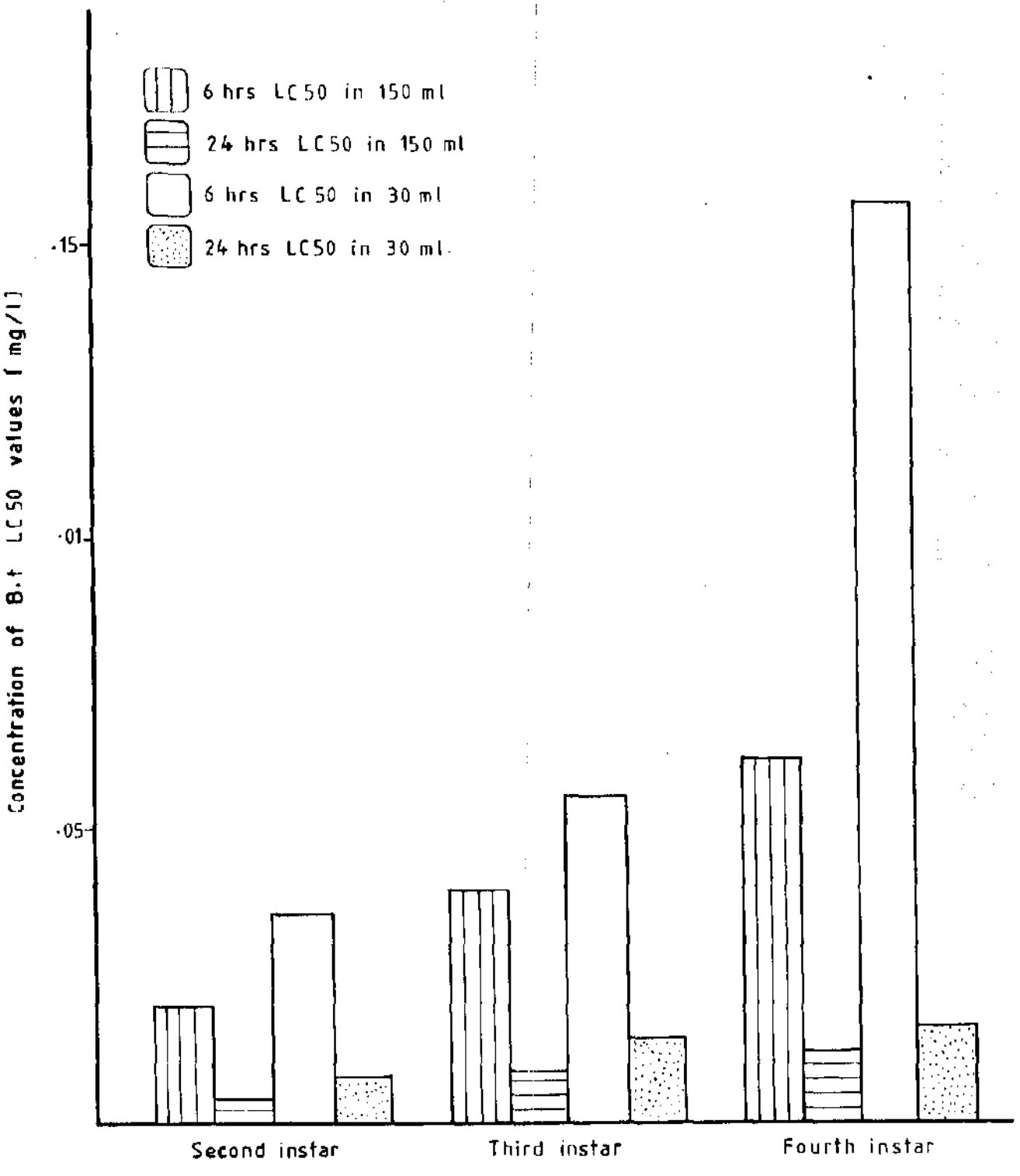


Fig. 9 Lethal concentration of B.t H-14 to 50% of the different larval instars.

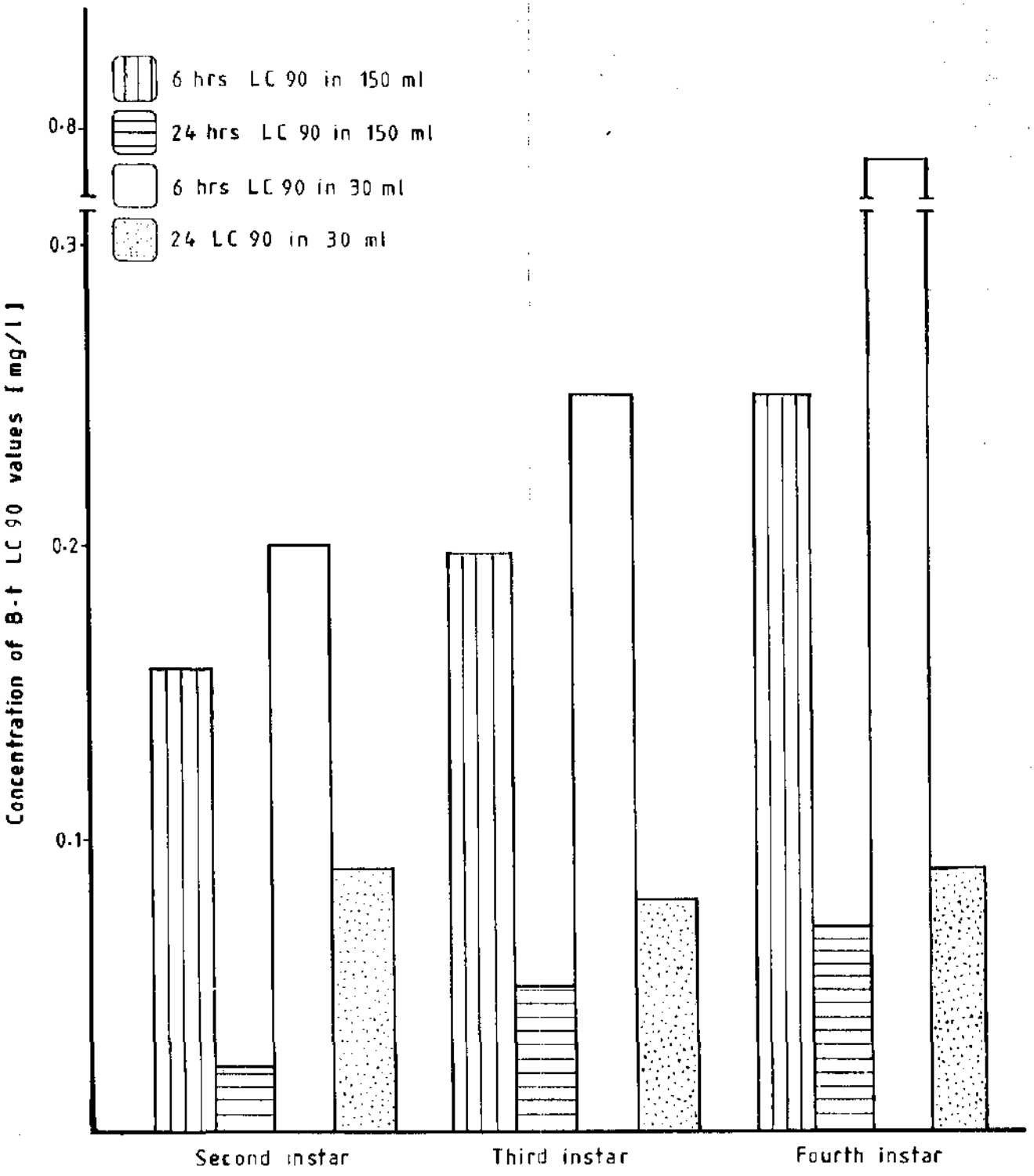


Fig. 10 Lethal concentration of B. t H-14 to 90% of the different larval instars.

at the 5% significant level ($P > 0.05$). The correlation coefficient 'r' was also calculated for each instar during 6 and 24 hours exposure periods. These r-values were found to be greatest within the second instar larvae and least for fourth instar group.

4.2 EFFECT OF WATER VOLUME AND DEPTH ON MORTALITY

Mortality of mosquito larvae was found to increase with the increase in water volume and depth of container. Bioassays carried out in 250 ml beakers with 150 ml bacterial suspensions resulted in higher mortalities than those performed in 50 ml beakers with only 30 ml of bacterial suspensions.

Higher average percentage mortality results of 63.33 and 97.50 percent; 40.83 and 90.0 percent; 31.67 and 79.20 percent were recorded in 250 ml beakers for second, third and fourth instar larval groups respectively after 6 and 24 hours exposures.

On the other hand, mortality readings observed in 50 ml beakers were 54.16 and 80.0 percent; 34.16 and 73.83 percent; 28.33 and 71.16 percent after 6 and 24 hours for second, third and fourth instar larvae respectively (Appendix, 1 -6).

Plotting the mean percentage mortalities against concentrations of bioassays for these two sets of beakers

showed steeper regressions lines with higher mortality rates for experiments carried out in 150 ml suspensions than for bioassays performed in 30 ml bacterial suspensions for second, third and fourth larval groups (Figs. 11, 12 and 13). The mean lethal concentrations (LC50 and LC90) were also graphically determined. These values decreased with increase in water volume and depth.

The 50% lethal concentrations for second, third and fourth larval stages obtained after 6 and 24 hours in bigger (250 ml) containers with 150 ml bacterial suspensions were 0.02 mg/l and 0.0036 mg/l; 0.04 mg/l and 0.009 mg/l; 0.063 mg/l and 0.013 mg/l respectively. These values are smaller than 0.035 mg/l and 0.008 mg/l, 0.055 mg/l and 0.014 mg/l; 0.158 mg/l and 0.017 mg/l estimated for bioassays carried out in 50 ml beakers for second, third and fourth instars after 6 and 24 hours respectively.

Similar trends were observed for 90% lethal concentrations where the values in 250 ml containers after 6 and 24 hours for second, third and fourth instars, 0.158 mg/l and 0.022 mg/l; 0.199 mg/l and 0.050 mg/l; 0.251 mg/l to 0.071 mg/l were lower than 0.211 mg/l and 0.093 mg/l; 0.251 mg/l and 0.079 mg/l; 0.791 mg/l and 0.089 mg/l obtained for bioassays performed in 50 ml beakers after 6 and 24 hours exposure periods.

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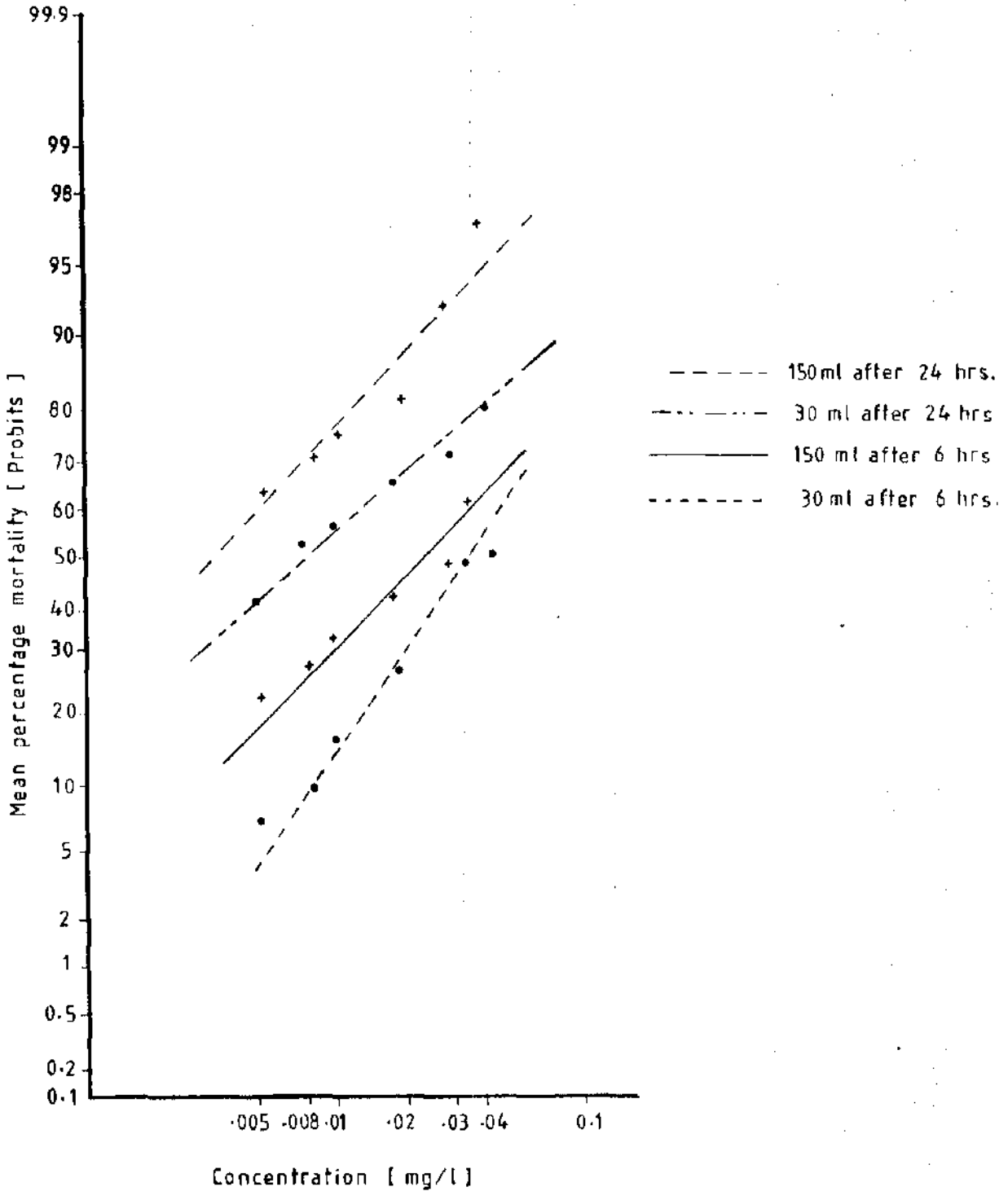


Fig. 11 Influence of water volume on the activity of Bacillus thuringlensis H-14 on second instar larvae of Culex pipiens.

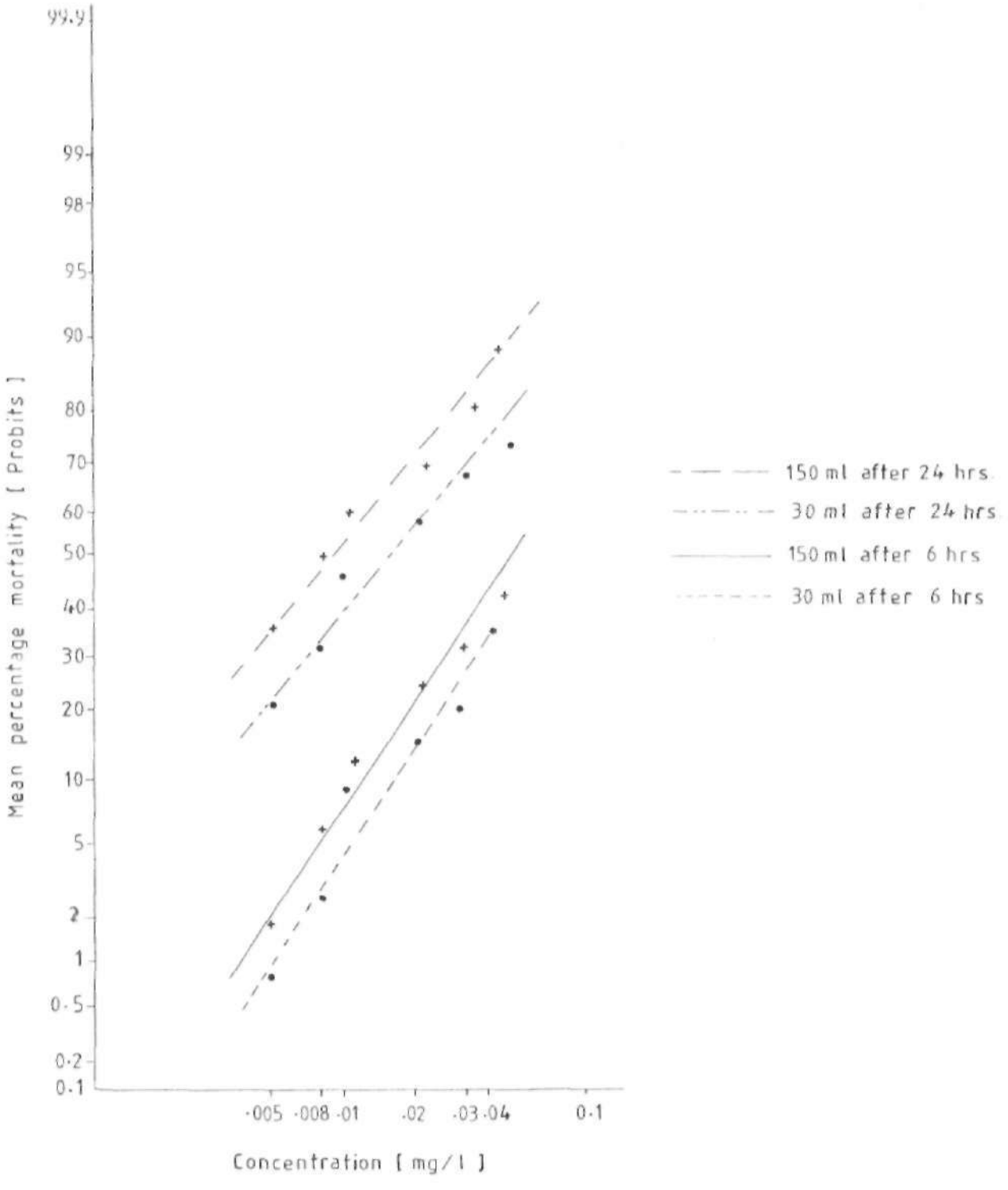


Fig.12 Influence of water volume on the activity of Bacillus thuringiensis H-14 on third instar larvae of Culex pipiens.

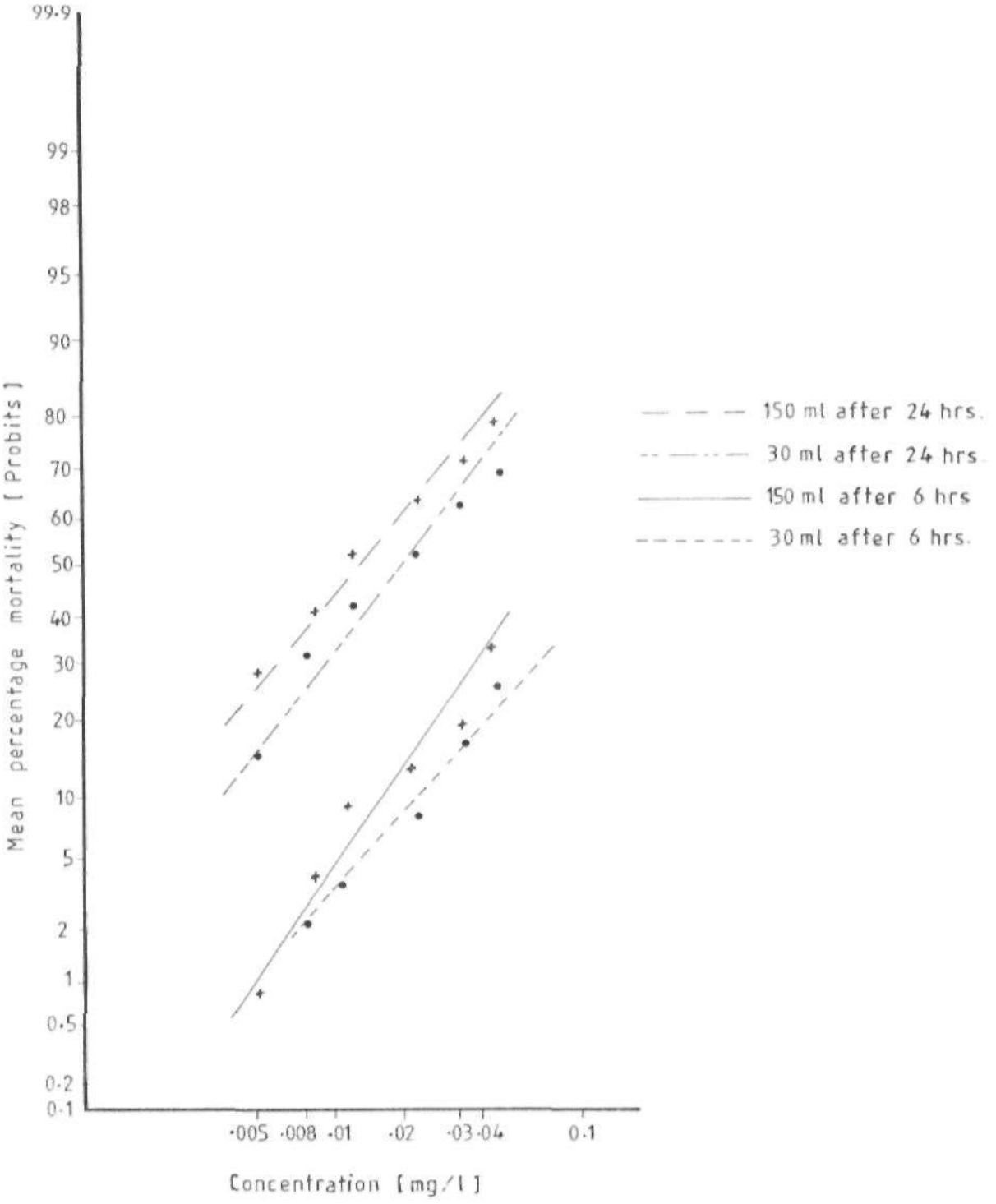


Fig. 13 Influence of water volume on the activity of Bacillus thuringiensis H-14 on fourth instar larvae of Culex pipiens

On the whole, the susceptibility of mosquito larvae of the same instar at a given time in these two types of beaker are significantly different ($P > 0.05$).

4.3 EFFECT OF EXPOSURE TIME

Mortality results within the same beaker with the same instar larvae diferred with exposure time. More deaths occurred after 24 hours exposures than during the first 6 hours (Appendix, 1 - 6). The mean percentage mortalities of Culex pipiens larvae treated with B.t H-14 over 24 hours observation period followed a linear relationship when plotted against concentration on probit-log scale (Figs. 1 - 6). There was also a progressive decline in lethal concentration values (LC50 and LC90) from 6 hours to 24 hours indicating that more larvae died at the end of 24 hours exposures to bacterial suspensions (Figs. 14 and 15).

The determined LC50 values after 6 hours exposures in 150 ml and 30 ml of B.t H-14 suspensions were 0.02 mg/l and 0.038 mg/l for second instars, 0.04 mg/l and 0.056 mg/l for third instars while 0.063 mg/l and 0.158 mg/l have been determined for the fourth instar larval group.

The 24hrs-LC50 values, on the other hand, have been found to be 0.0036 mg/l and 0.008 mg/l for second instars; 0.009 mg/l and 0.014 mg/l for third instars and 0.013 mg/l

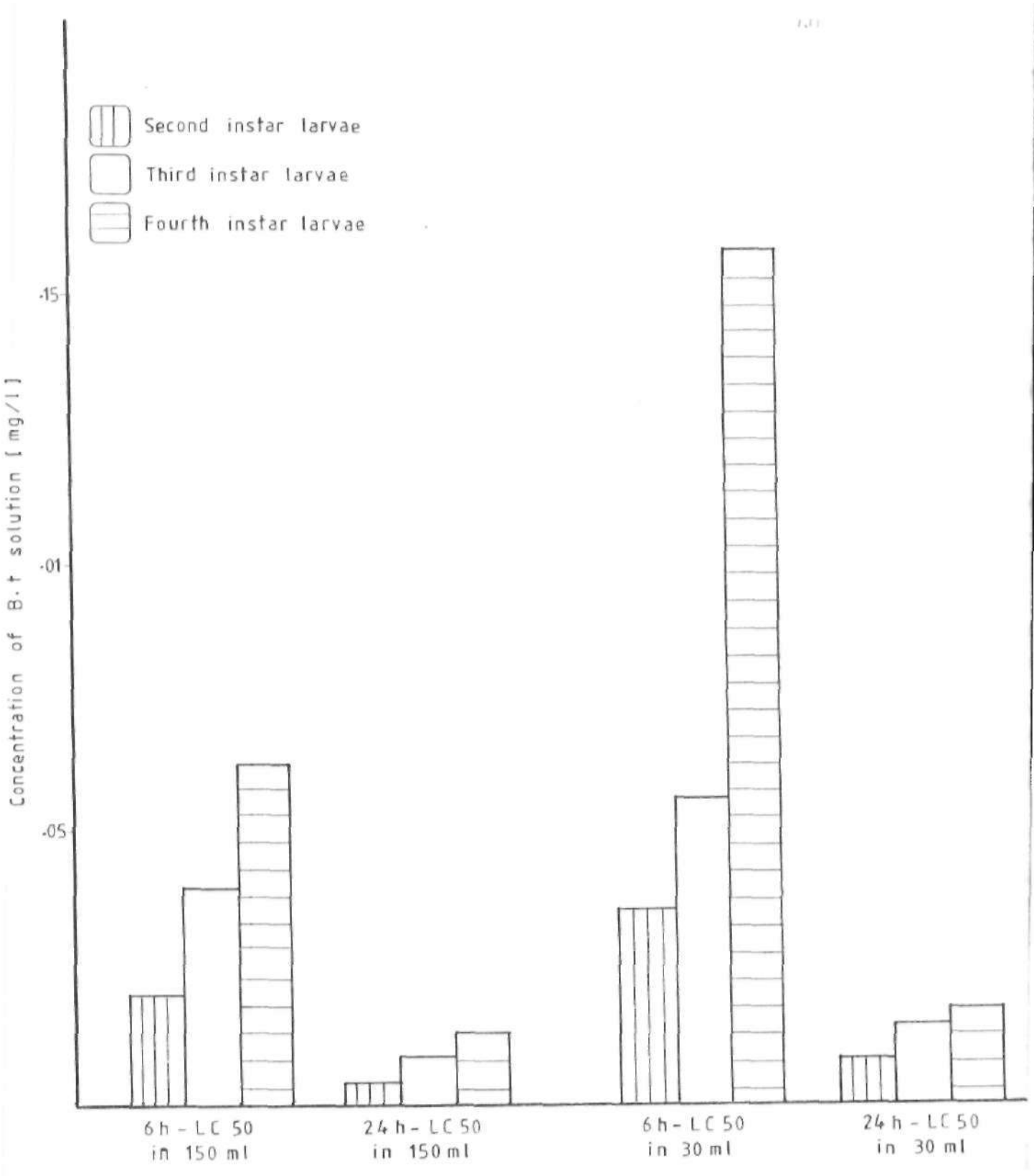


Fig. 14 Lethal concentration of B.t H-14 to 50% of larvae at various times.

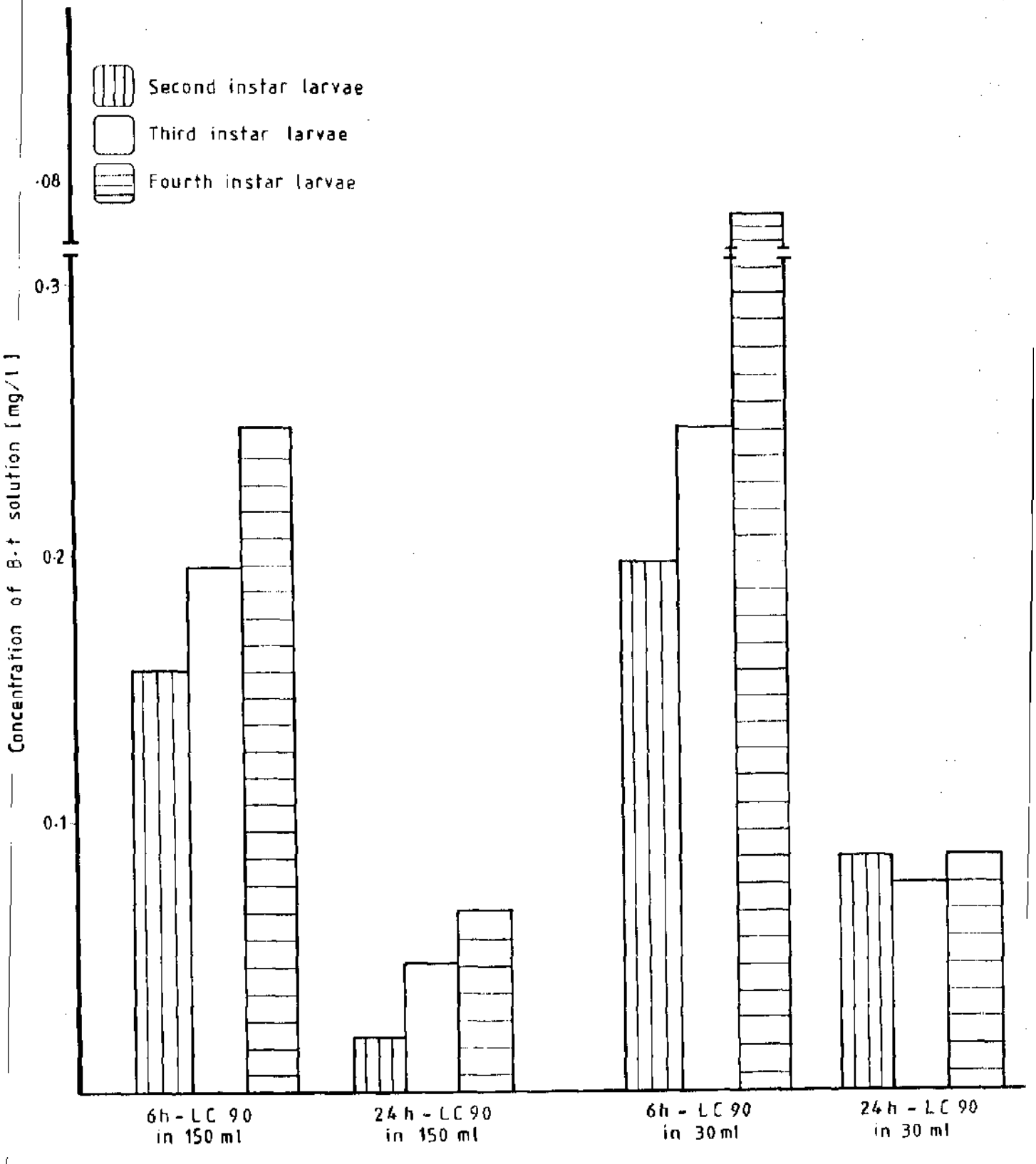


Fig. 15 Lethal concentration of B.t H-14 to 90% of the larvae at various times.

and 0.017 mg/l for fourth instars in 150 ml and 30 ml of bacterial suspension respectively (Fig. 14).

Six-hrs-LC90 values of 0.158 mg/l and 0.211 mg/l were determined for second instars while 0.199 mg/l and 0.251 mg/l; 0.251 mg/l and 0.791 mg/l were estimated for third and fourth larval instars in 150 ml and 30 ml suspension of B.t H-14 respectively.

Twenty-four-hrs-LC90 values of 0.022 mg/l and 0.093 mg/l; 0.0501 and 0.079 mg/l; 0.071 and 0.089 mg/l were estimated for second, third and fourth instars in 150 ml and 30 ml bacterial suspensions (Fig. 15).

The lethal concentration values decrease with the increase in exposure time. Lower values were obtained for bioassays exposed for 24 hours than those exposed for only 6 hours. It has been observed during the course of present studies that the second larvae were about 5.7 and 4.7 times more more susceptible after 24 hours than after 6 hours exposures in 150 ml and 30 ml of bacterial suspensions respectively. The third instar larvae were 4.4 and 3.7 times more sensitive while the fourth were 2.1 and 8.8 folds more susceptible after 24 hours exposures in 150 ml and 30 ml of bacterial suspensions.

Test statistics (analysis of variances) carried out showed that there existed a significant difference between these two (6 and 24 hours) exposure times.

The standard errors (S.E) which give the bioassays more precision were calculated for each lethal concentration value (Tables 1 - 4).

CHAPTER FIVE

5. DISCUSSION

5.1 EFFECT OF B.t H-14 ON LARVAL INSTARS

The results of present laboratory evaluations showed that all the three larval stages (second, third and fourth) of C. pipiens used for this experiment were sensitive to B.t H-14. However, comparison of the determined LC50 values obtained here with previous findings showed that Culex pipiens larvae are less susceptible to B.t H-14 than the larvae of Aedes aegypti. The estimated 24 hours LC50 value for second instar larvae in 150 ml of bacterial suspension was 0.0036 mg/l. This value is higher and indicates lower sensitivity when compared with 0.0034 mg/l estimated for Aedes aegypti by de Barjac and Larget (1979) even with the higher laboratory temperature (30 $\pm$ 1°C) where these present bioassays were carried out. Culex pipiens larvae are, however, more sensitive to bacterial suspension than mosquito larvae of Anopheles species. The 24 hours-LC50 for second instar larvae of Anopheles stephensi 0.066 mg/l in 150 ml of bacterial suspension reported by de Barjac and Larget (1979), indicates less susceptibility when compared with 0.0036 mg/l estimated for C. pipiens larvae here. Nicolescus (1982) also reported that C. pipiens larvae are more susceptible than Anopheles atroparus. The larvae of Anopheles spp have been reported to be about 20 times less toxic to B.t H-14 toxin than Culex and Aedes larvae (Tyrell et al., 1979). Such differences in

susceptibility can be attributed to differences in modes of feeding of these various larvae. Less sensitivity of Anopheles larvae is due to its surface feeding habits while Culex and Aedes spp larvae are bottom feeders.

The second instar larvae were found to be the most susceptible of the three different instars used for these bioassays. It is followed by the third larval instars and then the fourth instar group which showed the least response. This is reflected in the lower LC50 and LC90 values of the second instar larvae when compared with that of the rest instar groups (Tables 1 - 4). This finding is in agreement with a similar experiment conducted by de Barjac and Larget (1979), who reported that the second instar larvae of Aedes aegypti were more susceptible than the fourth instar groups. They estimated the 24hrs-LC50 values at 0.0034 mg/l and 0.024 mg/l for second and fourth instars respectively. This trend is similar to the values (0.0036 mg/l and 0.0126 mg/l) obtained during this study for second and fourth instars.

During the course of this work, the second instars were found to be about 3.75 times more sensitive than the fourth instar larvae while the third instar were about 1.5 times more susceptible than the fourth in 150 ml of bacterial suspensions after 24 hours exposures. This work is not in agreement with previously reported study where the second instar

larvae of Aedes aegypti were found to be about 7 folds more susceptible to B.t H-14 than fourth larval group while the second instars of Anopheles stephensi were about 1.67 times more susceptible than the fourth instar larvae. (de Barjac and Larget, 1979). These variations in susceptibility to B.t H-14 suspension can be attributed to differences in larval species used for experiments. Aedes aegypti and Anopheles stephensi larvae were used by the previous workers while Culex pipiens larvae were exposed to B.t H-14 during present investigations. The degree of susceptibility (number of folds) observed during this experiment do agree with that of Sinegre et al. (1979) who worked with Culex pipiens quinquefasciatus and found that early stages (1st and 2nd instars) are 4 times more sensitive than last stages (3rd and 4th instars). This little difference may be due to the fact that C. pipiens were used here while Sinegre and co-workers used a particular subspecies of this mosquito (Culex pipiens quinquefasciatus).

From the present investigations, it appears that susceptibility gradually decreases with increase in larval age (from second to fourth instar groups). This is because as larvae approach pupation they become less active. Second instar larvae were killed most by B.t H-14 because they were very active and so feed actively thus taking in more of bacterial toxins which are effective only when eaten. The late instars (3rd and 4th) which are close to developing into

pupae, feed less actively thus consuming less biocide. Pupae were not affected by bacterial suspension because they do not feed.

Culex pipiens larvae reared in the laboratory appeared to be more sensitive to B.t toxins than field collected species. The 50% lethal concentrations, of 3rd - 4th instars of C. pipiens larvae ranging from 0.009 mg/l - 0.0160 mg/l after 24 hours exposure is lower than previously reported values of 0.01 - 0.035 mg/l for field collected species. (Sinegre et al., 1990). Higher LC50 values or less susceptibility among field collected larvae may be due to lack of acclamatization to laboratory coaditions when brought from the field.

The estimated 50% lethal concentrations determined in 150 ml of bacterial suspensions after 24 hours exposures, generally indicated higher susceptibility when compared with previous findings. Ignoffo et al. (1981) estimated the LC50 value for second instar larvae of C. pipiens as 0.11 mg/l Vankora (1981) determined the LC50 value for third instar as 0.10 mg/l; while Lebrun and Vlayen (1981) estimated the LC50 value of fourth instar larvae of the same mosquito species as 0.018 mg/l. These values are generally higher when compared with 0.0036 mg/l estimated for second instars, 0.009 mg/l estimated for third instar larvae; and 0.0126 mg/l

estimated for fourth instar larvae. Differences in susceptibility to bacterial suspensions may be explained by different laboratory temperatures where experiments were conducted. While present bioassays were conducted at a temperature of 30 ± 1°C, these previous ones were performed at laboratory temperatures of 26 ± 1°C.

5.2 EFFECT OF WATER VOLUME AND DEPTH ON ACTIVITY OF B.t H-14

From the present work, it is quite evident that there exist a significant difference in bioassays carried out in 250 ml and 50 ml beakers. Shallow beakers with 30 ml of delta-endotoxin suspension induce less mortality than deeper ones with 150 ml of bacterial suspensions. This is reflected in the lower LC50 and LC90 values estimated for bioassays performed in 250 ml than in 50 ml containers. These lower lethal concentration values estimated for deeper beakers indicate higher susceptibility and mortality. The LC50 values for second instar larvae in 150 ml and 30 ml of bacterial suspensions recorded during this experiment after 24 hours were 0.0036 mg/l and 0.009 mg/l respectively. The pattern here is similar to the estimated LC50 for Aedes aegypti after 24 hours exposure which were determined by de Barjac and Larget (1979) as 0.0078 ITU in 150 ml and 0.024 ITU in 30 ml of bacterial suspensions. These workers also showed that the influence of depth was significant even for Anopheles larvae which are top feeders. The determined LC50 values

after 24 hours in 150 ml and 30 ml of bacterial suspensions for Anopheles stephensi have been reported at 0.010 ITU and 0.052 ITU respectively. Higher susceptibility in deeper beakers may be due to increase in metabolic activities of larvae (de Barjac and Larget, 1979). Deeper containers induce increase feeding rates which result in ingestion of greater quantities of toxin per unit time (WHO, 1982). The difference in activity of Culex pipiens larvae in these two sets of beakers could be due to temperature factor. Deeper containers with greater volume (150 ml) of suspensions may be slightly warmer than shallow ones (30 ml of B.t suspension). Temperature, according to Burges and Thomson (1971), not only result in higher metabolism and feeding of larvae but also results in faster multiplication of bacterium in the case of B.t var thuringiensis.

On the whole, the susceptibility of mosquito larvae of the same instar at a given time in these two types of beakers is significant. The effect of water volume was found to be much more less significant in this experiment than that of exposure time (6 and 24 hours). This is in contrast with the report by de Barjac and Larget (1979), where the effect of depth was found to be greater than that of exposure time. This dissimilarity may be due to the difference in types of shallow containers used. Here, small 50 ml beakers were used while petridishes were used by de Barjac and Larget (1979). The difference in mortality here may have been more

than that induce by time, if petridishes which are shallower were used instead of the 50 ml beakers which are smaller in diameter but deeper than petridishes.

5.3 EFFECT OF EXPOSURE TIME ON ACTIVITY OF B.t H-14

The results of toxicity tests using B.t H-14 against second third and fourth instar larvae of C. pipiens showed a direct and positive relationship between exposure period and mortality. Deaths occurred as early as after 6 hours with little differences in mortality among the various larval stages. This is found to be in agreement with report by Hertlein et al. (1980) who observed that the activity of B.t H-14 on a susceptible host starts within 2 - 10 hours after ingestion. Goldberg and Margalit (1977) reported that toxins of B.t H-14 take less than 12 hours to act, with little difference between 1st - 2nd and late 3rd - 4th instar larvae of mosquitoes. Similarly, Anderson and Rogoff (1965) reported the onset of rapid paralysis within 20 - 30 minutes after consumption of bacterial suspension in susceptible insects like Silkworm, Tobacco and Tomato hornworms. The paralysis rapidly spreads to all parts of the body within 1 - 7 hours which eventually results in death after 24 hours.

In the case of B.t H-14, mortality of larvae have been recorded as early as after 6 hours, however, paralysis occurred much earlier than this period. This also agrees

with WHO (1982) report which showed that the fourth stage larvae of A. aegypti and C. tarsalis became moribund and lerthagic within two hours when placed in 0.1 mg/l concentration of B.t H-14. This work is also in agreement with the fact that depending on the dose consumed and type of pathogenic action induced, a susceptible host dies in a few hours to about 3 weeks after ingestion of B.t H-14 (WHO, 1982).

Furthermore, a steady increase in the number of deaths of larvae was observed with increase in exposure time. More deaths occurred after 24 hours than after 6 hours exposures in all the bioassays (Appendix, 1 -6). This fact is reflected in the lower values of lethal concentrations(LC50 and LC90) determined after 24 hours when compared with that after 6 hours. This indicates higher sensitivity during 24 hours exposure, because the chances of a larva coming into contact with bacterial toxin increases with the increase in time. These findings are in accordance with that by Frommer et al. (1981) who reported a sharp decrease in LC50 from 0.01 mg/l - 0.32 mg/l when exposure time was increased from 30 - 60 minutes. Similarly de Barjac and Larget (1979) showed that the determined 40hrs-LC50 (0.0064 mg/l) was lower than the 24hrs-LC50 (0.0078 mg/l) for A. aegypti. Even though the exposure times used by these previous workers (30min - 60mins and 24hors - 40hrs) were different from the ones chosen in the present studies (6 - 24 hours), mortalities followed the same trend, they increase with increase in time. Almost all the 24hrs-LC50 values

estimated during the present studies fell within the 0.001 mg/l and 0.01 mg/l range reported by Larget and de Barjac (1981).

The LC50 value of second instar larvae of Culex pipiens after 6 hours in 150 ml of bacterial suspension was about 5.7 times greater (i.e less susceptible) than that observed after 24 hours. This is in contrast with the observations made by de Barjac and Larget, 1979) where the LC50 for A. Aegypti after 24 hours in 150 ml of bacterial suspension were found to be about 1.25 times greater than that of 48 hours. Higher folds observed in present investigation may be due to differences of mosquito larvae used (Aedes sp and Culex sp); exposure periods chosen (24 hours - 48 hours and 6 - 24 hours) and differences in laboratory temperatures (26 $\pm$ 1^{o}C and 30 $\pm$ 1^{o}C) where assays were carried out.

The present investigations revealed that mortality due to exposure time has more significance than that caused by water depth. This finding does not agree with that by de Barjac and Larget (1979) where the effect of water depth was said to be more than that of time. Dissimilarities in the two findings can be attributed to differences in exposure time chosen and larval species used for bioassays. In this experiment, the exposure time used were 6 and 24 hours while 24 and 48 hours were used by de Barjac and Larget (1979).

While C. pipiens larvae were exposed to bacterial suspensions here, A. aegypti were used for bioassays by these previous workers.

5.4 EFFECT OF FOOD ADDITION ON ACTIVITY OF B.t H-14

During the early part of the present experiment when food was not added to bacterial suspensions, the rate of mortality was very high. About 10 - 15% deaths were even recorded in controls after 24 hours exposures. This increase in death rates is due to starvation which increases susceptibility to B.t H-14 toxin and ensures that all larvae of sensitive host feed (Burges and Thomson, 1971). The condition, quality and amount of food were found to interfere with the efficacy of B.t toxins (Angus, 1965).

Addition of food to B.t H-14 suspensions during this experiment have greatly reduced the efficiency of B.t H-14. This finding agrees with that of Ali et al. (1981) who reported that food additions significantly dilute the effectiveness of bacterial formulations. Using Culex pipiens quinquefasciatus, they estimated the LC50 values of solutions without food to be 0.11 mg/l - 0.30 mg/l and the LC50 of bacterial suspensions with food at 0.35 - 0.59 mg/l after 24 hours exposures.

Food addition also can be used to explain the little erratic results obtained for 24-hours-LC90 values in 30 ml of bacterial suspension during this study. The LC90 value

obtained for second instar (0.093 mg/l) is higher than 0.079 mg/l and 0.081 mg/l estimated for third and fourth instar larvae respectively. This is because food particles added to bacterial suspensions were not quantified.

SUMMARY AND CONCLUSION

From the toxicity tests, it can be concluded that larvae of Culex pipiens are sensitive to B.t H-14 with highest deaths occurring in 0.04 mg/l bacterial concentrations. Second instars larvae were found to be the most susceptible of all the three (second, third and fourth) instar groups tested. These larvae are about 1.9 and 2.57 times more susceptible than third instar larvae in 30 ml and 150 ml of bacterial suspensions after 24 hours exposures. Second instar larvae are also about 2.10 to 3.75 folds more sensitive than fourth instar larvae in 30 and 150 ml of bacterial suspensions after 24 hours.

Exposures of mosquito larvae to B.t H-14 toxins for 24 hours resulted in higher mortalities than 6 hours exposures in all bioassays conducted. The 24hours-LC50 values (0.0036 - 0.008, 0.009 - 0.014, and 0.013 - 0.017 mg/l) and LC90 values (0.022 - 0.093, 0.050 - 0.079 and 0.071 - 0.089 mg/l)were smaller than the 6hours-LC50 (0.02 - 0.035, 0.04 - 0.056 and 0.063 - 0.058 mg/l) and LC90 (0.158 - 0.211, 0.199 - 0.251 and 0.251 - 0.791 mg/l) values for bioassays carried out in 150 ml and 30 ml of bacterial suspensions respectively.

Depth and volume of water in 250 ml containers induced more deaths than shallow (50 ml) ones. Lower 6 - 24 hours

LC50 (0.02 - 0.0036, 0.04 - 0.009 and 0.063 - 0.013 mg/l) and LC90 (0.158 - 0.022, 0.199 - 0.0501 and 0.251 - 0.071 mg/l) values were determined for second, third and fourth instar larvae respectively in 250 ml beakers when compared with the 50 ml - LC50 (0.035 - 0.008, 0.056 - 0.014 and 0.158 - 0.017 mg/l) and LC90 (0.211 - 0.093, 0.251 - 0.079 and 0.791 - 0.039 mg/l) for these three instars.

Mortalities of C. pipiens larvae were significantly different ($P > 0.05$), for the two types of beakers (250 - 50 ml), two exposure periods (6 - 24 hours) and the three larval instars (second, third and fourth).

The effect of exposure time of B.t H-14 on C. pipiens was the most significant factor than that of instars and two types of beakers used (250 ml and 50 ml). Addition of food to bacterial suspensions has greatly reduced excessive mortality by diluting the effect of bacterial efficacy.

Further research should be conducted in the fields to evaluate the efficacy of the bacterium against this mosquito.

REFERENCES

1. Adadevoh, B.K. and Fayiba, H.M. (Eds) (1978) Malaria in Nigeria: An author bibliography from 1950 - 1975. Nig. Ins. Med. Research Publ. pp. 55.

2. Ali, A., Beggs, R. and Stewart, J. (1981). Susceptibility of some Florida chironomi and mosquitoes to various formualtions of Bacillus thiringiensis serovar. Israelensis. J. Econ. Entomol., 74: 672-677.

3. Allen, N. (1961). Bacillus thuringiensis controls the tobacco budworm. Tobacco Sci., 5: 141-150.

4. Alvarado, C.A. and Bruce-Chwatt, L.J. (1962). Malaria. Scientific American 284-295.

5. Anderson, R.F. anf Rogoff, M.H. (1965). Crystalliferous bacteria as insect toxicants. In natural pest control agents. pp. 65-79. American chemical society, Publ.

6. Angus, T.A. (1965). Association of toxicity with protein crystalline inclusions with Bacillus Sotto Ishiwata. Can. J. Microbiol., 2: 122-131.

7. Angus, T.A. (1971). Bacillus thuringiensis as a microbial insecticide. In naturally occurring insecticides. (Jacobson and Crosby, eds). M. Dekker, New York. pp. 463-477.

8. Angus, T.A. and Luthy, P. (1971). Formulation of microbial insecticides. In Microbial control of insects and mites. (Burges and Hussey, eds). Academic Press. London. pp. 623-636.

9. Anonymous (1969). Editorial. Chemical week. 104, April 26, 36-38.

10. Anonymous (1973). Mosquito control: Some prospectives for developing countries. A report of an Ad Hoc panel of the advisory committee on Technological innovation board on science and technology for international development. National Academy of Science. 63 pp.

11. Aragon, J.H. (1964). In use of bacteria for microbial control. Microbial control of insects and mites. (Burges, H.D. and Hussey, N.W. eds) Academic Press. London. pp. 33.

12. Bailey, L. (1971). The safety of pest-insect pathogens for beneficial insects: In microbial control of insects and mites (Burges and Hussey, eds). Academic Press. London. pp. 491-503.

13. de Barjac, H. (1981). Identification of H-serolypes of B.t In Microbial control of pest and plant diseases, 1970-1980. (H.D. Burges ed.) Academic Press. London. 949 pages.

14. de Barjac, H. and Bonnefoi, A. (1963). Classification of Bacillus thuringiensis. Entomophaga., 18: 5-17.

15. de Barjac, H. and Larget, I. (1979). Proposals for the adoption of a standardized bioassay method for the revaluation of insecticidal formulations derived from serotype H-14 of Bacillus thuringiensis Mimeographed document WHO/VBC/79.744, 15 pp.

16. Beggs, J.A. (1964). In use of bacteria for Microbial control. Microbial control of insects and mites. (Burges, H.D. and Hussey, N.W. eds). Academic Press. London. pp. 83.

17. Bond, R.P.M., Boyce, C.B.C., Reggoff, M.H. and Shich, T.R. (1971). The thermostable exotoxin of Bacillus thuringiensis. In Microbial control of insects and mites. (Burges and Hussey, eds). Academic Press. London. pp. 275-302.

18. Bulla, L.A. (1981). Purification and characterization of entomocidal protoxin of Bacillus thuringiensis. J. Biol. Chem. 256: 3000-3004.

19. Bulletin of World Health Organisation (1980). Standardized method for bioassay of the larvacidal action of Bacillus thuringiensis serotype H-14. 58: 231-232.

20. Bulletin of World Health Organisation (1981). Biological control of insects vectors of diseases. 59:(5): 669-705.

21. Burgejon, A. and G. Galichet, P.F. (1965). The effectiveness of the heat-stable toxin of B.t var thuringiensis Berliner on larvae of Musca domestica. J. Invert. Pathol. 273-

22. Burgejon, A. and Martouret, D. (1971). Determination and significance of the host spectrum of B. thuringiensis. In Microbial control of insects and mites (Burges and Hussey). Academic Press. London. pp. 305-322.

23. Burges, H.D. and Thomson, E.M. (1971). Standardization and assay of microbial insecticides. In Microbial control of insects and mites. (Burges and Hussey, eds). Academic Press. London. pp. 687-708.

24. Bursell, E. (1964). Environmental aspects: Temperature in the physiology of insects. (Rockstein, ed). Academic Press. London. 1: 283-321.

25. Clement, A.W. (1963). The physiology of mosquito. Pergamon Press. Oxford, London, New York, Paris. 393 pp.

26. Colbo, M.H. and Undeen, A.H. (1980). Effect of B.t var israelensis on non-target insects in stream trials for control of simulidae. Mosquito News. 40: 368-371.

27. Couch, T. (1980). Mosquito pathogenicity of B.t var israelensis. Dev in Industrial Microbiol. pp. 22.

28. Damme, E.N.G. van and La'an P.A. van (1968). Some observation on the effect of E-58 powder. (B.t Berliner) on the Malacosoma neustria. Entomophaga. 4: 221-225.

29. Dulmage, H.T. and Rhodes, R.A. (1971). Economics of microbial control. In Microbial control of insects and mites. (H.D. Burges and N.W. Hussey, eds). Academic Press. London. pp. 581-589.

30. Elson, P. (1982). Nature and size of particles ingested by simuliid larvae, in relation to the use of larvicides. Procs. Bri. Soc. Parasitol. 84: 40.

31. Falcon, L.A. (1971). Use of bacteria for microbial control of insects. In Microbial control of insects and mites. (H.D. Burges and N.W. Hussey, eds). Academic Press. London. pp. 67-90.

32 Falcon, L.A., van den Bosch, R., Ferris, G.A., Stromberg, L.K., Etzel, L.K., Stinner, K.E. and Leigh, T.F. (1968). In the use of bacteria for microbial control. Microbial control of insects and mites. (Burges, H.D. and Hussey, N.W. eds). Academic Press. London. pp. 79).

33 Finney, D.J. (1964). Probit analysis: A statistical treatment of the sigmoid response curve. Cambridge University Press. 318 pp.

34. Fisher, R.A. (1963). Bioassay of microbial pesticides. In Analytical Methods for pesticides and food additives. Academic Press Inc. New York, pp 425-442.

35. Frommer, R., Hembree, S., Nelson, J., Remington, M. and Gibbs, P. (1981). The evaluation of Bacillus thuringiensis var israelensis in reducing Simulium vittatum (Diptera: Simuliidae) larvae in their natural habitat with no extensive aquatic vegetative growth. Mosquito News, 41: 339-347.

36. Goldberg, L.J. and Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against Anopheles sergentii, Uranotaenia unguiculata, Culex pipiens. Mosquito News, 37: 355-358.

37. Goldberg, L.J., Goldberg, E.M. and Margalit, J. (1967). Potential application of a bacterial spore ONR-60A to mosquito larval control: Demonstrated rapid larvicidal activity against Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti and Culex pipiens (Complex) WHO document WHO/VBC/77.662, 7 pp.

38. Hannay, C.L. (1953). Crystalline inclusions in aerobic spore forming bacteria. Nature 172 1004.

39. Heimpel, A.M. (1967). A critical review of Bacillus thuringiensis var. thuringiensis. Berliner and other crystalliferous bacteria. Ann. Rev. Entomol. 12: 287-322.

40. Heimpel, A.M. (1971). Safety of insect pathogens for man and vertebrates. In: Microbial control of insects and mites. (H.D. Burges and N.W. Hussey, eds). Academic Press. London. pp. 469-487.

41. Heimpel, A.M. and Angus, T.A. (1963). Diseases caused by certain spore forming bacteria. In: Insect pathology, an advanced treatise. (Steinhaus, ed). Academic Press. London. 2: 21-73.

42. Hertlein, B.C., Hornby, J., Levy, R. and Miller, T.W. (1980). Prospects of spore-forming bacteria for vector control with special emphasis on their local production potential. Mineographed document. WHO/VBC/80.791. 11 pp.

43. Ignoffo, C., Couch, T., Garecia, C. and Kroha, M. (1981). Relative activity of Bacillus thuringiensis var kurstaki and B. thuringiensis var israelensis against larvae of Aedes aegypti, Culex quinquefasciatus, Trichoplusia ni Heliothiszea and Heliothis virescens In: data sheet on the biological control agent. Document WHO/VBC/79.750 45 pp.

44. Informal consultation on the development of Bacillus sphaericus as a microbial larvicide (1985) UNDP/World Bank/WHO special programme for research and training in tropical diseases. Mineographed document. TDR/BCU/Sphaerueys/85.3.

45. Jensen, F.L. (1966). In use of bacteria for microbial control. Microbial control of insects and mites. (Burges, H.D. and Hussey, N.W. eds). Academic Press. London. pp. 86.

46. Kellen, W.R., Clark, T.B., Lindegren, J.E., Ho, B.C., Rogoff, M.H. and Singer, S. (1965). Bacillus sphaericus Neide as a pathogen of mosquitoes. Journal Invert. Pathol. 7: 442-448.

47. Klowden, M.J., Held, G.A. and Bulla Jnr. L.A. (1983). Toxicity of Bacillus thuringiensis subsp. israelensis to adult Aedes aetypti mosquitoes Applied Environ. Microbiol. 46(2): 312-315.

48. Knight and Stone (1977). In a guide to Medical Entomology. The MacMillan Press. London. 226 pp.

49. Krieg, A. (1969), In use of bacteria for Microbial control. Microbial control if insects and mites. (Burges, H.D. and Hussey, N.W. eds). Academic Press. London. pp. 86.

50. Krishnamurti, B.S. and Pal. R. (1958). A note on the comparative effect of different blood feed on egg production of Culex p. fatigans. Bull Nat. Soc. Ind. Mal. Mosq. Dis. 7: 21-26.

51. Krywienzyk, J. and Fast, P. (1980). Serological relationships of the crystals of B.t var israelensis J. Invert. Pathol. 36: 139-140.

52. Lebrum, P. and Vlayer, P. (1981). Comparative study of the bioactivity and secondary effects of Bacillus thuringiensis H-14: In data sheet on the biological control agent. Document WHO/VBC/79.750 pp. 45

53. Lacey, L.A. and Mulla, M.S. (1977). Evaluation of B.t as a biocide of Blackfly larvae. (Diptera: Simuliidae) Journ. Invert. Pathol. 30: 46-49.

54. Lacey, L., Escaffre, H., Philippon, B., Seketeli, A. and Guillet, P. (1982). Large river treatment with Bacillus thuringiensis (H-14) for the control of Similium damnosium s.l. in the Onchocerciasis control Programme: In data sheet on biological control agent. Document WHO/VBC/79.750. 45 pp.

55. Larget, I. (1982). Report on the study of IPS-78 stability after exposure at 50°C: In data sheet on the biological control agent. Document WHO/VBC/79.750 45 pp.

56. Larget, I. and Barjac de, H. (1981). The serotype H-14 of Bacillus thuringiensis. Parasitology. 82 117-118.

57. Luthy, P., Raboud, C., Delucchie, V. and Kuenzi, M. (1980). Field efficacy of Bacillus thuringiensis var israelensis: In data sheet on the biological control agent. Document WHO/VBC/79.750. 45 pp.

58. Lysenko, O. and Kurera, M. (1971). Micro-organisms as sources of new insecticide chemicals: Toxins In: Microbial control of insects and mites: H.D. Burges and N.W. Hussey, eds). Academic Press. London. pp. 205-226.

59. McConnell, E. and Richards, A.G. (1959). The production of B.t Berliner of a heatstable substance toxic for insects. Can. J. Microbiol. 5: 161-168.

60. Molloy, D., Gaugler, R. and Jamnback, H. (1981). Factors influencing efficacy of Bacillus thuringiensis var israelensis as a biological control agent of blackfly larvae: In data sheet on the biological control agent. Document WHO/VBC/79.750, 45 pp.

61. Newmark, P. (1993). What chance a Malaria vaccine (News). Nature. 302(5903): 473.

62. Nicolescu, G. (1982). The action of two formulation containing B. thuringiensis serotype H-14 on some autochthonous mosquito species (Diptera; culicidae). Archives Roumaines de pathologie experimentals et de Microbiologie. 4(1): 67-72.

63. Norris, J.R. (1964). The classification of Bacillus thuringiensis. J. Appl. Bacteriol. 27: 437-447.

64. Norris, J.R. (1971). The protein crystal toxin of B. thuringiensis: Biosynthesis and physical structure. In: Microbial control of insects and mites. (H.D. Burges and N.W. Hussey, eds). Academic Press. London. pp. 229-246.

65. Pantuwatana, S. (1980). Laboratory evaluation of various formulations of B.t H-14. In: Data Sheet on the Biological Control Agents: Mineographed document WHO/VBC79.750. 45 pp.

66. Pinder, R.M. (1973). Malaria: The design, use and mode of action of chemotherapeutic agents. Bristol: Scientechnica Ltd. pp 1-19.

67. Prasertphon, S. and Rishikesh, N. (1979). Progress report on biological control activities carried out from April through June at WHO/VBCU-1, Kaduna Nigeria. In: Data sheet on the biological control agent. Mineographed document WHO/VBC/79.750. 45 pp.

68. Ouraishi, M.S. (1977). Biochemical insect control: It's impact on economy, environment and natural selection. A Wiley interscience publications. New York. 280 pp.

69. Ramakrishnan, N. (1968). In crystal toxin; Biochemistry and mode of action. Microbial control of insects and mites. (Burges, H.D. and Hussey, N.W. eds). Academic Press. London. pp. 270.

70. Ramoska, W.A., McCollum, W.A., Quickenlon, K.L. and Seckinger, A. (1983). Field test of two commercially formulation of B. thuringiensis serotype H-14 against Aedes mosquito larvae in Montana pasture land. Mosquito News 43(2): 251-254.

71. Raun, E.S. (1963). Cornborer control with B.t Berliner. Iowa State Journ. Scien. 38: 141-150.

72. Rishikesh, N. and Quelennec, G. (1983). Introduction to a standardize method for the evaluation of potency of B.t serotype H-14 based products. Bull. World Health. Org. 6(1): 93-97.

73. Sebesta, K. and Horska, K. (1968). In the thermostable exotoxin of Bacillus thuringiensis microbial control of insects and mites. (Burges, H.D. and Hussey, N.W.) Academic Press. London. pp 298.

74. Service, M.W. (1976). Mosquito ecology: Field sampling methods. Applied science publishers. London. 583 pp.

75. Service, M.W. (1980). A guide to Medical Entomology. The MacMillan Press. London. 226 pp.

76. Sinegre, B., Gaven, B. and Jullien, J.L. (1979). Essais de titrage de deux poudres primaires experimentales du serotype H-14 de Bacillus thuringiensis par comparaison avec la formulation de regerence IPS.72 en employant de larves de Culex pipiens et d' Aedes caspius. Mimeographed document WHO/VBC/79.745. 18 pp.

77. Sinegre, S., Gaven, N.B., Jullien, J.L. et Crespo, C. (1979). Activite du serotype H-14 de Bacillus thuringiensis vis-a-vis de principales espèces de moustiques. Anthropophiles due littoral mediterranean Francais. Mimeographed document WHO/VBC/79.743. 7 pp.

78. Singer, S. (1973). Insecticidal activity of Recent bacterial isolates and their toxins against mosquito larvae. Nature. 244(5411): 110.

79. Subra, R. (1980). Biology and control of Culex pipiens quinquefasciatus say, 1823 (Diptera, culicidae) with special reference to Africa. Mimeographed document WHO/VBC/80.781.

80. Tanada, Y. and Reiner, C. (1962). In use of bacteria for microbial control. Microbial control of insects and mites. (Burges and Hussey, eds). Academic Press. London. pp. 82.

81. Toumonoff, C., and Le Conroller, Y. (1959). Contribution á l'etude de Bacillus cereus Frankland and Frankland cristallophoves et pathogenes pour los larvae de lepidopteres. In a critical review of Bacillus thuringiensis var thuringiensis Berliner and other crystalloferous bacteria. Ann. Rev. Entomol. 12: 287-322.

82. Tyrell, D., Davidson, L., Bulla, L. and Ramosta, W. (1979). Toxicity of parasporal crystal of B.t subsp. israelensis to mosquitoes. Appl. Environ. Microbiol. 38: 656-658.

83. Undeen, A. and Nage, W. (1978). The effect of Bacillus thuringiensis ONR 60A strain (Goldberg) on Simulium larvae in the laboratory: In Dats Sheet on the biological control agent. Document WHO/VBC/79.750. 45 pp.

84. Undeen, A. and Berl, A. (1979). Laboratory studies on effectiveness of B.t var. israelensis de Barjac against Simulium damnosum. (Diptera: Simuliidae) larvae. Mosquito News. 39: 742-745.

85. Undeen, A. and Colbo, M. (1980). The efficacy of Bacillus thuringiensis var israelensis against blackfly larvae (Diptera: Simuliidae) in their natural habitat: In data sheet on the biological control agent. Document WHO/VBC/79.750. 45 pp.

86. Undeen, A., Takaoka, H. and Hansen, K. (1981). A test of Bacillus thuringiensis var israelensis de Barjac as a larvicide for Simulium ochraccum the Central American vector of onchocerciasis. In data sheet on the biological control agent. Document WHO/VBC/79.750. 45 pp.

87. Van Essen, F. and Hembree, S. (1980). Laboratory bioassay of Bacillus thuringiensis var israelensis against all instars of Aedes taeniomynbus. larvae. Mosquito nNews. 40: 424-431.

88. Vankova, J. (1981). House-fly susceptibility to Bacillus thuringiensis var israelensis and a comparison with the activity of other insecticidal bacterial preparations In data sheet on biological control agent. Document WHO/VBC/79.750. 45 pp.

89. WHO. (1982). Data sheet on the biological control agent. (Bacillus thuringiensis serotype H-14, de Barjac). Document WHO/VBC/79.750. 45 pp.

90. Yamamota, T. (1982). Identification of entomocidal toxins of B.t by high performance liquid chromatography. Journ. Gen. Microbiol. 129: 2595-2603.

91. Yoshimura, K., Yamamota, O., Seki, T. and Ochuma, Y. (1982). Distribution of heterogenous and homologous plasmid in Bacillus spp Appl. Environ. Microbiol. 46(6): 1268-1275.

92. Youstein, A.A., Jones, M.E. and Benoit, R.E. (1983). Development of selective/differential bacteriological media for the enumeration of Bacillus thuringiensis serovar. israelensis (H-14) and B. sphaericus 1593. Document WHO/VBC/82.844. 7 pp.

APPENDIX 1

RESULTS OF BIOASSAYS

Toxicity of *Bacillus thuringiensis* on second instar larvae of *Culex pipiens* in 150 ml.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 6 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	12	13	13	14	10	14	63.33
0.03	20	8	9	12	13	8	10	50.00
0.02	20	6	7	10	11	7	8	40.80
0.01	20	6	6	8	10	5	5	33.33
0.008	20	5	5	7	9	3	5	28.33
0.005	20	3	5	4	6	3	4	20.83

No deaths in control.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 24 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	20	19	20	20	18	20	97.50
0.03	20	19	18	18	18	18	19	91.67
0.02	20	18	16	16	15	16	17	81.70
0.01	20	16	15	15	13	14	16	74.00
0.008	20	15	15	14	12	13	15	70.00
0.005	20	12	13	14	11	12	14	63.00

No deaths in controls.

APPENDIX 2

Toxicity of B.t on second instar larvae of Culex pipiens

30 ml

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 6 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	10	11	13	10	11	10	54.16
0.03	20	9	10	11	10	10	10	50.00
0.02	20	6	7	6	5	4	5	27.50
0.01	20	2	3	4	3	4	3	15.83
0.008	20	2	2	2	1	3	2	10.00
0.005	20	1	2	1	1	2	2	7.50

No deaths in controls.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 24 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	16	15	17	16	15	17	80.00
0.03	20	14	14	16	14	13	15	71.66
0.02	20	12	13	15	13	12	13	65.00
0.01	20	10	11	13	11	12	11	56.66
0.008	20	10	11	13	10	11	10	54.16
0.005	20	8	9	7	6	9	8	39.16

No deaths in controls.

APPENDIX 3

Toxicity of B.t on third instar larvae of Culex pipiens in 150 ml

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 6 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	9	7	10	8	9	6	40.80
0.03	20	7	5	8	5	6	5	30.00
0.02	20	5	4	6	4	5	3	20.50
0.01	20	2	3	3	2	4	3	14.20
0.008	20	2	-	2	1	1	2	6.67
0.005	20	-	-	-	-	1	1	1.67

No deaths in controls.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 24 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	20	18	18	13	17	17	90.00
0.03	20	18	15	17	16	16	15	80.80
0.02	20	16	13	15	13	15	13	70.80
0.01	20	13	10	14	11	12	12	60.00
0.008	20	13	7	10	9	10	12	50.35
0.005	20	7	4	5	6	8	10	35.80

No deaths in controls.

APPENDIX 4

Toxicity of B.t on third instar larvae of Culex pipiens in 30 ml

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 6 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	6	7	6	7	7	8	34.16
0.03	20	4	5	4	4	4	3	20.00
0.02	20	2	5	3	3	3	3	15.83
0.01	20	2	2	2	2	2	2	10.00
0.008	20	-	-	1	-	1	1	2.50
0.005	20	-	-	-	-	-	1	0.83

No deaths in controls.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 24 hours						Mean % mortality
		Experiment						
		1	2	3	4	5	6	
0.04	20	14	16	16	15	14	16	73.83
0.03	20	13	14	14	14	12	14	67.50
0.02	20	12	13	12	12	10	11	58.33
0.01	20	8	12	10	9	9	8	46.67
0.008	20	6	7	7	4	7	5	30.00
0.005	20	4	5	5	3	4	3	20.00

No deaths in controls.

APPENDIX 5

Toxicity of B.t on fourth instar larvae of Culex pipiens in 150 ml

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 6 hours							Mean % mortality
		Experiment							
		1	P 2	P 3	P 4	P 5	P 6	P	
0.04	20	5	2 6	1 8	2 6	2 7	2 6	1	31.67
0.03	20	3	2 4	2 5	1 3	1 4	1 4	3	19.17
0.02	20	2	1 3	1 3	1 2	2 3	3 3	1	13.39
0.01	20	1	2 2	5 2	- 2	3 2	1 2	1	9.17
0.008	20	-	1 1	5 2	2 1	1 1	- -	3	4.17
0.005	20	-	- -	1 -	1 -	1 1	2 -	1	0.85

P = pupae.

No deaths in controls.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 24 hours												Mean % mortality
		Experiment												
		1	P	2	P	3	P	4	P	5	P	6	P	
0.04	20	15	3	17	2	17	3	15	3	16	3	15	2	79.20
0.03	20	14	4	16	3	15	2	14	3	16	3	13	3	73.30
0.02	20	12	5	15	2	14	2	12	2	14	4	11	4	65.00
0.01	20	6	6	12	5	13	2	11	3	13	3	9	3	53.30
0.008	20	4	5	10	6	11	3	11	3	9	3	9	3	43.30
0.005	20	2	4	8	2	7	2	6	2	7	2	4	2	28.30

P = pupae

No deaths in controls.

APPENDIX 6

Toxicity of B.t on fourth instar of Culex pipiens in 30 ml

Concentration in ng/l	Number of larvae in each beaker	Total number of dead larvae after 6 hours — Experiment							Mean % mortality
		1	P 2	P 3	P 4	P 5	P 6	P	
0.04	20	4	- 7	2 5	- 5	1 6	2 7	2	28.33
0.03	20	3	3 5	1 3	1 4	2 3	1 4	2	18.33
0.02	20	-	1 2	- 2	1 2	1 2	3 2	1	8.33
0.01	20	1	1 1	1 -	2 1	1 1	- 1	3	4.17
0.008	20	-	- 1	- 1	- -	2 1	2 -	1	2.50
0.005	20	-	1 -	1 -	1 -	1 -	2 -	1	-

P = Pupae

No deaths in controls.

Concentration in mg/l	Number of larvae in each beaker	Total number of dead larvae after 24 hours												Mean % mortality
		Experiment												
		1	P	2	P	3	P	4	P	5	P	6	P	
0.04	20	14	1	15	3	15	3	14	4	13	5	15	3	71.16
0.03	20	13	3	14	1	13	2	12	3	12	2	14	3	65.00
0.02	20	8	3	11	2	12	2	11	5	11	3	12	2	54.16
0.01	20	4	2	9	2	9	2	9	2	10	2	11	3	43.33
0.008	20	2	4	5	2	8	4	7	4	8	3	9	2	32.50
0.005	20	-	2	2	2	4	3	5	3	3	3	4	3	15.00

P = Pupae

No deaths in controls.