Efficacy and residual activity of *Bacillus sphaericus* granules and *Bacillus thuringiensis* var. *israelensis* wettable granules for controlling *Culex quinquefasciatus* in Dar es Salaam, Tanzania.

BY:

NDARO JOHNSON
(DEHS. University of Dar es Salaam)

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DECLARATION

I, Johnson Colman Ndaro, hereby declare that this is my original work and has not been submitted for a degree in any other university.

Signature.................................... **Date** 01-10-2009..

Johnson Colman Ndaro

This thesis has been submitted for examination with our approval as supervisors:

Signature.. **Date** 1-10-2009..

Wolfgang Richard Mukabana, PhD.

Senior Lecturer, School of Biological Sciences, University of Nairobi, Nairobi, Kenya

and

Signature Gerry Killeen.................................. **Date** 1/10/2009......................................

Gerry F. Killeen, PhD

Research Scientist, Ifakara Health Institute, United Republic of Tanzania.

Reader, Liverpool School of Tropical Medicine, UK.

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Dedication

To my lovely wife Aisia Habiba and my little lovely daughter Anna for their patience and encouragement in the course of my study and your endless prayers for me so as to complete my work with success. May God bless you.

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List of Abbreviations

ALB	Albendazole
Bs	*Bacillus sphaericus*
Bti	*Bacillus thuringiensis* var. *israelensis*
CG	Corn cob granules
Cx	*Culex*
DEC	Diethylcarbamazine
EPB	Expanded polystyrene beads
IVM	Integrated Vector Management
LF	Lymphatic Filariasis
UMCP	Urban Malaria Control Programme
WG	Wettable Granules
WHO	Word Health Oranization

Abstract

The microbial larvicides *Bacillus sphaericus* and *Bacillus thuringiensis* have recently proven to be effective tools for integrated mosquito control programmes in Africa. This study assessed the efficacy and duration of activity of *Bs* and *Bti* for controlling *Culex quinquefasciatus* Say in the pit latrines and cesspits of urban Dar es Salaam, Tanzania. Larval sampling was conducted on a weekly basis for six weeks prior to treatment and sixteen weeks post treatment in both habitats categories. Three different dosages (low, medium and high) of *Bs* and *Bti* were randomly assigned to 70 of each habitat type following stratification within pit latrine and cesspit categories according to larval density, while ten of each habitats type served as controls. *Bti* at all doses of application achieved very short-lived and weak levels of control of immature stage of *Culex* in both habitats types. The medium and high doses of *Bs* achieved complete control of *Cx quinquefasciatus* late-stage larvae for approximately five weeks in pit latrines while the low dose provided only one week of complete control. In contrast, all three doses of *Bs* achieved complete control of late-stage larvae for only one week in cesspits. Using a minimum threshold of 80% control, as the criterion for acceptable operational control, *Bti* at all doses did not achieved acceptable partial control for any extended period in pit latrines and cesspits. The low dose of *Bs* in pit latrines provided six weeks of acceptable partial control of late-stage larvae while the medium and high dose achieved twelve and eight weeks of partial control, respectively. For both low, medium and high doses of *Bs* in cesspits attained partial control of late-stage larvae of not more than six weeks. The extended control provided by *Bs* therefore great advantages to any control programme, such as the Dar es Salaam Urban Malaria Control Programme, that could correspondingly reduce the cost of product procurement and of application and monitoring which are often higher than the cost of material in operational programmes.

CHAPTER 1.0: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Lymphatic filariasis is a parasitic disease caused by *Wuchereria bancrofti* and *Brugia malayi* or *B. timori* (Boyd *et al.*, 2004). It affects an estimated 120 million people throughout the tropics and sub-tropics in at least 80 countries, with one billion people considered to be at risk of becoming infected (Michael and Bundy, 1997). The disease is transmitted by various mosquito species (de Almeida and Freedman, 1999) and is a major cause of morbidity affecting all ages, one third of whom suffer from chronic manifestation of the disease resulting from damage to lymphatic vessels, swelling of the limbs and genitals and painful bacterial infections (Partono, 1985). These symptoms worsen as the disease progresses, making it difficult for victims to carry out their domestic and livelihood-related activities comfortably (Gyapong *et al.*, 1996; Anne and Kestler, 2000).

The global elimination of lymphatic filariasis (LF) programme has been launched in many countries including Tanzania and country-level elimination activities are underway across the tropical world. The primary strategy of the global LF programme is to administer an annual single dose of diethylcarbamazine (DEC) and albendazole (ALB) to the target population, irrespective of the level of endemicity, for an extended period so that the cumulative effect of the drug may lead towards the elimination of the disease (Ottesen, 2000).

Night blood tests for Bancroftian filariasis by new antigen detection kits and better methods for combinations of drugs for reducing the prevalence and density of microfilaria are now available and drug resistance remains absent from populations of the target organisms. Nevertheless, there is a concern that mass drug administration campaigns may fail to maintain sufficient treatment coverage to achieve lymphatic filariasis elimination (Ottesen *et al.*, 1997; Gyapong *et al.*, 2005). Thus, extra measures to limit transmission may be desirable to guarantee success of the Global Program for Elimination of Lymphatic Filariasis (Burkot *et al.*, 2006).

Integrated approaches that incorporate microbial larvicides for mosquito larval control (Maxwell *et al.*, 1999; Sunish *et al.*, 2002) might be an effective component of integrated vector management (IVM) strategies for reducing the transmission of mosquito borne diseases, including LF (Burkot *et al.*, 2002; Townson *et al.*, 2005). Larval control using microbial insecticides to kill aquatic stages of mosquitoes has a major advantage in that this approach controls mosquitoes before they depart from their habitats and scatter to bite people and transmit disease (Killeen *et al.*, 2002). In areas where a lot of larval habitats occur, large numbers of adult mosquitoes cause intense biting nuisance and disease transmission. Unless appropriately controlled at source through effective larval control, it can be difficult to control these adult mosquitoes after they disperse, particularly if they prefer feeding and resting outside. Mosquito larvae are a more desirable target for control efforts since mosquito larvae, unlike adults (Charlwood and Graves, 1987; Yohannes *et al.*, 2005), cannot change their behavior to avoid control activities targeted at larval habitats (Killeen *et al.*, 2002b). *Bacillus sphaericus* (*Bs*) has enjoyed widespread use in the control of mosquito larvae in both polluted and clear water. This is because it possesses the unique property of being able to survive in water that is rich in organic matter for long periods of time by actively propagating in mosquito cadavers (Lacey and Undeen, 1986). On the other hand *Bacillus thuringiensis* var. *israelensis* (*Bti*) use has typically been limited to relatively clean water in which it works most effectively (Lacey, 2007).

The results of an assessment of the efficacy of *Bs* granules applied directly by hand and of *Bti* wettable granules in aqueous mist applications for the control of *Culex quinquefasciatus* Say, a major LF vector in Urban Dar es Salaam, are reported in this thesis. Three different concentrations of *Bs* and *Bti* were tested against populations of *Cx. quinquefasciatus* developing in cesspits and pit latrines to establish the most effective dosage and the maximum length of time over which the densities of *Cx. quinquefasciatus* larvae and pupae were suppressed. Field experiments were conducted at Vingunguti ward in Ilala Municipality, Dar es Salam, Tanzania.

1.2. LITERATURE REVEIW

1.2.1. Major vectors of *Wuchereria bancrofti*

Culex quinquefasciatus is the principal vector of Bancroftian filariasis in areas where *Wuchereria bancrofti* has nocturnal periodicity, but, in other regions, other species of mosquitoes may serve as the local vector of the worm (White, 1989). Across the rural coast of East Africa *Anopheles gambiae* and *An. funestus* are considered to be the major vectors of *Wuchereria bancrofti* while in the towns and cities of this region *Cx. quinquefasciatus* dominate transmission (White, 1971; Hawking, 1977).

1.2.2. *Culex quiquefasciatus* in urban settings

Culex quiquefasciatus, a major nuisance biting mosquito and vector of Bancroftian filariasis (Kar *et al.*, 1997), typically undergoes larval development in water heavily polluted with organic material, such as refuse, excreta or decayed vegetation. Examples of such larval habitats are blocked drains, canals, abandoned wells and sanitation structures. Sanitation structures are important facilities constructed purposely for receiving and storing waste matter and effluent discharged from human habitations (Cairncross, 1988) such as sewers, pit latrines and cesspits.

Urban settings, owing to their high density of human settlement, have more sanitation structures per unit area than rural areas (Chavasse *et al.*, 1995). In Dar es Salaam, the largest city of the United Republic of Tanzania, sanitation structures are found in, or associated with, almost every house and they typically contain highly polluted water, hence providing ideal larval habitats for *Cx quiquefasciatus*, a markedly domestic species. The adult females feed mainly outdoors in the evening and at night, spending the daylight hours resting in cool, humid, and dark places. It is in the process of blood seeking that the mosquitoes prove to be a biting nuisance, making human victims restless and irritated. Also the continued bites expose people to the risk of contracting filariasis. In Dar es Salaam, *Cx quinquefasciatus* account for about 95% of biting mosquitoes (Bang *et al.*, 1975; Chavasse *et al.*, 1995). Experience to date suggests the need to control nuisance biting mosquitoes as an incentive for the community to support other disease control programs such as application of microbial larvicides to *Anopheles* habitats to prevent malaria (Khannady, Personal Communication).

1.2.3. Systematic larviciding as a means to prevent mosquito-borne diseases

Larviciding has a proven historical track record for control of African malaria vectors, and more rigorous contemporary evidence is now accumulating (Utziger *et al.*, 2001; Utziger *et al.*, 2002; Fillinger *et al.*, 2003; Fillinger *et al.*, 2004; Keiser *et al.*, 2005; Fillinger *et al.*, 2008; Fillinger *et al.*, 2009; Geissbuhler *et al.*, 2009). Larviciding can be implemented rapidly once the presence of the developing larvae or even merely potential aquatic habitat has been identified. Using safe formulations of mosquito-specific microbial insecticides, application can be effectively carried out by hand for small accessible habitats, machines for fairly large habitats and aircraft for very large areas that need to be treated quickly. Larvicides with such an excellent safety profile as *Bti* and *Bs* have the additional advantage that they can easily be adopted by community-based personnel who can be trained within a short time on application techniques using equipment which are simple and often readily available (Fillinger *et al.*, 2008; Geissbuhler *et al.*, 2009).

Although larviciding has largely been neglected as an option for malaria control, it is often considered the highest priority intervention for preventing other parasitic and viral diseases that are transmitted by mosquitoes (Mulla *et al.*, 2001). The challenge to success in resource-poor countries is to achieve effective, sustained implementation at affordable cost. The Urban Malaria Control Programme (UMCP) in Dar es Salaam, Tanzania, is a pilot programme to develop such systems which uses microbial larvicides for larval-stage control of malaria vectors. Microbial larvicides may be combined with other methods such as the domestic use of synthetic chemical pesticides and environmental management to form comprehensive mosquito control strategies that effectively reduce transmission rates of pathogens which cause a diversity of diseases. This means that, as a complementary approach to mainstream tools such as insecticide treated nets and indoor residual spraying, larval control has a useful place in the list of options for vector control specialists in Africa and provides an opportunity for integrated disease control targeted at such diseases as malaria and filariasis (WHO, 2004; Townson *et al.*, 2005). Such integration helps to reduce cost and ensure sustainability through support from more than one disease control programme budget.

The larviciding approach is sometimes easier for communities to accept or implement than environmental management of key resources such as water and land (Mutuku *et al.*, 2006). Additionally, larviciding can be feasible even in resource-poor countries since the equipment needed for larviciding is simple and inexpensive, and skills for larviciding can be easily acquired (Becker and Rettich, 1994; Mukabana *et al.*, 2006; Fillinger *et al.*, 2008). Microbial and non-microbial larvicides are promising and practical methods that communities may easily accept. Expanded polystyrene beads (EPB) are a non-microbial larvicide that could effectively reduce population densities of mosquito within a short time after application (Reiter, 1978; Curtis and Minjas, 1985). Expanded polystyrene does not require frequent reapplication to the habitat like most other agents; the beads remain floating on the surface of water for a long period of time, suffocate mosquito larvae and pupae and inhibit egg laying (Charlwood, 1994). Expanded polystyrene beads application is simple, cheap and does not cause any harm to the environment (Cook and Dunsby, 1978; Curtis and Minjas, 1985).

1.2.4. Microbial larvicides

Bacillus sphaericus (*Bs*) and *Bacillus thuringiensis* var. *israelensis* (*Bti*) are both soil-dwelling bacteria which produce toxins that, upon ingestion, kill specific insects of the sub-order Nematocera in the order Diptera, including mosquitoes. Currently, *Bs* and *Bti* are merely two of the many kinds of active ingredients which are routinely used in control programs in the field (Gammon *et al.*, 2006) but are particularly popular among programmes in the developed world because they are considered to be environmentally friendly. These naturally occurring bacteria are cultivated on industrial scales and can be sprayed as a liquid suspension or formulated as granules for hand application by mixing with small fragments of maize cob, clay or sand. Once in water, mosquito larvae eat the bacteria which destroy the gut lining, causing the mosquito to die (Ragoonanansingh *et al.*, 1992). These products do not cause any adverse effects to people, animals, birds or indeed the vast majority of other invertebrates so they are ideal for community-based mosquito control in Africa and other parts of the tropics (Mulla *et al.*, 1984; Karch *et al.*, 1992; Fererici, 1995; Fillinger *et al.*, 2003).

1.2.4.1. Characteristics of *Bacillus* species as microbial larvicides

Bacillus thuringiensis var. *israelensis* is an aerobic, gram positive, rod-shaped and spore-forming bacterium. Once sporulation takes place, it produces a crystalline inclusion body containing four different protein toxins, comprising the principal bioactive ingredients in its formulation (Gill *et al.*, 1992). Upon being consumed, proteins in the crystals are solubilized in the midgut of susceptible species, after being activated by enzymatic action in this alkaline micro-environment (Aronson *et al.*, 1986). The activated toxins destroy and disrupt the gut lining of the larvae so that the larvae stop feeding and starve to death.

Bacillus sphaericus is a spore forming aerobic bacterium and is also highly toxic to mosquito larvae (Davidson, 1984). It grows in culture as a rod of approximately 3μm length that forms spherical spores at the end of the rod. Its insecticidal protein is sited in the spore wall, also in a granule similar to the crystal inclusion of *B .thuringiensis*. *Bacillus sphaericus* crystals have two component proteins which act as binary toxins with both the proteins jointly required for toxicity (Baumann *et al.*, 1991). The mode of action of *Bs* is through its effect in the larval gut, similar to *Bti*. For *Bti*, proteinaceous toxins bind to the surface membranes of the epithelial cells in the larval midgut, interrupting osmotic balance of the cells. However, in the case of *Bs* these protein toxins bind to the cells of the gastric caecum and posterior midgut (Davidson, 1981; Boonserm *et al.*, 2005). During binding of the binary toxin to the midgut epithelium, mitochondrial and endoplasmic reticula swell and vacuoles enlarge. This is followed by lysis of epithelial cells, midgut puncturing and then death of larvae (Davidson and Titus, 1987; Charles *et al.*, 1996).

Laboratory evaluation of larvicide activity of the two *Bacillus* species against various mosquito larvae shows that *Aedes* larvae was the most sensitive to *Bti* followed by *Culex*, *Anopheles* and finally *Mansonia* (WHO., 1980; Foo and Yap, 1982). In the case of *Bs*, mortality is highest in *Culex* species followed by *Anopheles*, then *Mansonia* and then *Aedes* (WHO, 1980b; Lacey and Singer, 1982; Mulla *et al.*, 1984; Choeng and Yap, 1985). Species within a particular mosquito genus are not equally prone to a given toxin from different strains of *Bs* (Yap, 1987; Berry *et al.*, 1993). *Bacillus sphaericus* 2362, IAB59 and 2297 have the same effectiveness against *Cx. quinquefasciatus* and *Aedes nicromaculis* (Berry *et al.*, 1993) while *Bs* 1593 only affects *Ae. Nicromaculis* (Mulligan *et al.*, 1978). On the other hand *Bs* 2297 and IA59 have no effect on *Aedes eagypti* while *B. sphaericus* 2362, 2317.3 and 1593 are quite toxic to this species (Mulligan *et al.*, 1978; Yap, 1987).

1.2.4.2. Factors affecting activity of *Bacillus thuringiensis*

Numerous biotic and abiotic factors influence larvicidal activity of *Bti* including habitat characteristics, species of mosquito and their relevant feeding strategies, rate of ingestion, age and density of larvae, dosage, storage condition and method of application (Mulligan *et al.*, 1980; Aly *et al.*, 1988; Mulla *et al.*, 1990b; Becker *et al.*, 1992; Beck *et al.*, 1996; Nayar *et al.*, 1999; Christiansen *et al.*, 2004).

The impact and longevity of *Bti* larvicidal activity is shortened in habitats having organic matter because the bacterial toxins are rapidly bound to organic particles (Mulligan *et al.*, 1980; Hougard *et al.*, 1983; Karch *et al.*, 1991; Mulla *et al.*, 1993) thus enabling survival of larvae under these conditions. It has been demonstrated that even very high concentrations of *Bti* were unable to extend the duration of control of *Culex* species in organically enriched ponds (Mulligan *et al.*, 1980; Mulla, 1990). The rapid settling rate of *Bti* toxins can also reduce residual activity, especially against mosquito species that feed at the air-water interface, such as *Anopheles spp*, or in the water column itself (Hougard *et al.*, 1983; Mullen and Hinkle, 1988). Settling rates can be accelerated by turbidity, thus exacerbating this problem (Margalit and Bobroglio, 1984; Mulla *et al.*, 1993). Shortened longevity of larvicidal activity imposes the need for regular reapplication of larvicide where mosquito proliferation is continuous. However, various formulations of *Bti* achieve extended control of *Ae. aegypti* in fresh water tanks and other containers with more ideal conditions (Batra *et al.*, 2000; Mulla *et al.*, 2004). Control of mosquitoes breeding in such domestic water storage containers, *Ae. aegypti* in particular, requires slow release formulations that allow more extended availability of active ingredient and have no effect on the water quality or appearance (Mulla *et al.*, 2004; Valarinhos and Monnerat, 2004). More generally, the huge diversity of water bodies which mosquitoes exploit create the need to manufacture a diversity of larvicide formulations to target each of these habitats categories. Granules for instance, can be readily applied to habitats covered with vegetation like rice fields and easily penetrate to reach the water surface and target larvae underneath. Other formulations like aqueous suspensions (AS), flowable concentrates (FC) and aqueous suspensions of water dispersible granules (WG) can be applied using spray equipment and are suitable for controlling surface feeders such as *Anopheles* larvae (Yates, 1984; Sandoski *et al.*, 1985).

Larval density is a vital factor influencing effectiveness of microbial toxins in a diversity of habitats. Higher larval densities require higher rates of application. Likewise, deep bodies of water, as well as habitats having vegetation, need to be treated at higher rates than habitats without vegetation and shallow water. The larvicidal activity of *Bti* can be affected by storage condition and duration of storage. When stored properly at room temperature or cooler, *Bti* activity will not be altered for several months or even a year (Thiery and Hamon, 1998), but as the temperature and duration of storage increase, the larvicidal activity is reduced (Farghal and Darwazeh, 1988).

1.2.4.3. Factors affecting the efficacy of *Bs*

Effects caused by most of biotic and environmental factors on the efficacy of larvicidal activity of *B. thuringiensis* are similar for *B. sphaericus* (Mulligan *et al.*, 1980; Burke *et al.*, 1983; Lacey, 1984; Lacey *et al.*, 1988; Mulla, 1990). However, one clear difference is their *et al.*, 2004). Control of mosquitoes breeding in such domestic water storage containers, *Ae. aegypti* in particular, requires slow release formulations that allow more extended availability of active ingredient and have no effect on the water quality or appearance (Mulla *et al.*, 2004; Valarinhos and Monnerat, 2004). More generally, the huge diversity of water bodies which mosquitoes exploit create the need to manufacture a diversity of larvicide formulations to target each of these habitats categories. Granules for instance, can be readily applied to habitats covered with vegetation like rice fields and easily penetrate to reach the water surface and target larvae underneath. Other formulations like aqueous suspensions (AS), flowable concentrates (FC) and aqueous suspensions of water dispersible granules (WG) can be applied using spray equipment and are suitable for controlling surface feeders such as *Anopheles* larvae (Yates, 1984; Sandoski *et al.*, 1985).

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Bacillus sphaericus formulations often include live spores which enables self-propagation and long-lasting larvicidal effectiveness in the field (Des Rochers and Garcia, 1984; Karch *et al.*, 1992). In the larval midgut, spores germinate and proliferate, producing new spores which are discharged into the aquatic environment as the larval carcass breaks up (Mulligan *et al.*, 1980; Rochers and Garcia, 1984). This can give *Bs* a considerable extended residual effect against targeted mosquito species. Such residual activity is often easier to achieve in organically enriched habitats with correspondingly high densities of mosquito larvae, *Culex* in particular (Davidson, 1984; Lacey, 1984; Mulla *et al.*, 1984).

1.3. JUSTIFICATION AND SIGNIFICANCE OF THE RESEARCH

Mosquito control entails managing the population of mosquitoes to reduce the damage they cause to human health, the economy and public enjoyment of the environment (Metcalf and Novak, 1994). Mosquito control is a public health practice throughout the world, especially in the tropics where mosquitoes spread many debilitating diseases such as malaria, bancroftian filariasis and numerous arboviruses.

Mosquito control operations can be targeted against three different mosquito problems:

1. Nuisance mosquitoes which bother people with painful or irritating bites.
2. Economically important mosquitoes which reduce property values, adversely affect tourism, leisure and related business interests and even livestock or poultry production.
3. Public health is the focus when mosquitoes are the vectors of infectious disease agents.

Depending on the situation, source reduction, biocontrol, larviciding (control of larvae), adulticiding (control of adults) or physical protection against human-vector contact may be used to manage mosquito populations (Mulla *et al.*, 2001). These techniques are accomplished using habitat modification, pesticide application, biological control agents, trapping or physical barriers such as window screening. In Dar es Salaam it may be possible to affordably and effectively control *Cx. quinquefasciatus* if treatment regimes for microbial insecticide formulations can be identified which are long lasting and easy to use.

The information obtained from this study on application of *Bs* and *Bti* in the closed habitats against *Cx. quinquefasciatus* will be used (1) by any Urban Authority especially along coastal areas to plan for the control of *Cx. quinquefasciatus* proliferated in pit latrines and cesspits; (2) to provides information on the most effective dose among these microbial larvicides and the duration of application within pit latrines and cesspits; (3) to give way forward on how to implement large scale programmes which are sustainable and cost effective.

1.4. OBJECTIVES

1.4.1. OVERALL OBJECTIVE:

To assess the efficacy and duration of effect of *Bacillus sphaericus* granules as applied directly by hand and *B. thuringiensis* wettable granules in aqueous mist applications for the control of *Culex quinquefasciatus* in urban Dar es Salaam.

1.4.2. SPECIFIC OBJECTIVES.

1. To identify potential domestic larval habitats of *Culex quinquefasciatus* in Dar es Salaam.
2. To establish the most efficacious dosage of *Bs* granules and *Bti* mist against populations of *Cx. quinquefasciatus* developing in cesspits and pit latrines.

3. To establish the maximum length of time over which larvae of *Culex quinquefasciatus* are controlled by different dosages of *B. sphaericus* granules and *B. thuringiensis* mist.

1.5. HYPOTHESIS

Bacillus sphaericus and *Bacillus thuringiensis* can be used to effectively control *Culex quinquefascitus* mosquitoes in closed habitats.

CHAPTER 2.0. MATERIALS AND METHODS

2.1. Study site.

The study was conducted in Dar es Salaam, Tanzania. Dar es Salaam is the largest city and primary commercial centre of the United Republic of Tanzania with over 2.5 million inhabitants, covering a total area of 1400 km². It is situated on the shores of the Indian Ocean and is administratively comprised of three Municipalities namely Kinondoni, Temeke and Ilala. Each of these municipalities is divided into wards and each ward is divided into neighborhoods called *Mitaa*, literally meaning streets. The temperature in Dar es Salaam ranges from a mean of 32°C in the hot season of December to 18°C in the coolest month of June. It experiences two wet seasons in a year, with heavy rains between March and May and shorter, less intense rains between September and November with average annual rainfall of approximately 1000mm.

Field studies were carried out at Vingunguti ward in Ilala municipality. Vingunguti is an urban community located 25 km west of the city center (Figure 1). It comprises approximately 10,000 houses, occupied by approximately 89,000 inhabitants with high densities of shops and mostly modern house construction. It is a poorly planned area with inadequate infrastructure, resulting in a large squatter settlement with narrow pathways that hinder access to refuse and garbage collection, as well as other services requiring motorized transport. In the case of waste water management, houses have their own sanitation structures that are used to collect and store excreta and waste water. These structures include pit latrines, septic tanks and cesspits. Structures containing water and allowing accessibility to mosquitoes, including those which have been abandoned, provide larval habitats for *Cx. quinquefasciatus* mosquitoes, whose adults disperse to the environment to feed on people, causing substantial disturbance and irritation.

Figure 1: A map of Dar es Salaam showing Vingunguti ward and the other 14 wards included in the urban malaria control programme. Wards marked in light grey are those to which larviciding of *Anopheles* habitats were introduced in 2006 (Geissbuhler *et al.*, 2009).

Figure 2. Pit latrines of various designs.

Figure 3.Typical examples of cesspits.

2.2. Identification of potential domestic larval habitats of *Culex quinquefasciatus*

Two hundred sanitation structures (Figure 2 and 3) which continuously contained water were identified. Preliminary larval sampling was accomplished using a standard 250ml dipper to identify active larval habitats (habitats with larvae or pupae) and a subset of 140 habitats were selected on the basis of high larval density for the experiments. Larval searchers were protected by wearing gloves, masks, overalls and gumboots so as to prevent them from being contaminated with faecal matter. Lists of habitats in the study area from which 10 cesspits and 10 pit latrines were randomly allocated to each seven treatments were assembled. Septic tanks were intended to be included in the experiment as a third category of habitat, but were excluded because insufficient numbers of them were identified which consistently contained water and mosquito larvae.

2.2.1. Larval monitoring

Within all 140 habitats, sampling was conducted weekly using standard 250mls dippers, scooping from the upper layer at between one and ten points on different sides of habitats. Up to ten dips in each habitat were taken until approximately 100 larvae or more were obtained or 10 dips had been taken. The contents of the pooled dip samples were transferred to a white plastic tray where aquatic stages of mosquitoes were counted and categorized as early (instars 1 or 2) or late (instars 3 or 4) stage larvae or as pupae. Both the numbers of mosquito stages counted and the number of dips taken to obtain them were recorded on a sampling form and the surface area of the habitat was also measured and recorded. Sampling of aquatic stage mosquitoes proceeded for 6 weeks before application of larviciding treatments and for 16 weeks afterwards.

2.3. Application of larvicides in the active cesspits and pit latrines

Treatments were randomly assigned to the selected experimental units following stratification within each habitat categories (pit latrines & cesspits) according to larval density. A total of 140 habitats (70 pit latrines and 70 cesspits) were ranked by larval density within each of the two categories and then one from each stratum was randomly assigned to each of the 7 treatments (Table 1). Before application, *B. sphaericus* granules (Vectolex® CG) were weighed using a weighing scale in three different sized-single doses: (5.8 gm, 11.6 gm, and 23.2 gm) which represent doses of 1.0, 2.0 and 4.0 gms/m² based on the mean surface area of the pool of habitats (Figure 4). These doses were placed in polythene sachets which were subsequently opened and poured into each habitat, depending upon the assigned dosage rate. *Bacillus thuringiensis* var. *israelensis* water dispersible granules (Vectobac®WG) were applied by using a Stihl SR 420 motorized backpack mist blower (Figure 5). Dilutions in tap water were made to achieve doses of 0.29g, 0.58g and 0.87g per habitat based on field calibration of the mist volume output.

Figure 4.Measured, prepared doses of *Bacillus sphaericus* CG in polythene sachets.

Figure 5. The Stihl 420 motorized backpack mist blower.

Vectolex® CG at dosage rates 1.0gm/m², 2.0gm/m² and 4.0gm/m² were each assigned to 10 pit latrines and 10 cesspits. Similarly, Vectobac® WG at dosage rates 0.05gm/m², 0.1gm/m², and 0.15gm/m² was applied to 10 replicates per habitat type. Twenty sites were left untreated, with 10 of each habitat category to serve as controls. This means that 10 replicates of each treatment, including the controls were distributed equally across the two habitat categories (Table 1).

Table 1. Experimental design

Category	Treatments							Total
	Control	Bs-1	Bs-2	Bs-3	Bti-1	Bti-2	Bti-3	
Pit latrines	10	10	10	10	10	10	10	70
Cesspits	10	10	10	10	10	10	10	70
Total	20	20	20	20	20	20	20	140

2.4. DATA ANALYSIS

The sampling of immature mosquitoes was carried out weekly throughout the pre- and post-treatment period on specified, pre-scheduled days. The weekly data of all the dips taken from each pit latrine and cesspit were aggregated to estimate the average density per dip for that habitat on that week. Impact was assessed based on the elimination or degree of reduction of late-stage larvae and pupae in treated habitats compared to untreated controls of the same habitat category. Two different thresholds for acceptable levels of control were set based on the density of these immature stages:

1) Complete control, meaning complete absence of late-stage larvae or pupae.
2) Partial control, adequacy of which was deemed to be acceptable if a reduction in density of larvae or pupae was obtained that was 80% or more.

The percentage reduction in larval and pupal densities in the pit latrines and cesspits was calculated using Abbott's formula,

$$R (\%) = [1 - (T2 * C1)/(T1 * C2)] * 100$$

Where T1 and T2 are the pre and post-treatment population densities in the treated habitats and C1 and C2 are corresponding untreated control habitat population densities. Abbott's formula corrects for initial population differences and natural population changes over time to assure that differences seen are actually a result of treatment. Since Abbott's formula yields an output expressed as percentage control, we derived relative densities by subtracting the outcome of Abbott's formula, express as a proportion, from 1. In other words, 90% control is equal to relative density of 10% or 0.1. The relative density can be simply considered as a ratio of the density of larvae or pupae in the treated habitats to that in the untreated control habitats. The statistical differences between the mean rank of intervals of complete control of late larvae and pupae in the controls and each treatment in each-habitat categories were compared by non-parametric Mann-Whiteney U test. Significant differences between dosages of *Bs* or *Bti* in any particular habitat category was assessed by calculating the heterogeneity of variance between dosages for the mean rank of the intervals of absence of larvae or pupae, using the Kruskal-Wallis test. All statistical tests were conducted using SPSS 15.0 software and all graphs were prepared by using Microsoft Excel and PowerPoint packages.

CHAPTER 3.0. RESULTS

3.1 Overview of impact in pit latrines and cesspits

These studies showed that both the microbial larvicides *Bacillus sphaericus* and *Bacillus thuringiensis* caused mortality of immature stages of *Cx. quinquefasciatus* in pit latrines within 24 hours of application but achieved different levels of control (Figure 6). In all cases, except for the lowest dose of *Bti*, the population density of late instar larvae decreased after application, with greatest impact being recorded in the first day after treatment. All three *Bs* concentrations tested achieved successful control of immature stages of *Culex* (Figure 6). The medium and high doses had similar residual effects, both appearing to have a longer period of residual activity than the lowest dose (Figure 6). For at least one week post-treatment, the habitats treated with any of the three *Bs* dosages experienced complete absence of late instar larvae. In the second week, the early and late instars larvae were observed in the habitats treated with the low dose of *Bs*, showing that the larvicides activity in pit latrines with this dose had began to decline (Figure 6). From week three onwards, immature stages of *Culex* continued to recover. The medium and high dose both maintained complete control of late stage larvae at for 5 weeks and appeared to suppress densities to some degree for the full 16 weeks of follow up.

It is noted that the three doses of *Bti* tested manifested much lower levels of activity against *Cx. quinquefasciatus* in pit latrines (Figure 6). While the lowest dose of *Bti* had no obvious effect, there were substantial but incomplete reductions in the density and occupancy of larvae and pupae in the first week of treatment for the medium and high dose. One week later immature stages of *Cx. quinquefasciatus* started to recover. In summary, efficacy of *Bti* is much lower than *Bs* and has much less residual activity in these organically enriched habitats.

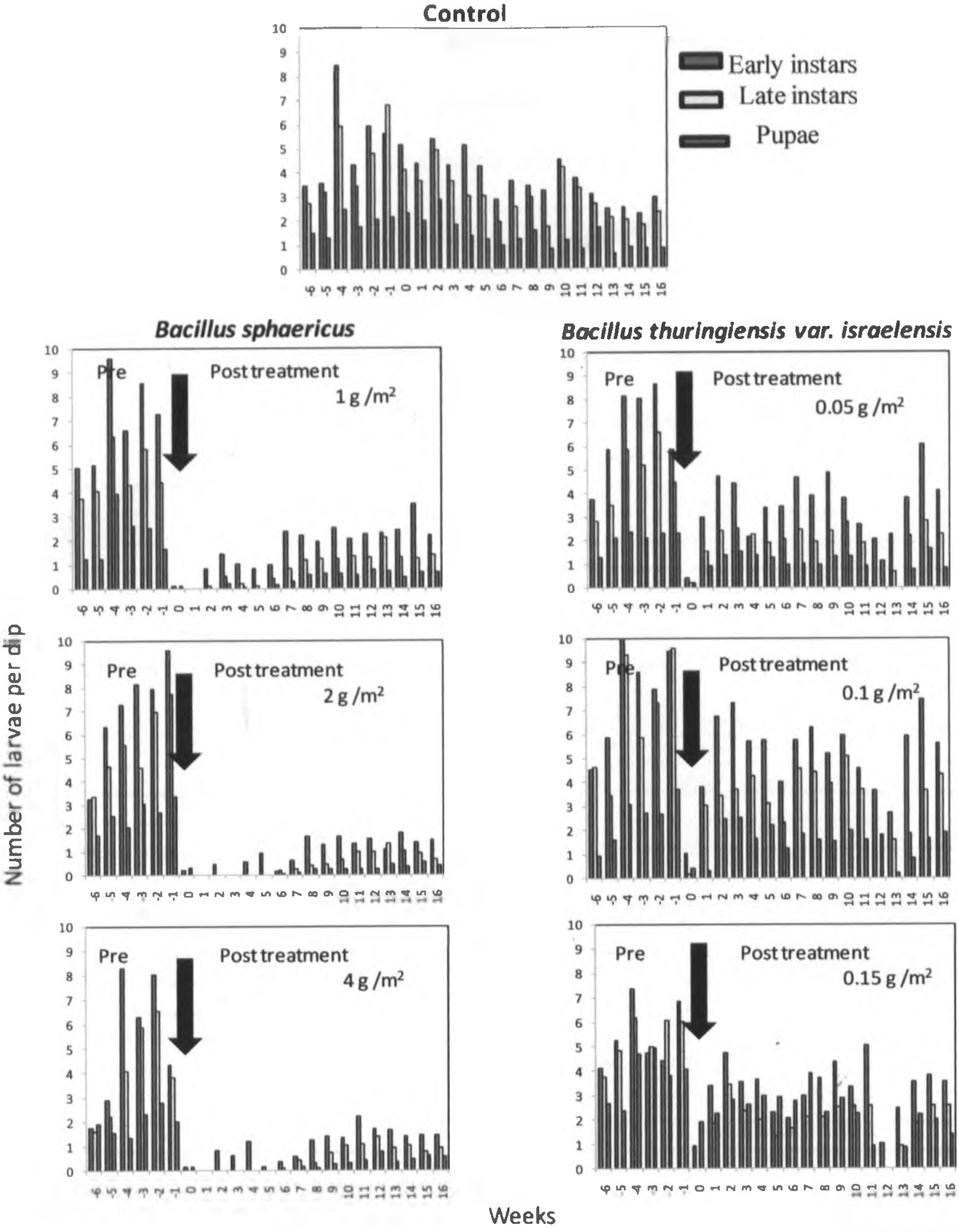

Figure: 6 Densities of aquatic stage *Culex quinquefasciatus* in pit latrines before and after treatment with variying doses of *Bacillus sphaericus* and *Bacillus thuringiensis*.

Figure 7 clearly illustrates the difference between the efficacy of *Bti* and *Bs* in controlling immature stages of *Cx. quinquefasciatus* in cesspits. *Bti* had only a brief impact upon immature density (Figure 7) post-treatment. After one week, larvae reappeared in almost all cesspits although not at the level observed before treatment. Perhaps *Bti* could be used to control *Cx. quinquefasciatus* in cesspits but this would necessitate weekly treatment control. In cesspits, *Bs* again consistently achieved better control than *Bti,* irrespective of the dose applied (Figure 7). The three doses of *Bs* achieved complete control of *Cx. quinquefasciatus* pupae in cesspits for at least two weeks post treatment. *Bs* larvicidal activity in cesspits started to decrease in the second week after application and late instar larvae began to reappear (Figure 7). Recovery of immature stages of *Cx. quinquefasciatus* continued slowly over the following weeks in medium *Bs* treated dose.

3.2 Duration of complete control

Bacillus thuringiensis did not provide adequate or durable control of immature stages of *Cx. quinquefasciatus* in either pit latrines or cesspits although the pit latrines treated with high dose of *Bti* attained zero occupancy with late instars one day after treatment (Figure 8). Within one week of treatment with any dose of *Bti,* all immature stages of *Cx. quinquefasciatus* reappeared in all pit latrines and cesspits. Incomplete and transient control of immature stages of *Cx. quinquefasciatus* within pit latrines and cesspits suggests weak and short-lived activity of *Bti.*

The proportion of habitats re-occupied by late-stage larvae and pupae in pit latrines and cesspits after treatment with *Bs* at low, medium and high doses is clearly illustrated in figure 8. In the second week after treatment with *Bs*, twenty percent of pit latrines treated with the low dose of *Bs* were again re-occupied by late instars. In contrast twenty percent of pit latrines treated with medium and high dose respectively were re-occupied by late instars within six weeks of treatment. Correspondingly, pupae started to reappear in the pit latrines treated with the low dose of *Bs* by the third week post-treatment. Twenty percent of pit latrines treated with medium and high dose were re-occupied by pupae in the sixth and seventh weeks post-treatment, respectively (Figure 8).

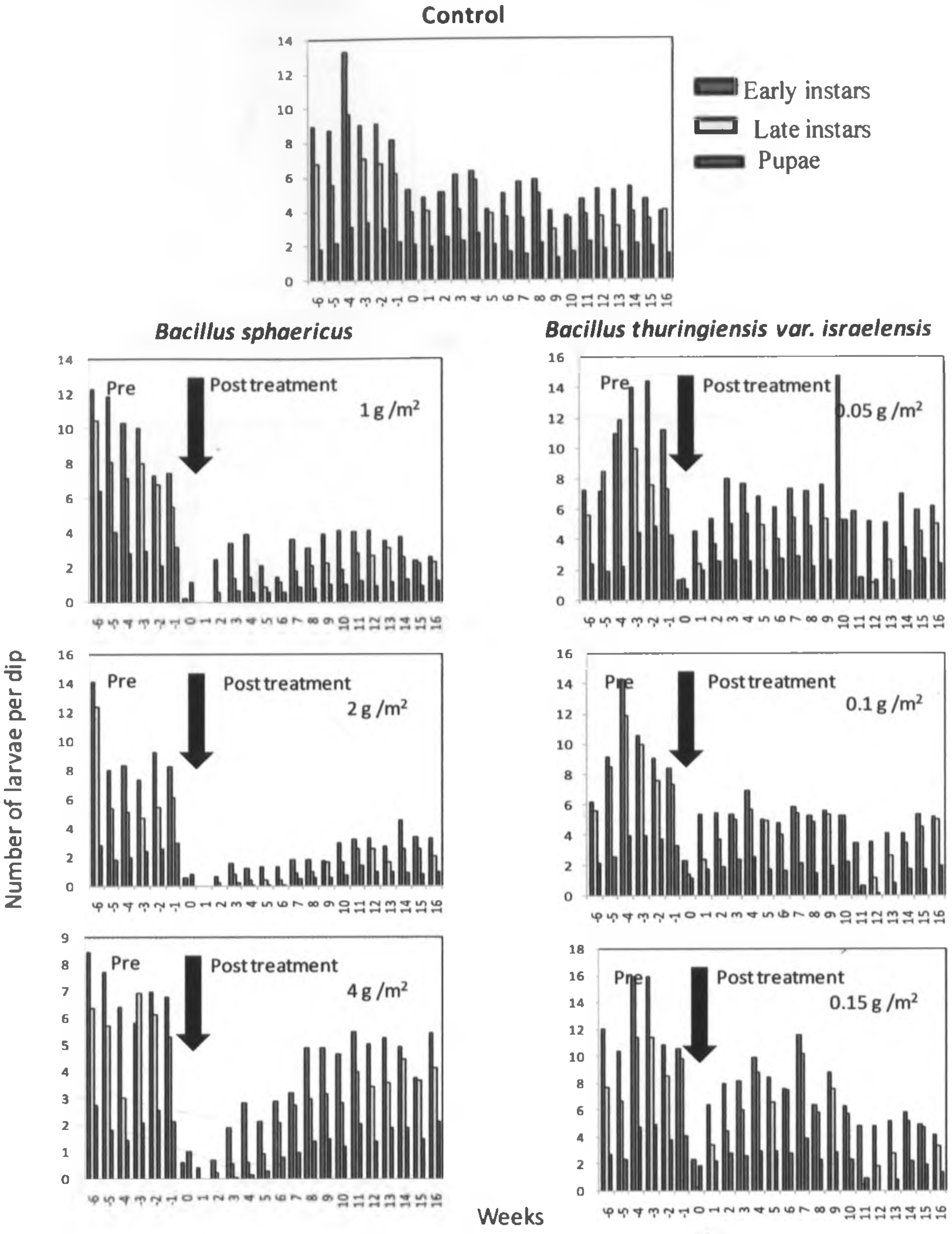

Figure: 7. Densities of aquatic stage *Culex quinquefasciatus* in cesspits before and after treatment with varying doses *of Bacillus sphaericus* and *Bacillus thuringiensis.*

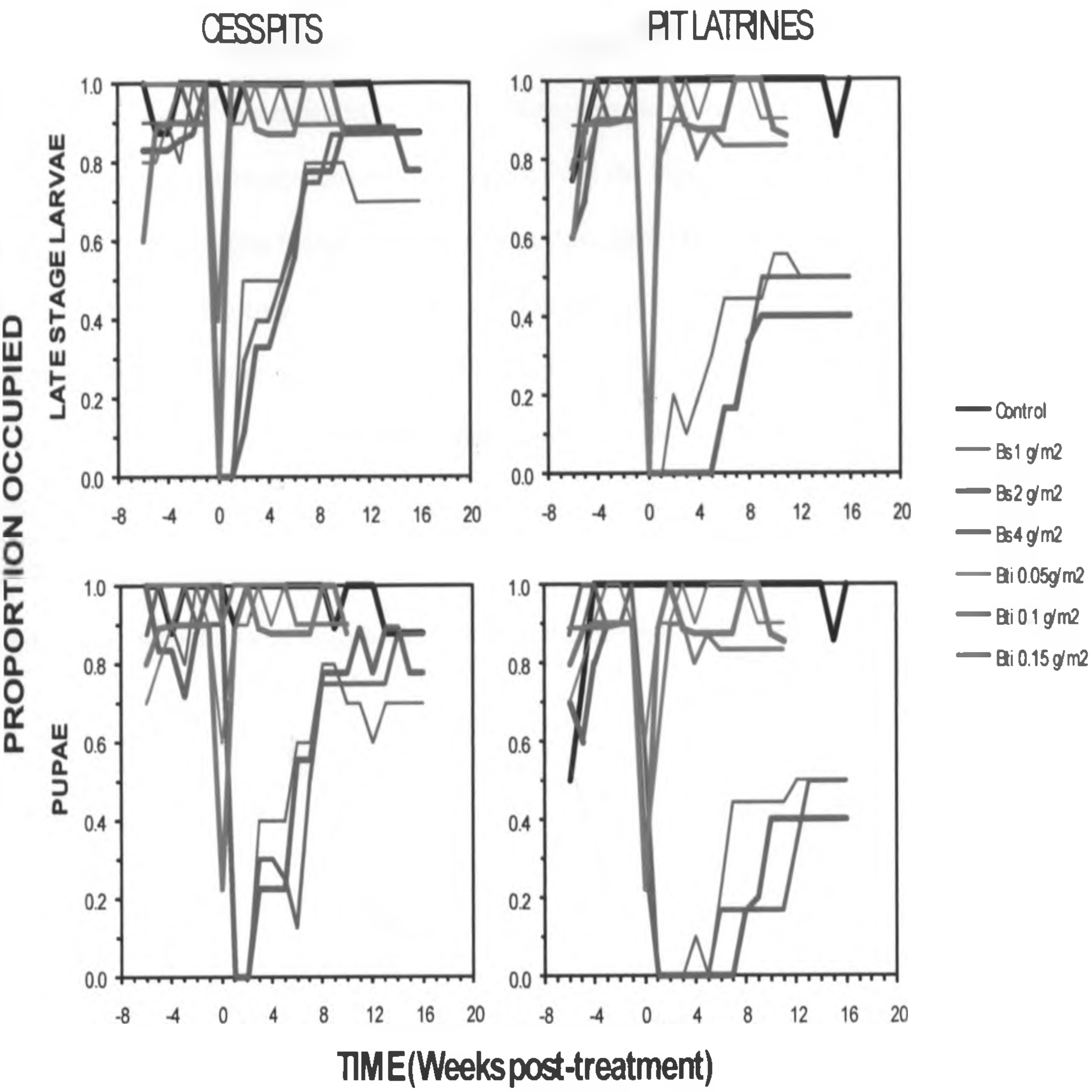

Figure 8. Proportion of cesspits and pit latrines occupied by late-stage larvae and pupae of *Culex quinquefasciatus* Say, before and after treatment with *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis* var. *israelensis* (*Bti*).

Figure 9 illustrates the mean, range and distribution of times taken for immature stages of *Cx. quinquefasciatus* to reappear in an individual pit latrines and cesspits after treatment. Late-stage larvae reappeared immediately in almost all the habitats treated with the three different doses of *Bti*. Only two pit latrines treated with medium and high dose took two weeks to recover. The interval between treatment and recovery for any dose of *Bti* in pit latrines or

By the second week after treatment. half of the cesspits treated with the low dose of *Bs* were re-occupied by late stage larvae. Similarly, the medium dose of *Bs* appeared to loose activity within two weeks of treatment by which time forty percent of cesspits were re-occupied with late stage larvae. No improvement was obtained with the highest dose of *Bs* in cesspits with thirty percent of cesspits being re-occupied with late-stage larvae within two weeks (Figure 8). Pupae consistently took one week more to reappear in the cesspits when compared to the late-stage larvae By the third week, forty percent of cesspits treated with the low dose were re-occupied with pupae, whereas thirty percent treated with medium and twenty five percent with high doses, respectively, were re-occupied with pupae (Figure 8). Thus the doses of *Bs* achieve similarly brief duration of complete control of *Cx. quinquefasciatus* in cesspits and any residual impact upon densities were for less obvious than in pit latrines.

cesspits was not significantly different when compared with the controls (Table 2). The results suggesting very brief activity of *Bti* in pit latrines and cesspits and the necessity for weekly re-application intervals.

Apparently habitats treated with the three dosages of *Bs* produced extended residual activity but with wide variation in the interval between treatment and reappearance of late stage larvae and pupae in individual pit latrines and cesspits (Figure 9). The activity of the low dose of *Bs* in pit latrines achieved complete control of larvae for one week after application suggesting reapplication interval of two weeks. However, medium and high doses of *Bs* produced a minimum of five weeks of complete control of late-stage larvae suggesting a reapplication interval of six weeks (Figure 9). The three different doses of *Bs* in cesspits achieved one week of complete control of late-stage larvae, suggesting a re-application interval of two weeks (Table 3) and differed significantly from the corresponding control habitats (Table 2)

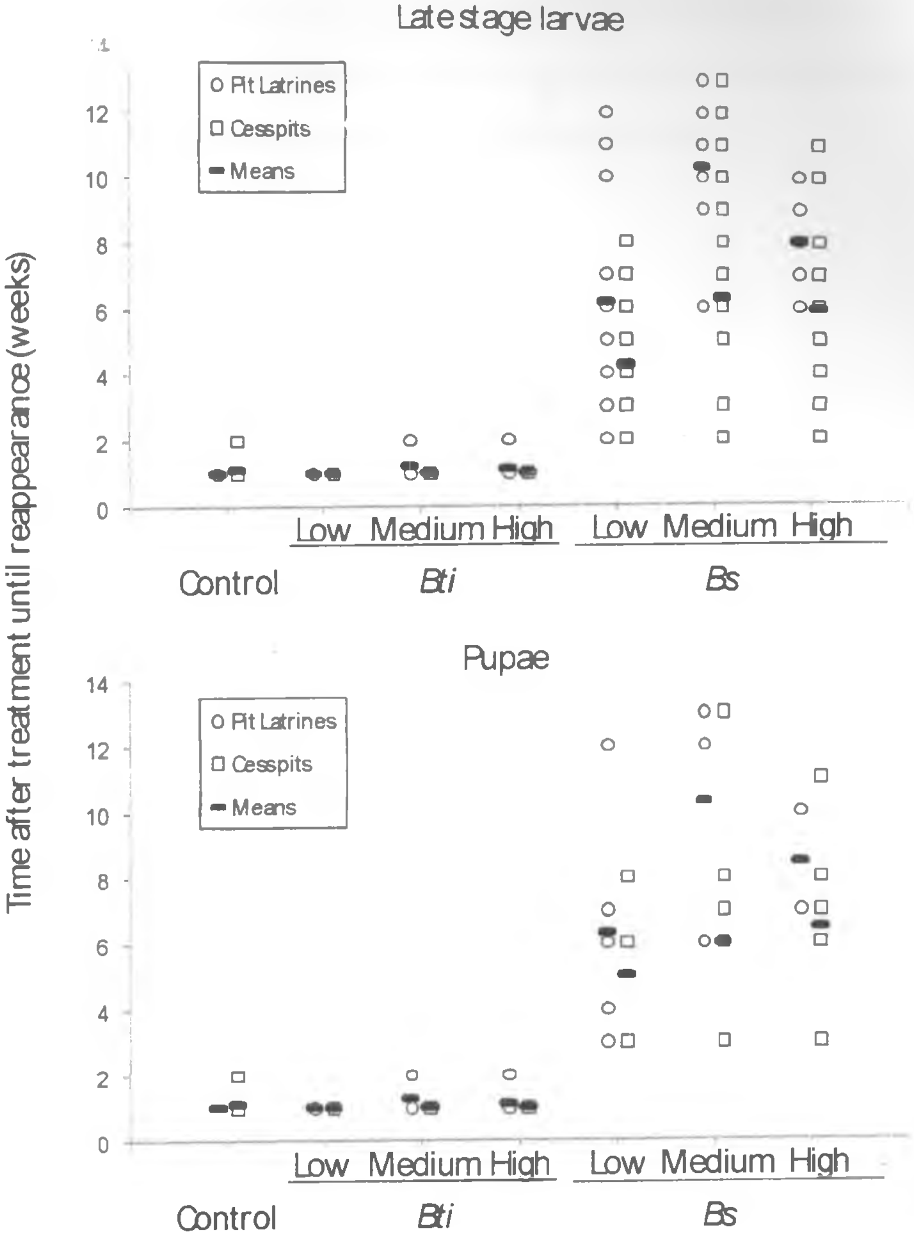

Figure 9: Mean, range and distribution of duration of activity of *Bs* and *Bti* post treatment

Table 2: Statistical comparison of the mean rank of the duration of the post-treatment interval during which larvae and pupae were absent from pit latrines and cesspits treated with each dose of *Bs* or *Bti* compared to the corresponding control habitats.

Treatment	Categories			
	Pit latrines		Cesspits	
	Late larvae P-value	Pupae P-value	Late larvae P-value	Pupae P-value
Low *Bti*	1.000	1.000	0.739	0.739
Medium *Bti*	0.481	0.408	0.739	0.739
High *Bti*	0.739	0.720	0.739	0.739
Low *Bs*	<0.001	<0.001	<0.001	<0.001
Medium *Bs*	<0.001	0.007	<0.001	<0.001
High *Bs*	0.001	0.030	<0.001	<0.001

NB: In all cases variance in the rank of the interval associated with the different dosages was tested using Mann-Whitney test, treating the control as a dosage of zero.

The wide variation in the time to reappearance of immature stages of *Cx. quinquefasciatus* in individual *Bs*-treated habitats prompted us to examine the mean times until the reappearance following each treatment. (Figure 9) *Bti* in all doses produced a similarly short mean duration which was not significantly different from the mean of the control (Table 2). The pit latrines treated with the medium dose produce a higher mean duration (P = 0.008 by Kruskal-Wallis test for heterogeneity of the mean rank of the interval to reappearance of late-stage larvae between the 3 *Bs* treatments) of eleven weeks compared to low and high doses, both of which achieved a mean duration of six weeks and eight weeks, respectively (Figure 9). In cesspit, treatment with medium and high doses of *Bs* produced a similar mean duration of four weeks and these appear their superiority to the low dose approaches significance (P = 0.082 by Kruskal-Wallis test for heterogeneity between the 3 *Bs* treatments in terms of the mean rank of time to re-appearance of late-stage larvae). This mean duration of appearance can be a useful process indicator for mosquito control programms for estimating and planning the reapplication interval of larvicides.

The reappearance of the pupae in habitats treated with *Bti* produced similar results to the reappearance of larvae with only one week more required for reappearance compared to that observed from *Bs* doses (Figure 9). It is essential to decide whether the re-application interval for these larvicides should be based on the re-appearance of late-stage instar larvae or pupae as indicators (Table 3). The persistence of *Bs* in against *Cx. quinquefasciatus* larvae is normally based on the appearance of pupae because pupae, rather than late instar larvae, are the best proxy measure for adult mosquito production (Mutuku *et al.*, 2006b).

Table 3: Persistence of *Bs* in pit latrines and cesspits

	Pit latrines		Cesspits	
Dose g /m²	Reappearance of late larvae	Reappearance of pupae	Reappearance of late larvae	Reappearance of pupae
Bs 1	Week 2	Week 3	Week 2	Week 3
Bs 2	Week 6	Week 6	Week 2	Week 3
Bs 4	Week 6	Week 7	Week 2	Week 3

3.3 Duration and extent of partial control

Figure 10 shows the reductions in densities of late-stage larvae and pupae caused by three doses of *Bs* and *Bti* in pit latrines and cesspits compared to corresponding control habitats. Reappearance of immature stages of *Cx. quinquefasciatus* occurred immediately after treatment with *Bti* in both pit latrines and cesspits, so *Bti* did not achieve acceptable partial control for any extended period. Continuous weekly application is probably required to achieve acceptable levels of control with *Bti* (Figure 10). Application of the low dose of *Bs* to pit latrines provided six weeks of acceptable partial control of late-stage larva density while the medium and high dose achieved twelve weeks and eight weeks, respectively. For both low and medium doses of *Bs* in cesspits, extended partial control was achieved, suggesting a reapplication interval of six weeks or more. Curiously highest dose produced partial control for only 4 weeks, perhaps because complete elimination of the larval population prevented extended recycling.

In the pit latrines, seven weeks of acceptable partial control of pupal density was attained by the low dose of *Bs*. The median and high dose of *Bs* achieved a partial control of pupae for 10 weeks. In cesspits, treated with low and medium doses of *Bs*, pupae were partially controlled for seven and eight weeks, respectively. Again, the high dose extended partial control of pupae density for only five weeks, suggesting this may constitute an overdose which self-limits the residual activity of the active ingredient. By ignoring low densities of immature stages of mosquitoes observed, extended reapplication interval of *Bs* is increased to a minimum of 6 weeks for both habitat categories (Figure 10).

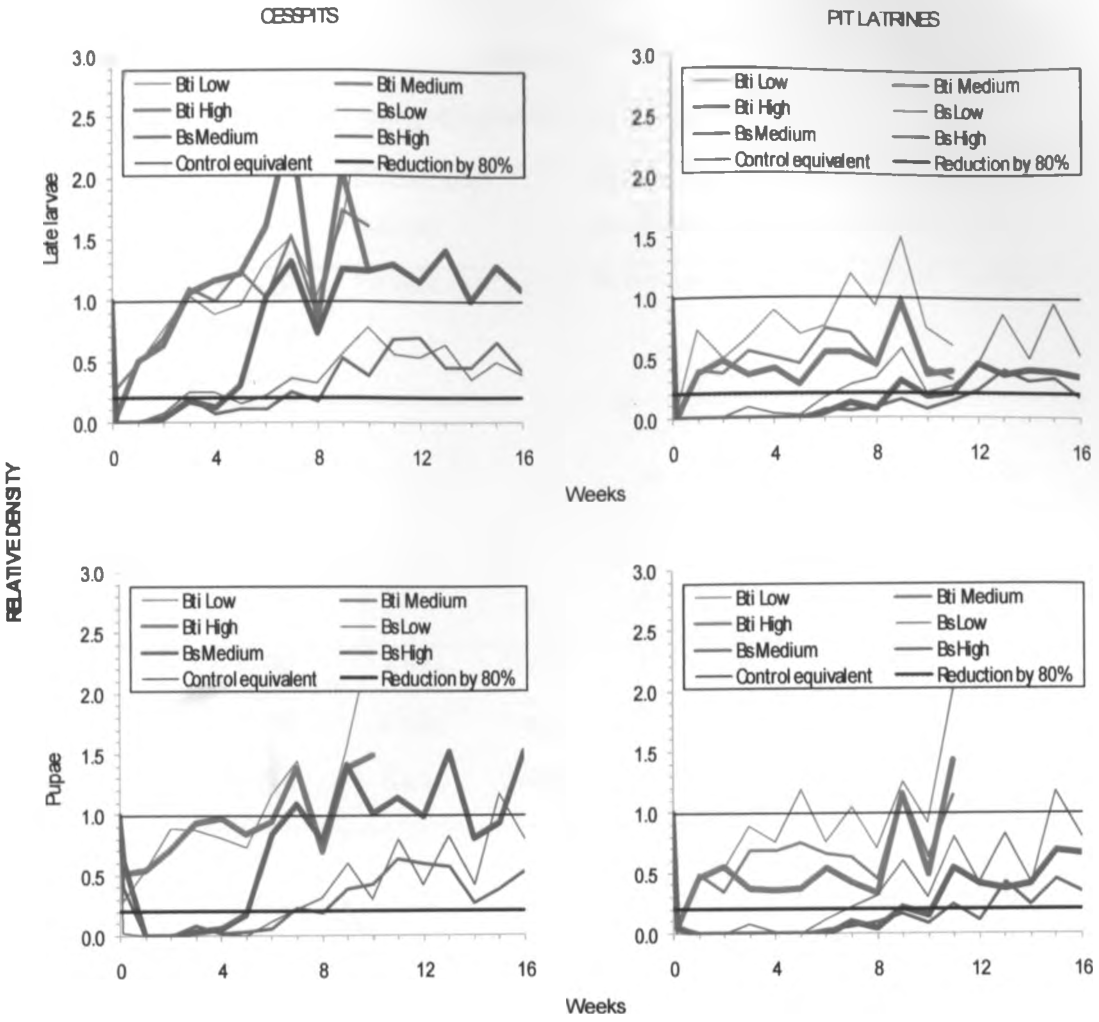

Figure 10: Eighty per cent reduction of immature stages of *Culex*.

Table 4: Estimated annual product procurement costs for application of various dosages of microbial larvicides required to achieve complete (100% reduction) and acceptable partial control (80% reduction) of *Culex quinquefasciatus* in pit latrines and cesspits.

Insecticides	Dosage g/m²	Cost per Habitat per treatment	Re-treatment interval		Treatment per year per habitat		Cost per year per habitat in 2009 US $	
			Pit latrines	Cesspits	Pit latrines	Cesspits	Pit latrines	Cesspits
100% cont.								
	0.05	0.009	1 wk	1 wk	56	56	0.504	0.504
Bti WG	0.1	0.017	1 wk	1 wk	56	56	0.504	0.504
	0.15	0.026	1wk	1 wk	56	56	0.504	0.504
	1	0.029	2 wks	2 wks	26	26	0.754	0.754
Bs CG	2	0.058	6 wks	2 wks	9	26	0.522	1.508
	4	0.115	6 wks	2 wks	9	26	1.035	2.99
80% cont.								
	0.05	0.009	1 wk	1 wk	56	56	0.504	0.504
Bti WG	0.1	0.017	1 wk	1 wk	56	56	0.504	0.504
	0.15	0.026	1wk	1 wk	56	56	0.504	0.504
	1	0.029	6 wks	6 wks	9	9	0.261	0.261
Bs CG	2	0.058	12wks	6 wks	4	9	0.232	0.406
	4	0.115	8 wks	4 wks	7	13	0.805	1.495

NB: Bs CG = 5 $ per kg

Bti WG = 30 $ per kg

100% cont = One hundred percent control

80% cont = Eighty percent control

Mean habitat area = 5.77 m

CHAPTER 4.0. DISCUSSION

Experiments indicated that *Bti* at all doses of application achieved short-lived and very weak levels of control of immature stages of *Cx. quinquefasciatus* in pit latrines and cesspits This is unsurprising as *Bti* is not normally expected to have a long term effect because it lacks the ability to recycle in polluted water (Mulla *et al.*, 1993). This is because in organically enriched habitats, toxins released by *Bti* are easily destroyed or bound to organic matter, thus shortening and weakening its larvicidal activity (Mulla *et al.*, 1993). Such poor persistence of *Bti* has even been observed in other studies done in relatively clear water (Das and Amalraj 1997). To our knowledge, there are no previous reports on the effect of *Bti* on mosquito larvae in pit latrines or cesspits in a developing African city to compare with the results of this present study. However, recovery of mosquito larval populations within 5 to 7 days in stagnant water in ponds has been observed (Mulla, 1985), suggesting that weekly application is essential in almost any habitat type. Our results discourage the routine use of *Bti* for the control of *Cx. quiquefasciatus* in pit latrines and cesspits, particularly when compared with the more encouraging results achieved with *Bs* (Fig 8).

The three different concentrations of *Bs* tested against *Culex quinquefasciatus* in pit latrines and cesspits all proved to be efficacious with varying levels of control and persistence. It is interesting that the longest intervals of partial control are achieved with the medium dose, presumably by extending the duration of recycling in reduced but sufficient mosquito populations. This optimum appears to be surpassed by highest dose which appears to eliminate larvae for a long enough period to curtail extended recycling.

The medium dose of *Bs* applied every 6 weeks to both habitat types appears to be the most practical and affordable way to achieve acceptable partial control. Prolonged residual activity and reduced application intervals achieved with such a treatment regime can correspondingly reduce the labour costs of treatment and monitoring which are often higher than cost of materials in operational programmes (Warall, 2007). The extended control produced by *Bs* therefore great advantages to any control programme, even before considering product procurement costs.

It was interesting to note that the reappearance of immature stages of *Cx. quinquefasciatus* in both pit latrines and cesspits after treatment was slow (Figure 7 & 8). It is interesting and important to note that once populations do recover, their density can surpass the controls, presumably because of accumulated nutrients and lack of competition. This suggest that rigorous implementation of treatment schedules are essential to prevent rapid recovery of the adult mosquito population.

However, the present study suggests that the re-treatment intervals suggested for operational larval control should be based on the re-appearance of late instars larvae because the main aim of any mosquito larval control is to target larvae before they become pupae which rapidly metamorphosize into flying, biting adults. The pupa is the stage at which mosquito cease to feed so microbial larvicides cannot kill them before they emerge. Hence, pupae are the best indication of failure of control but the appearance of the late instars is probably the best indicator of the need to reapply.

From this outcome it can be established that a reapplication interval of two weeks is needed for complete control of *Cx. quinquefasciatus* larvae in cesspits if the most rigorous level of control are needed. Since low, medium and high dose of *Bs* achieved the same level of control of immature stages of *Cx. quinquefasciatus* in cesspits, it is best to opt for the low dose that can be operationally used to treat pit latrines and cesspits at the same time, using the same manpower. In terms of product procurement cost alone it might be best to treat pit latrines and cesspits with separate treatment schedules, using the medium dose of *Bs* in pit latrines and a reapplication interval of six weeks but the low dose in cesspits and a reapplication interval of two weeks (Table 3). However, in practice organizing such separate schedules and correspondingly separate teams to handle sets of habitats which are found beside each other might prove impractical and prohibitively expensive so combined single schedules for treating both habitats categories with the lowest dose of *Bs* (or even *Bti* for pit latrines) every week might be optimal in terms of cost-effective (Table 4).

Complete control (meaning 100%) of immature stages is ideal under any circumstances but especially under operational conditions where such absolute control requirements can be unambiguously monitored. However, slightly less rigorous levels of control may be a more practical and cost-effective method for reducing the mosquito abundance to acceptable levels because it necessitates less frequent re-application of larvicides. Partial control may be more realistic to sustain so long as it makes a useful contribution to disease and nuisance biting control. We therefore set a minimum threshold of eighty percent control as the criteria for acceptability of partial control.

The increased re-application interval resulting from this relaxation of control requirements (Figure 10) could be of significant advantage in mosquito control in practical and economic terms because the reduced number of applications required per year could drastically reduce the product procurement cost for larvicides (Table 4).The labour cost would also be corresponding reduced because it is presumably proportional to the annual treatment frequency. Note that relaxing the threshold of acceptable control had no impact on the re-treatment interval or cost of *Bti* application so the medium or perhaps low dose of *Bs* is probably optimal for this active ingredient in terms of cost, effect and practicality (Table 4).

4.1 Conclusion and recommendations

In the present study *Bs* corn cob granules appear to be more efficacious than *Bti* water-dispersible granules for controlling *Cx. quinquefasciatus* proliferating in pit latrines and cesspits. The medium dose of *Bs* at the rate of 2 g/m^2 appeared to be the most cost-effective, affordable and practical option, assuming that 80% reduction of aquatic-stage *Cx. quiquefasciatus* is acceptable.

Recommendations

1. There is a need to compare the most promising treatment regimes identified in this study in terms of impact on the adult mosquito densities.
2. Before planning for application of *Bacillus sphaericus* survey should be conducted to identify and list all pit latrines and cesspits in the community.
3. *B. sphaericus* should be subsequently applied to wet pit latrines and cesspits only.
4. Application of Bs in pit latrines and cesspits to control Cx, quinquefasciatus may integrated with malaria vector control to reduce the cost the cost of intervantion.

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