KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF SCIENCE

DEPARTMENT OF THEORETICAL AND APPLIED BIOLOGY

MPHIL ENTOMOLOGY

THE EFFECTIVENESS OF *Bacillus sphaericus* FORMULATION FOR MALARIA VECTOR CONTROL IN KUMASI

SANDRA BAFFOUR-AWUAH

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, (K.N.U.S.T.) IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MPHIL DEGREE IN ENTOMOLOGY

BY

SANDRA BAFFOUR-AWUAH

BSc (HONS) BIOLOGICAL SCIENCES

DECLARATION

I, Sandra Baffour-Awuah, author of this thesis, "The Effectiveness of *Bacillus sphaericus* formulation for malaria vector control in Kumasi," do hereby declare that, apart from references to past and current literature duly cited in this thesis, the entire research work presented in this thesis was done by me as a student of the Department of Theoretical and Applied Biology, K.N.U.S.T.

It has neither in whole nor in part been submitted for a degree elsewhere.

SANDRA BAFFOUR-AWUAH	…….....	……
Student	Signature	Date
PROF. K. OBIRI-DANSO	…….....	……
Academic Supervisor	Signature	Date
DR. ELLIS OWUSU-DABO	…….....	……
Academic Supervisor	Signature	Date
REV. STEPHEN AKYEAMPONG	…….....	……
Head of Department	Signature	Date

DEDICATION

This work is dedicated to my parents, Richard Kofi Awuah and Rosemary Adu for their love and support.

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ABSTRACT

The vast majority of malaria deaths, about 90%, occur in Africa where malaria also presents major obstacles to social and economic development. Mortality from malaria results from severe infection caused by *Plasmodium* and transmitted by *Anopheles* mosquito. In recent times, attempts at curbing malaria include: vector and parasitic control which involves drugs, insecticides and biological control measures. There have been reported cases of mosquito resistance against major insecticides throughout various parts of the world. Biological control agents do not kill non-target organisms. The ecosystem is therefore not disturbed and predators of the larvae can feed on the rest of the larvae that hatch after the effect of the biolarvicide has worn out. This project aimed at using an environmentally safe microbial control agent against the *Anopheles* larvae, the vector of malaria. The microbial control agent that was used was in the form of Water Dispersible Granular formulation (WDG) of *Bacillus sphaericus* (*Bs* Vectolex®, Valent BioSciences Corporation Illinois, USA). The study was divided into two main parts: phase 1and phase 2. Phase 1 involved laboratory studies where the optimum lethal laboratory concentration of the biolarvicide was determined. In phase 2, there were controlled field trials during the rainy season and repeated in the dry season, where optimum lethal field application dosage of the biolarvicide was determined. The residual effect of the biolarvicide was determined. The study was conducted over a one and half year period. At the end of the laboratory study it was observed that mosquito larvae were highly susceptible to the biolarvicide (*Bs* Vectolex®) with LC_{50} and $LC_{95}$of 0.0027mg/l and 0.0086mg/l respectively after 24 hours of exposure. The controlled field trial revealed that 0.5mg/l was as effective and efficient as the 1.0mg/l concentration during both the rainy season ($p=0.2820$) and the dry season ($p=0.8578$). The residual effect of the *Bs* formulation during the rainy season lasted for 12days while that for the dry season was 10days for both concentrations. The biolarvicide proved to be an effective tool for reducing malaria vector population therefore potential for reducing malaria prevalence. Its cost effectiveness coupled with its efficacy and existence of residual effect makes it ideal for vector control via killing of larvae. This will lead to a reduction in the need for chemical applications such as insecticides, thereby achieving considerable improvement in public health and in the economy.

TABLE OF CONTENTS

LIST OF TABLE

LIST OF PLATES

LIST OF FIGURES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Globally, malaria is an important parasitic infection and ranks among the major health and developmental challenges facing large parts of the world. The health and wealth of nations and individuals alike are affected. It is estimated that 1 million deaths occur annually throughout the world as a result of malaria (Roll Back Malaria, 2010). The majority of malaria mortality occurs in Africa, where malaria also presents major obstacles to social and economic development (White *et al.,* 1999).

The year 2005 saw the launch of the antimalarial drug policy in Accra. It was during this programme that it was confirmed that 15,000 children under the age of 5 had died of malaria the previous year. A quarter of child mortality cases in Ghana were attributed to malaria (Ghana Health Digest, 2004). According to the Malaria Operational Plan Report (2011), malaria claimed 43% of total deaths of children in Ghana under age five in the year 2008.

Mortality from malaria results from severe infection transmitted by the *Anopheles* mosquito, the vector of *Plasmodium* (Sachs and Malaney, 2002). Malaria is an acute or chronic disease caused by the presence of *Plasmodium* in the red blood cells. *Plasmodium* is transmitted from an infected to an uninfected individual by the bite of female *Anopheles* and characterized by periodic attacks of chills and fever that coincide with mass destruction of red blood cells and the release of toxic substances into the blood stream of the human host by the parasite at the end of each reproductive cycle of malaria parasites (Matteelli *et al*.,1997).

Currently, methods at controlling malaria involve vector and parasitic control and these include drugs, insecticides and biological control interventions (Chandre *et al*., 1999).

Drug and insecticide resistance is always resurfacing in infectious disease control programmes. Malaria as an infectious disease is no exception; there is widespread resistance to some classes of drugs (Hayton and Suz, 2004) and insecticides (Chandre *et al.*, 1999; Harvargreaves *et al.*, 2000).

Vector control can focus on the various stages of the mosquito but in Africa, it has focused mostly on adult control based on indoor residual house spraying and on the use of ITNs (Roberts *et al*., 2000). Biological control although successful is a much neglected approach (Fillinger and Lindsay, 2006) comprising of source reduction and larviciding. Larviciding refers to killing the mosquito in its larval stage. It is advantageous because the mosquito larvae are easily accessible since they occupy minimal habitat and are not at the flying stage. Larvicidal agents are also easy to handle and safe for the environment and the user (Becker and Rettich, 1994; Killeen *et al*., 2002). The predators of mosquito larvae are not killed when larvicidal agents are used thus these predators will feed on the newly hatching mosquito larvae after the treatment.

1.2 Justification

Malaria still remains a challenge in sub-Saharan African countries and continues to be the leading cause of morbidity and mortality in Ghana (Ronald *et al*., 2006). Over the years, there has been a gradual increase in malaria cases in Ghana. From 1985 to 2003 for example, the annual reported cases of malaria increased from 37.1% to 44.7%. In 2008, 38% of all out patient illnesses and 36% of all admissions were as a result of malaria and 14,000 died (MoH, 2011). In 2010, the Statistics Department of the Kumasi Metropolitan Assembly (KMA) reported of substantial increase from 10.5%, 7.2%, 12.3% and 8.7% of malaria cases reported in 2006 to 37%, 25.2%, 34.5%, 24.7% and 30.4% within Manhyia North, Manhyia South, Bantama, Subin and Asokwa respectively (KMA, 2007; KMA, 2012).

Worldwide, a number of studies have been undertaken to help control malaria. These include interventions that focused on controlling its vector (larviciding and adulticiding). Malaria control is however still in the state of evolution (Floore, 2006).

There was the use of chemical larvicides before the introduction of DDT (dichlorodiphenyltrichloroethane) especially using petroleum oils (Gratz and Pal, 1988) and Paris green. Paris green was used in the 1940's (Rozendaal, 1997) on the breeding sites of the mosquitoes. Although these chemolarvicides were effective, it had high levels of toxicity and posed a risk to non-target organisms (Coosemans and Carnevale, 1995).

Chemical larviciding was replaced by DDT in the 1950's and became a widely used intervention for malaria vector control. It was used both for larviciding and adulticiding. Its use however declined because it is a persistent organic pollutant which is toxic to non-target organisms and thus pose a threat to the ecosystem (Rozendaal, 1997; Curtis, 1994). Countries that used DDT as vector control considered the use of other chemicals. Some of the replacements made included organophosphates and carbamates. Organophosphates and carbamates after a while were also found to be acutely toxic (Gratz and Pal, 1988) and were known to harm crabs, shrimps and zooplanktons (FCCMC, 1998). Also there were growing resistance by the mosquitoes to these chemicals (Majori *et al.*, 1987; Chevillon *et al.*, 1999). Synthetic pyrethroids have also been used but are toxic to non-target organisms and there are reports of mosquitoes growing resistance to it (Chavasse and Yap, 1997).

In Ghana, various interventions have been introduced to control malaria. These include the WHO supported Indoor Residual Spraying (IRS) using DDT, chloroquine as an anti malarial drug and the recent Roll Back Malaria (RBM) programme which is also a Global Strategy (WHO, 2001).The RBM programme includes adopting multi prevention strategies, that is, the use of

Insecticide Treated bed Nets(ITNs), Intermittent Preventive Treatment for pregnant women (IPT) and environmental management. In the year 2000, Ghana switched to the use of pyrethroids in ITNs and Artemisinin-Combination Therapy (ACTs) due to increasing resistance of the *Anopheles* species to DDT and the *Plasmodium* parasite's resistance to chloroquine respectively (Ehrhardt., 2002). From 2006 till date, the campaign on malaria continues with emphasis on free distribution and use of ITNs especially among children below the age of five.

Although developing a vaccine for malaria in Ghana has been proven feasible by Basu (2002) it is progressing at a rather slower pace (Trape, 2001).

Interest is now being shifted to alternative biological control methods that are sustainable so as to complement existing malaria vector control programmes. Biological control agents seem to have an added advantage to that of chemicals because they do not persist or accumulate in the environment and are not toxic to non-target organisms which include predators of mosquito larvae. The predators that occur naturally in the mosquito larval habitat regulate *Anopheles* population and could serve as a control tool (Kweka *et al*., 2011).

However, scanty work has been done so far in Ghana to reduce the incidence of malaria by reducing the population of the mosquito larvae using a biolarvicide.

Bacillus sphaericus (Bs),a mosquito biolarvicide has been known since the 1960s when the first strain with larvicidal activity was discovered (Becker *et al*., 2004). Over the years *Bs* has proven to be effective and selective in its operation (Charles and Neilsen-LeRoux, 2000) therefore making it environmentally safe to other organisms.

In eradicating malaria it is always important to have a varied control programme referred to as an Integrated Vector Management programme (IVM).The IVM is not a new concept and has been used in the United States for mosquito control. In 2004, the WHO adapted this programme globally for control of vector-borne diseases, however the potential benefit of it in national health in sub-Saharan Africa is not fully realised (Beier *et al*., 2008;WHO, 2004). IVM utilises environmentally friendly control measures including *Bs* as well as ITNs and Indoor Residual Spraying (IRS). A programme in Zambia (Utzinger *et al.*, 2001), the United States, Europe and the Middle East (Hays, 2000) that incorporated biological control in an IVM yielded high results.

1.3 General Objective

The main objective was to assess the contribution of *Bacillus sphaericus*, a microbial larvicide, as a tool in the reduction of malaria vector population in the Kumasi Metropolis of Ghana.

1.4 Specific Objectives

The specific objectives were to:

- obtain an F1 generation from wild *Anopheles* larvae
- determine the median (LC_{50}) and (LC_{95}) optimum effective dosages of *Bs* formulations against *Anopheles* larvae under laboratory conditions.
- determine the optimum effective dosage of *Bs* formulations against *Anopheles* larvae under controlled field conditions.
- determine the residual effect of *Bs* formulation.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Morbidity Pattern of Malaria

In 2000, the reported incidence of malaria was between 300-500million people (Guerin *et al.*, 2002) and accounted for 2.05% of the total global deaths (WHO, 2002) and 8% in 2008 (WHO, 2010). Malaria as a single disease accounts for 3% of the global disease burden (WHO, 1999).

In developing countries, one of the top ten leading causes of death is malaria, (Mather *et al*., 2007). The infection rate of malaria in Africa is relatively high amounting to 10-15% of all admissions and 20–40% of all outpatients in heath care centres. Malaria leads to 9.0% of mortality in Africa (WHO, 2002) and presents a major hindrance to the financial growth on the continent. A country with high rate of malaria has only 33% of the income level of those without malaria (WHO, 2004).

Malaria is the leading cause of death in children under 5 years of age. This disease can lead to cerebral complications among others. It is also established that 2% of children who recover from cerebral malaria suffer from brain damage. A survey conducted in 1996 by Koram *et al*. (2000) in the Kassena –Nankana District of Ghana diagnosed 22% of children 6-24 months old as having malaria that led to anaemic conditions. Repeated malaria infections in children make them more susceptible to other common childhood sicknesses, such as diarrhoea and respiratory infections, which can indirectly contribute to death (Adams *et al*., 2004). The occurrence of malaria causes anaemia, miscarriage, still birth, low birth weight (LBW) babies and maternal deaths in pregnant women (Mills and Shillcut, 2004; Klinkenberg *et al*., 2006).

A case study in Dar es Salam, Tanzania revealed that the annual prevalence rate of malaria is 1418million with 100,000 to 125,000 deaths occurring, resulting in economic loss and thus serves

as obstacles to foreign investments. Morbidity occurs in 70,000-80,000 infants below the ages of 5years (Castro *et al*., 2004). In Gambia, 40% of visits and 20% of antenatal consultations in maternal and child health service is due to malaria (D'Alessandro *et al*., 1995). Over the last ten years, the prevalence of LBW deliveries attributable to malaria in women at health facilities in rural Gambia varied between 18 and 30% (Malaria Situational Analysis Report, 2002).

Malaria is hyper endemic in various parts of Ghana. Its incidence follows a particular ecological zoning, the highest occur in the forest areas followed by the coastal zone and then the northern savannah which has the lowest occurring incidence (Afari *et al*., 1992). The estimated total Ghanaian population of 24.2million are at risk (Ghana Statistical Service, 2002). There are 3.13.5million clinical cases of malaria per annum, of which 900,000 are in children below five years (MoH, 2011). According to the Ghana Health Service (GHS) health facility data, malaria is the number one cause of morbidity, accounting for 38% of all outpatient illnesses and 36% of all admissions (MoH, 2011). A household survey carried out in Ghana discovered that malaria accounted for 43% of all deaths in children aged 29days to 5 years (MoH, 2011).

2.2 Pathogenesis of Malaria

The parasite of malaria, the *Plasmodium* completes its life cycle in two hosts (Figure 1), the primary host that is the female mosquito of the genus, *Anopheles* where sexual reproduction occurs and the secondary host, the human body where asexual multiplication occurs (Talman *et al*., 2004).

2.2.1 *Plasmodium* in Mosquitoes

Plasmodium is a parasite belonging to the sub-kingdom, Mitozoa of the kingdom Protozoa (Howe, 1992). The mosquito becomes infected with this parasite when it takes blood meal from an infected

human and it is at this point that the travel of the parasite in the mosquito begins (Vlachou *et al*., 2006). The mosquito first ingests the parasite and becomes infected. The parasites' gametocyte that is taken up during the blood meal further differentiates into male and female gametes which fuse to produce ookinete in the mosquito gut. This ookinete penetrates the gut lining and forms an oocyst in the gut wall. It ruptures to release sporozoites that move to the salivary gland ready to infect the new host (Pimenta, 1994). The mosquito during a blood meal takes up gametocytes which forms part of its meal whiles transferring *Plasmodium* in a form of sporozoites to the human (Talman *et al*., 2004).

2.2.2 *Plasmodium* in Humans

In humans, malaria is caused by *Plasmodium falciparum*, *P malariae*, *P.vivax, P. ovale* and *P. knowlesi* (Mueller *et al.*, 2007). *P. falciparum* is the most predominant cause of infection and mortality in Africa, contributing 80-90% of all malaria infections (Mendis *et al*., 2001). An individual infected with *P. malariae* and not well treated can remain infected for many years (Vinetz *et al*., 1998). It is distributed all over the world but more common in sub-Saharan Africa (Khim *et al*., 2012). *P. vivax* is widely distributed (Genton *et al*., 2008) but only 10-20% of the cases in the world occur in Africa (Mendis *et al*., 2001). *P. ovale* is the rarest, less than 0.5% of all malaria infection in Africa (Breman, 2001). *P. knowlesi* is the fifth and newly discovered in Malaysian Borneo (Singh *et al*., 2004). It has from that time onward been reported in Thailand, China, Malaysia and the Philippines (Jongwutiwes *et al*., 2004: Zhu *et al*., 2006: Cox-Singh *et al*., 2008: Luchavez *et al*., 2008).

In the Ashanti Region of Ghana, *P. falciparum* was found to be the most common cause of malaria infection (89-92%) followed by *P. malariae* (10.4-22.8%)and then *P. ovale* (2.6-15.5%) (Browne *et al*., 2000).

The bite of an infected mosquito releases saliva containing sporozoites into the blood stream of the human host. The sporozoites continue to the liver, infecting the hepatocytes where it multiplies asexually to form merozoites between 8-30days (Bledsoe, 2005). These merozoites move to the blood stream and infect the red blood cells. In the red blood cells asexual multiplication occurs to release more merozoites (Cowman and Crabb, 2006) (Figure 1). This period is accompanied by the periodic breaking out of some of the merozoites to attack uninfected red blood cells thus accounting for the incidence of wave of fever evident in a malaria infected individual. Some of the merozoites however develop into male and female gametocytes. The bite from a mosquito will potentially result in it ingesting these gametocytes which are within the blood of the infected human (Sturm *et al*., 2006) and infect another human during the next blood meal.

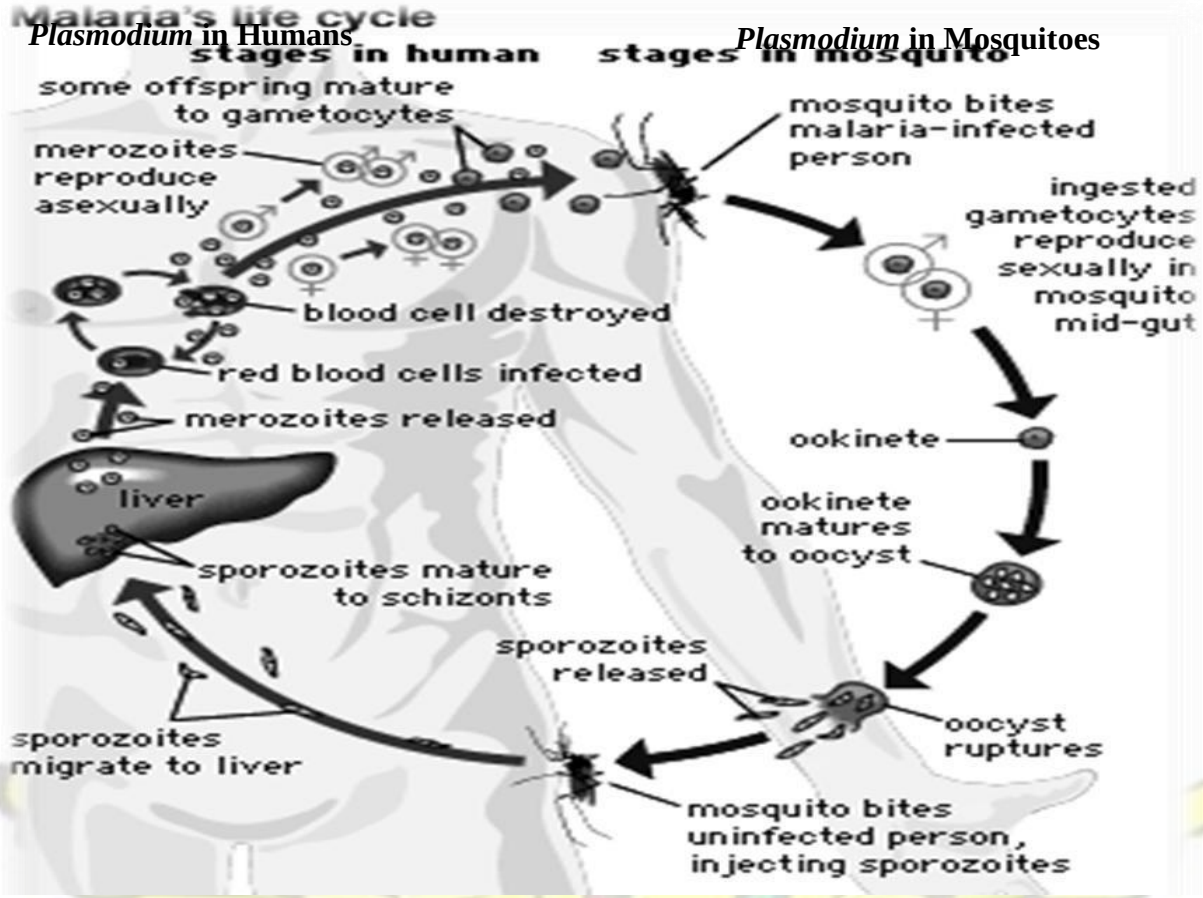

Figure 1: Life Cycle of Malaria Parasite **Source: Encyclopaedia Britannica Inc.**

2.3 Vector of malaria

There are over 2500 different species of mosquitoes throughout the world of which about eight are of economic importance (Singh *et al*., 2004). The only known mosquito responsible for the transmission of malaria is *Anopheles* (Ottaviani *et al*., 1998). It contains more than 400 species, but about 25 species are of economic importance as vectors of malaria. However for a particular geographical location 4 to 5 primarily become adapted (WHO, 2005).

Anopheles gambiae is the most widespread in sub-Saharan Africa (Audibert *et al.*, 1990).). In Ghana, distribution of *Anopheles* is based on ecological conditions. There are 4 documented

Anopheles species that transmits malaria in Ghana and they are *A. gambiae sensu stricto* (*s.s.*) distributed throughout the country, *A. melas* along the coast, *A. gambiae arabiensis* in the north (Appawu *et al*., 1994) and the *A. funestus* throughout the country (MoH, 2001).

Anopheles gambiae adults bite primarily between the periods from dusk to dawn. They may bite during the daylight hours in an area that is heavily shaded or in a room that is dark. They usually rest and feed with the body at an angle of 45°-75° to the surface (Robinson, 2005; Gupta *et al*., 2004). Only the female adult mosquitoes bite animals and suck blood. They need the blood meal to develop their eggs. Male adult mosquitoes do not bite, but feed on nectar of flowers and other sources of sugar (Foster, 1995). They breed in clear, sunlit, temporary water bodies such as swampy areas for agriculture, foot and hoof print, gold mining sites, edges of boreholes, road side puddles, drainage ditches and other man-made shallow water bodies (Minakawa *et al*., 1999; Gimnig *et al*., 2001; Mutuku *et al*., 2006; Kweka *et al*., 2011). The breeding site should have presence of algae, chlorophyll A and should not be too deep (Kweka *et al*., 2011). The larvae lie parallel to the surface of the water (Merritt *et al*., 1992). The larvae of mosquitoes feed on microorganisms whilst the pupa does not feed at all (Kweka *et al*., 2011).

2.4 Vector Management for Malaria Control

In order for the disease to be curbed, vector abundance should be controlled (Kweka *et al*., 2011). Mosquito populations have to be managed to reduce their damage to the health and wealth of nations. Depending on the situation, adulticiding and larviciding may be used to manage mosquito populations. These techniques are accomplished using several methods.

2.4.1 Adulticiding

Control of adult mosquitoes is the most familiar aspect of mosquito control (WHO, 2009). It may include the following measures: preventing mosquitoes from entering human dwelling by, personal protective measures such as netting doors and windows, wearing protective clothing and using mosquito nets and also Indoor Residual Spray (IRS) which involves using insecticidal spray and mosquito coils among others.

2.4.1.1 *Insecticide Treated Mosquito Nets (ITNs)*

Mosquito nets are the best and safest means of protection against mosquito bites at night (Stauffer, 2003). Many types of nets are available depending on the size, material and treatment with insecticides.

Bed nets that have not been treated with insecticides are available and serve as a partial form of protection around persons using them. These nets can develop small holes through continuous use and mosquitoes can feed on people through nets with even few small holes.

The application of an insecticide to the bed nets greatly enhances the protective efficacy of bed nets (Faulde *et. al*., 2010). This insecticide kills mosquitoes and other insects (WHO, 2010) and also has repellent properties (Mathenge *et al.,* 2001). ITNs also prevent mosquito bites by serving as a mechanical barrier to them (Ghana Health Digest, 2004). ITNs have consistently proven to reduce severe disease and mortality due to malaria in endemic areas. It has been documented to reduce the episode of malaria by half throughout the world (Lengeler, 2004)

In community trials in several African settings, ITNs have been shown to reduce mortality from 3% in 2003 to 33% in 2008 (MoH, 2011). In Ghana, the Government of has made ITNs more accessible and affordable to the Ghanaian by reducing taxes on its importation. There are other

companies, example Mobil Oil (Ghana) Limited who have also subsidized the prices for pregnant women and children below the ages of five (Adarkwa, 2009). There was 17% efficacy in preventing malaria mortality in children below the ages of 5 from 1993-1995 in the Northern part of Ghana using ITNs for malaria control (Binka *et al*., 2002).

2.4.1.2 *Indoor Residual Spray (IRS)*

A study conducted in Equatorial Guinea, Mozambique and Zambia confirmed the success in the use of IRS as a means of malaria vector control (Sharp *et al.,* 2007; Contoh *et al.,* 2004; Sharp *et al*., 2002).

Organophosphate insecticides are a group of insecticides that act irreversibly on inactivating the enzyme acetylcholinesterase, which is essential for nerve function in mosquitoes and thus in their potential to serve as poison to the mosquito exposed to it (Carlier, 2008).

Dichlorodiphenyltrichloroethane (DDT) and pyrethroids have been some of the most successful insecticides ever developed (Davies *et al*., 2007). They act on the voltage-gated sodium channel proteins found in insect nerve cell membranes. The correct functioning of these channels is essential for the normal transmission of nerve impulses. This process is however disrupted by binding of the insecticide to these channels leading to paralysis and eventual death of the vector (Davies *et al*., 2007).

2.4.2 Larviciding

Larviciding refers to the process of killing mosquito larvae. Biological control agents are tools used for larviciding.

2.4.2.1 *Biological Control Agents*

Biological control is the use of natural enemies to manage mosquito populations. It involves the direct introduction of parasites, pathogens and predators to target mosquitoes. Also used as biological control agents are microbial control agents such as *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis israelensis* (*Bti*) (Karch *et al*., 1992). These two biological control agents in a number of laboratory and field studies have proven to be excellent larvicides of a variety of mosquito species world-wide (Davidson *et al.,* 1981*;* Lacey *et al*., 1984; Ali and Nayar, 1986). *Bti* and *Bs* 2362 are environmentally friendly microorganisms that effectively control the larval stage of the mosquitoes (WHO 1999). They have minimal effect on non-target organisms and are safe to the user (WHO, 1999). It has been documented that *Bs* in general is more toxic to some mosquito species than *Bti* and has an advantage of longer persistence in the treated habitat (Hougard *et al*., 1990).

2.4.2.2 *Bacillus sphaericus (Bs)*

Bacillus sphaericus is an endospore forming, aerobic, rod-shaped, gram positive soil bacterium. It has a terminal round spore found in a swollen sporangium (Tanada and Kaya, 1993). There are several biochemical pathways lacking in *Bs* hence it cannot use sugars as metabolites. It becomes active metabolically when substrates that are suitable for its growth are available but forms spores when nutrients become exhausted (Australian Pesticides and Veterinary Medicines Authority, 2004).

The first reported *Bs* strain with mosquitocidal activity was isolated from moribund mosquito larvae (Kellen *et al*., 1965). Afterwards several *Bs* strains have been isolated and identified from a variety of sources all over the world. A study conducted in Devakottai of Tamil Nadu in South

India isolated *Bs* from sewage bed, river bed, pond bed, agricultural land and forest in and around the town. The bacilli also settle in the upper crust of soil in mosquito breeding habitats because spores are known to settle rapidly in water bodies (Surendran and Vennison, 2011).

The strains of *Bs* can be divided into two main groups, based on their toxicity to mosquito larvae. Strains with high toxicity make a parasporal crystal, whereas strains with low toxicity lack a crystal (Baumann *et al*., 1991). The parasporal inclusions are made up of crystal structures released into the medium in which it find itself along with the spore after completing the sporulation process.

The mosquitocidal strains of *Bs* have a number of similarities in their properties. All are aerobes and are unable to ferment glucose, denitrify nitrate to nitrite. They all lack extra-cellular enzymes such as amylase, gelatinase, chitinase, and lecithinase. None are able to utilize pentoses, hexoses, or disaccharides as sources of carbon and energy but are able to utilize gluconate. Most strains utilize a variety of carbon compounds, which include fatty acids
(Alexander and Priest, 1990). Mosquitocidal activity have been studied for strains, 2362 (Weiser, 1984), 2297 (Kalfon *et al*., 1984) and 1593 (Myers *et al*., 1979). New strains of *Bs*, strain 2362 were isolated from an adult black fly in Niger (Weiser, 1984).

There are two (2) different kinds of toxins reported to account for mosquitocidal activity of the *Bs*; crystal and Mtx toxins. Their differences lie in their composition and the time they are synthesised.

The crystal toxins are present in all highly toxic strains and are produced during sporulation. It is made up of two proteins synthesized in amounts that are equimolar. These toxins are arranged in crystal structures (Charles *et al*., 1996). The crystal toxins are proteins (protoxins) designated as P51 and P42 on the basis of their predicted molecular masses of 51.4kDa and 41.9kDa respectively. The mode of action of these crystal toxins have been studied extensively in mosquito

larvae (Charles *et al*., 1996). Alterations in the midgut start in the mosquito larvae as quick as 15minutes after ingestion of the *Bs* spore-crystal complex (Charles *et al*., 1996). The protoxin dissolves in the lumen of the anterior part of the stomach (Charles 1987) as a result of the action and presence of midgut proteinases and the high pH (Charles *et al*., 1996). These crystal toxins when ingested are activated in all species, even in non-susceptible species such as *Aedes aegypti*. Some studies have reported the susceptibility differences in *Bs* crystal toxins between mosquito species not to result from differences in activation of the crystal toxin but rather to the direct binding with specific cell receptors in susceptible insects (Charles *et al*., 1996).

The hypothesis that a specific receptor was involved in the toxin binding was confirmed by Charles *et al*. (1996) invitro binding assays using 1251-labeled activated crystal toxins and midgut brush-border membrane fractions (BBMFs) isolated from susceptible *Culex pipiens* and nonsuceptible mosquito larvae, *Aedes aegpti* (Nielsen-LeRoux and Charles, 1992). These direct binding experiments with the *Culex pipiens*' BBMFs indicated that the toxin binds to a single class of specific receptor. The characteristics of the toxin-receptor binding involves a dissociation constant (Kd) of 20 f 5nM toxin and a receptor concentration of 7 f 4pmol toxin/mg of BBMF protein. Both crystal toxin components, P51 and P42 were bound to the membranes of the susceptible species and that the binding of P42 was based on the binding of P51 (Charles *et al*., 1996).The P51 binds specifically to the caecum and posterior stomach, whereas the binding of the P42 is nonspecific throughout the midgut. After the experiments there were no significant specific binding detected with BBMFs from *Aedes aegypti* consistent with the lack of specific binding in fluorescence labelling studies conducted by Davidson and Yousten (1990).

The P51 does not bind to the midgut cell lining of *Aedes aegypti*, whereas P42 is weakly bound and this binding is nonspecific in this species. This leads to a damaging effect in that there is an

appearance of large vacuoles in *Culex pipiens*' midgut cells, whereas large areas of low electron density appear in *Anopheles* midgut cells. A symptom that occurs generally is swelling in the mitochondria, described for *Culex pipiens* and *Anopheles*, as well as for *Aedes aegypti* when intoxicated with a very high dose of crystals toxins (Charles, 1987). The midgut cells, especially the gastric caecum and cells of the posterior section of the stomach are the most severely damaged by the toxin, and Singh and Gill (1988) also report damage in skeletal muscles and in neural tissue. There have been reports on the condensation of the mitochondria1 matrix and the swelling of the endoplasmic reticula of the larvae (Davidson and Titus, 1987). The overall disturbance is that of regulation of the osmotic processes of the cell membrane at the site of binding which leads to swelling and bursting of the cells (Becker, 1995).

The Mtx toxins however are responsible for the toxicity of most of the weakly active strains synthesized during the vegetative phase of the *Bacillus*. There have been two (2) reported types of Mtx toxins; Mtx and Mtx2, with molecular masses of 100kDa and 30.8kDa respectively. Although there seem to be no similarities between the Mtx and Mtx2 toxins or to the crystal proteins, the Mtx toxins have not been extensively studied as the crystal toxins of the *Bs*. Their modes of action probably differ but it is still not very well understood (Charles *et al*., 1996).

Several formulations of *Bs* have been produced for the control of mosquito larvae and includes, Water Dispersible Granules (WDG), wettable powder (WP), water dispersible concentrate (WDC), emulsifiable concentrate (EC), flowable concentrate (FC) and dust (D) (Surendran and Vennison, 2011).

This opens up the possibility of a successful and cost effective control of malaria. Certain mosquito species, such as *Culex quinquefasciatus, Culex pipiens, Anopheles stephensis* and

Anopheles gambiae are highly susceptible whereas *Aedes aegypti* larvae are less susceptible (Fillinger *et al*., 2003; Surendran and Vennison, 2011). Most recent studies indicates that *Bs* only kills mosquito larvae, whereas black fly larvae as well as other insects, mammals and other nontarget organism are not susceptible to *Bs* (Das and Amalraj, 1997).

Bs strain 2362 is an active constituent which is generated by propagation from a seed culture and formulated into the product VectoLex WDG Biological Larvicide in an integrated process and is native to the United States. *Bs* strain 2362 is from the family Bacillaceae, genus, *Bacillus*, species, *sphaericus*, serotype, H5a5b and strain, 2362. It has an appearance of brown Water Dispersible Granules with a characteristic musty odour. Its bulk density is 0.3-0.5 g/cm^3 with a pH of 4.0-6.0 (10% slurry). It is not corrosive and stable for at least two (2) years when stored below 25°C. *Bs* can cause slight eye irritation and a slight skin sensitization but it is not a skin irritant (Australian Pesticides and Veterinary Medicines Authority, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in the Kumasi Metropolis (Figure 2b) which is on latitude N 06°41.37' and longitude W 001°36.65'. It is the second largest city in Ghana and the capital of the Ashanti Region (Figure 2a). Kumasi lies in the tropical forest zone and has two main seasons (dry and rainy). The rainy season normally occurs from June to July and from September to October and the dry from November to January (Meteorological Statistical Department, 2011). The research was carried out at the Kumasi Centre for Collaborative Research (KCCR) on the campus of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi.

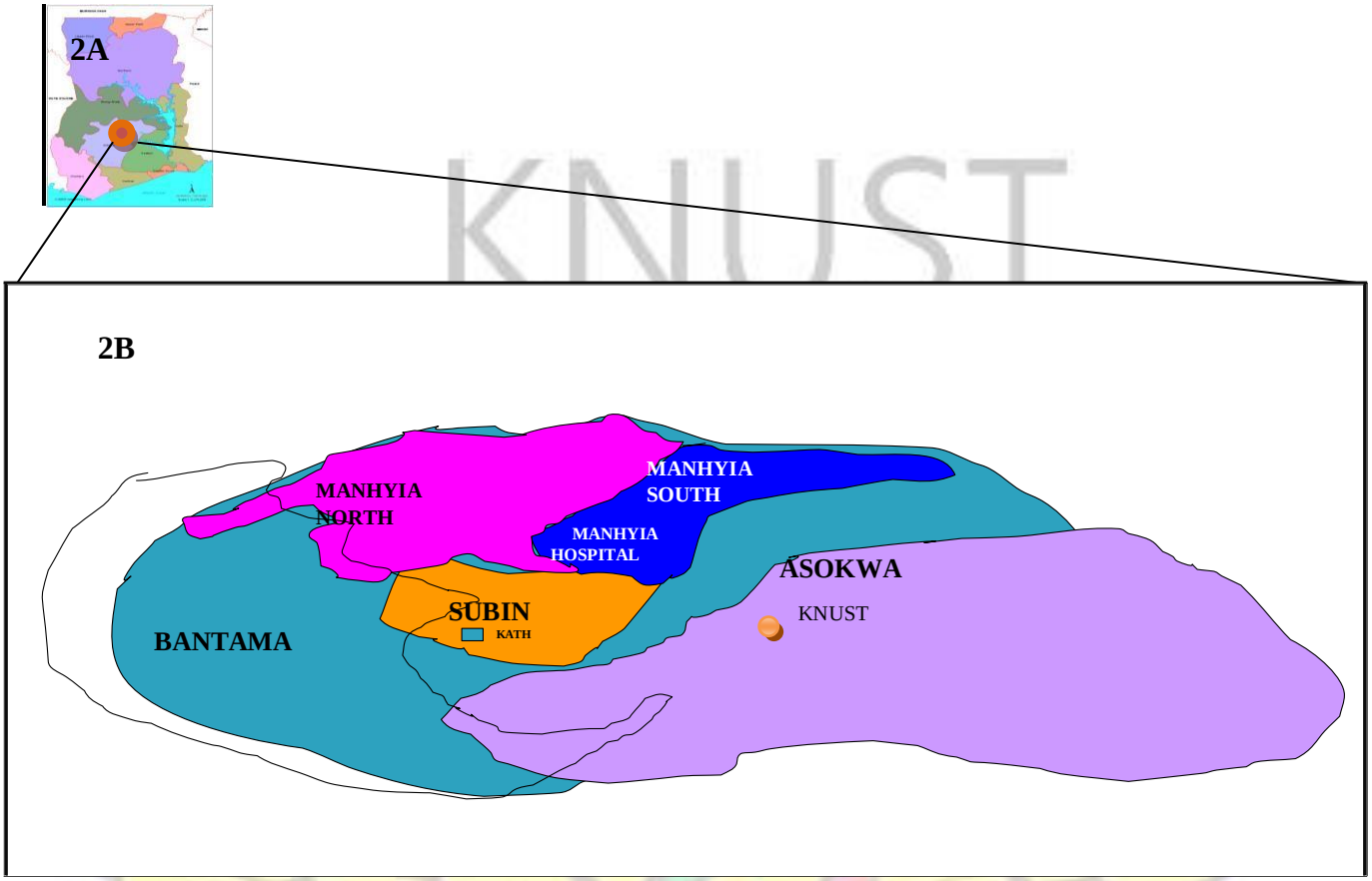

Figure 2A: Map of Ghana **Figure 2B: Health Map of Kumasi Metropolis**

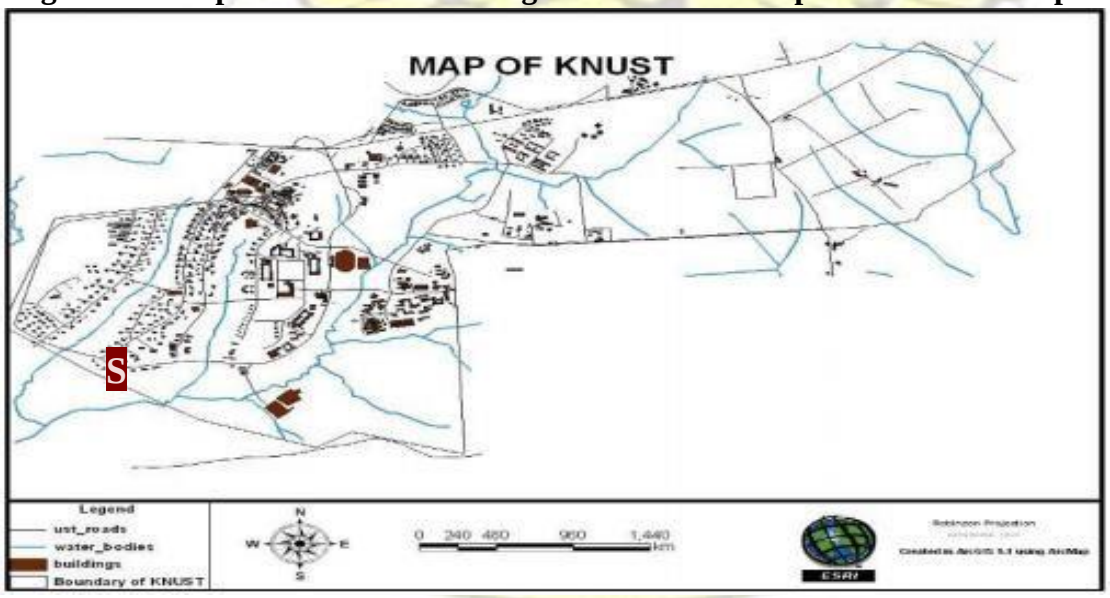

Figure 3: Map of Kwame Nkrumah University of Science and Technology showing larval sampling site (S) KNUST

3.2 Larval Sampling

The characteristics of a breeding site of *Anopheles*, aquatic, little to no pollution (Bruce-Chwatt, 1985), temporal, not more than 2km from human settlements, presence of vegetation, stagnant, shallow and well sunlit conditions were noted which made it easy for identification and mapping by walking through the catchment area (Figure 3).

In many areas within the University (KNUST) campus are wetlands, which are largely used by encroachers for vegetable gardening. Most of these farmers create pools of water between the ridges of the beds on their farms to provide easy access to water for irrigation. These water compartments between the ridges serve as suitable breeding sites (Plate 1) for the *Anopheles gambiae*, the predominant vector of malaria in the study area (Agyepong, 2008). *Anopheles gambiae* also breed in fresh shallow pools of water on untarred roads (Plate 2), in footprints (Plate 3) and well that has been dug out manually on farms (Plate 4).

Larvae were collected from sites shown in Plate 1, 2, 3 and 4 and reared in the laboratory for the first generation (F1) larvae and also for morphological identification using the keys of Gillies and DeMeillon (1968).

Plate 1: *Anopheles* breeding sites in between 2 ridges on a vegetable farm

Plate 2: *Anopheles* breeding sites on an untarred road

Plate 3: *Anopheles* breeding sites in footprints

Plate 4: *Anopheles* breeding site in a well dug out for irrigation

3.3 Study Design

The toxicity test was divided into two phases; laboratory studies and the controlled field trials.

The laboratory assays were performed to derive the optimum lethal concentration of the biological larvicide in the laboratory and the controlled field trials were conducted to obtain the optimum lethal concentration of the biolarvicide in the field.

3.4 Biological Larvicide

The biolarvicide used was the *Bs* serotype H5a5b, strain 2362 which has been commercialized into WDG formulation as Vectolex®, lot number 183-371-pg, with a potency 650 *Bs*ITU/mg; Valent Biosciences Corporation, Illinois, USA.

3.5 Laboratory studies

3.5.1 Rearing Mosquitoes

3.5.1.1 *Rearing Mosquito Larvae and Pupae*

The larvae were sampled from their natural breeding sites with a 500ml plastic dipper. They were collected with their habitat water into covered buckets and transported to the insectary at KCCR.

The door to the insectary was always firmly closed after entering or leaving it. The floor and insect rearing cages were cleaned daily to restrict access of predators (ants and spiders) to the insectary. The cages were kept on a shelf which had its legs standing in petri dishes containing palm kernel oil to prevent ants and other predators from climbing.

The larvae brought from the field were reared at one side of the insectary whiles the F1 larvae that had developed from the eggs of the adults mosquitoes being reared in the insectary, were kept and reared at a different side. The field larvae were reared in their original habitat water since a sudden change in their environment would have caused increase in mortality. They were reared in a 26x24x5cm well labelled white bowls (Plate 5**)** filled with 1L of habitat water at a temperature of

25±1°C and 70% relative humidity. All the larvae were subjected to 12 hours of light and 12 hours of darkness and fed with Tetramin® (Tetra Germany) fish meal which was ground and spread evenly on the surface of their water habitat every day.

Plate 5: Bowl for rearing *Anopheles* larvae from the natural breeding site.

The mosquito larvae moulted through the four stages, 1st, 2nd, 3rd and 4th instar. After the 4th instar stage, it pupated. All pupae were collected with a Pasteur pipette early in the morning and late in the evening into petri dishes filled with water and placed in the rearing cages to emerge into adult in the cage (Plate 6).

3.5.1.2 *Rearing Adult Mosquitoes*

These adults, both males and females immerged in the cages and were fed on cotton wool soaked with 5% sugar solution. The cotton wool was changed every 2days to prevent it from fermenting. The females were fed also on blood from guinea pigs which were sedated. The females were allowed to feed on the blood meal twice a week, early in the morning with three days interval.

Feeding was allowed until the abdomen of the female was full. The female mosquitoes started laying eggs after 72hours of feeding. A filter paper soaked with water in a petri dish was placed in the cages after feeding for the females to lay on them. These filter papers were inspected every day for eggs and contamination in order to change them. The cages were labelled with the type of mosquito, the source of its larvae and the date it emerged as adults.

Plate 6: Rearing cages for adult *Anopheles* species arranged on a shelf

3.5.1.3 *Rearing Eggs of Mosquitoes*

The egg stage was the most fragile stage of the mosquito life cycle, so extreme care was taken to minimize mortality. The eggs were washed into the larval bowls, gently shaking or using the Pasteur pipette to prevent damage of the eggs. The larval bowls were immediately covered with sewn nets to prevent straying mosquitoes from laying in them. Each of the larval bowls was labelled with the species of mosquito and the date of collection. These eggs hatched into larvae after two days. The larvae that hatched formed F1 larvae.

3.5.1.4 *Rearing First Generation Mosquito Larvae (F1)*

These F1 larvae were reared in tap water and covered with sewn nets (Plate 7). The larval water was changed biweekly by sieving to avoid contamination of the larvae. The larvae were allowed to grow to the third and fourth instar larvae which were then used for the laboratory assays.

Plate 7: Rearing first generation (F1) *Anopheles* larvae

3.5.2 Bioassay

The procedure for the bioassay was conducted according to the method described by WHO (1999).

3.5.2.1 *Preparing Stock Solution*

A 1% fresh stock solution was prepared in a 50ml falcon tube each day a bioassay was carried out. A 200mg of the solid product was weighed and added to 20ml deionized water. This suspension was vigorously homogenized on a shaker. It was from this that subsequent test concentrations were made.

3.5.2.2 *Optimization*

The bioassay was started by exposing the larvae to a wide range of test concentrations and a control to determine the range of activity of the *Bs* formulation under investigation. The range finding concentrations were 0.05mg/l, 0.04mg/l, 0.03mg/l, 0.02mg/l, 0.01mg/l, and 0.0015mg/l. The mortalities of the larvae were determined and a narrower range of concentrations; 0.008mg/l, 0.005mg/l, 0.004mg/l, 0.0035mg/l, 0.003mg/l, 0.0025mg/l and 0.002 mg/l, were used to determine LC_{50} and LC_{95} values.

The following procedure was followed in determining the wide range concentrations and the narrower range concentrations.

Deionized water of 100ml was measured into plastic cups. Twenty-five *Anopheles gambiae* larvae were added to each cup using micropipettes. A control set-up was made consisting of 25 *Anopheles gambiae* larvae in 100 ml deionized water. Four replicate cups were used for each concentration. The entire experiment was carried out on three different occasions under similar environmental conditions. The experiment was run for 24 hours. During this period larval mortality records were taken at 25±1°C. The experiment was discarded and repeated when mortality for the control exceeded 10%. This was to ensure that mortality in the larval population was due solely to the activity of the biolarvide and not an external factor.

Moribund larvae and dead larvae were counted for mortality calculation. Dead larvae were those that could not be induced to move when touch with a pipette.

Plate 8: Laboratory Assay Procedure.

3.6 Climatological Data

The controlled field trials were carried out from 23rd June to 29th July (rainy season) and in 27th October to 30th November (dry season) 2011.

During the rainy season, the experiment was set up on the 23rd of June 2011 but the formulation was added on the 7th of July 2011. From the 7th to the 29th of July, the average number of larvae and pupae per dip that were alive were recorded. The period was characterised by frequent rains, on 15 different occasions. The peak rainfall, 58.6mm occurred on the 25th of July 2011 which was on the 19th day post-treatment with the biolarvicide (Figure 4). Temperatures during the rainy season ranged from a minimum of 19.2°C to a maximum of 29.5°C (Figure 4).

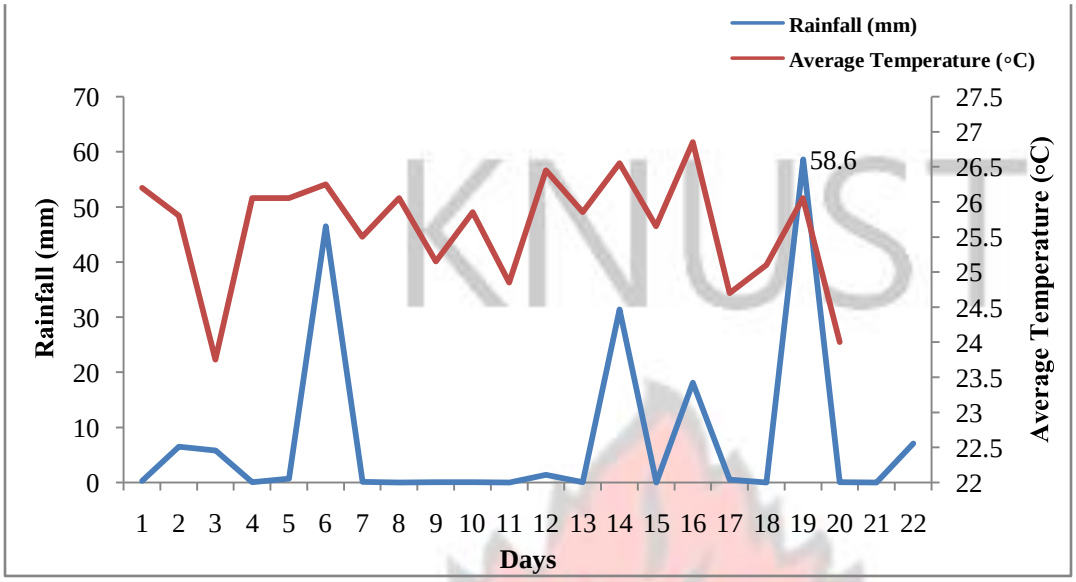

Figure 4: Daily rainfall and temperature pattern after application of the *Bs* formulation in the rainy season

The experiment for the dry season was set up on the 27th of October 2011. The *Bs* formulation was however added on the 9th of November 2011. The number of larvae and pupae per dip were recorded from the 9th to the 30th of November 2011. This period was characterised by less rainfall. It rained five times after application of formulation and the peak rainfall was 11.6mm on the 10th November 2011 which was the second day post-treatment(Figure 5). Temperature for the dry season ranged from a minimum of 21.6ºC to a maximum of 33.5ºC (Figure 5).

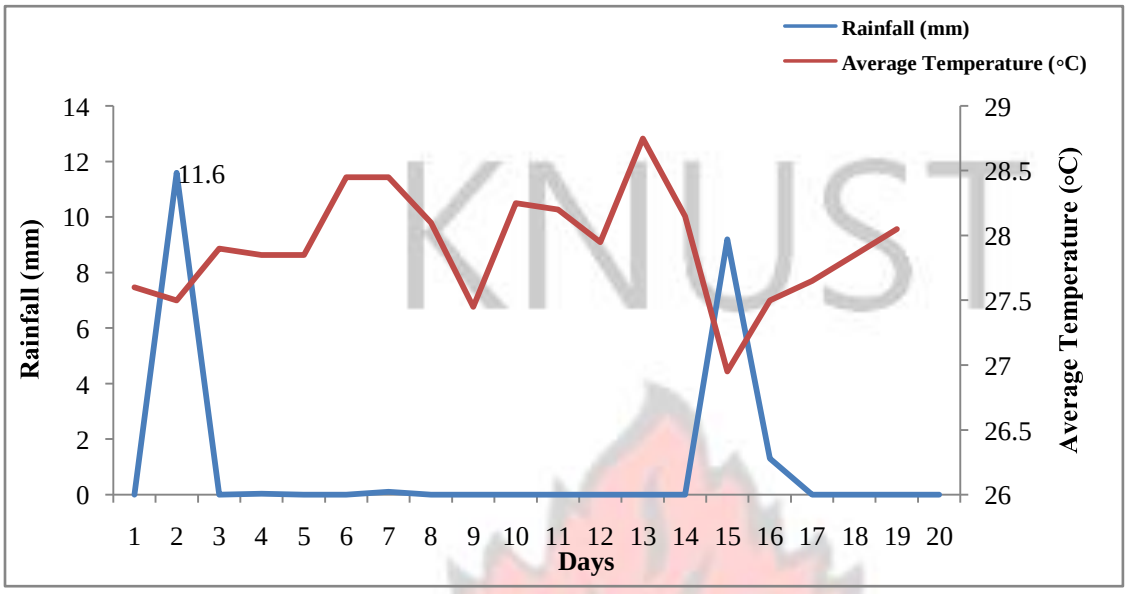

Figure 4: Daily rainfall and temperature pattern after application of *Bs* formulation in the dry season

3.7 Controlled Field Trial

Field trials were performed on a controlled field at KCCR, KNUST where the *Bs* formulation was used against the target mosquito larvae in a controlled but open field. These experiments were designed following one described in Kenya (Fillinger *et al.*, 2003).

On a well sunlit area, two beds were raised with 9 holes dug on each bed. A total number of 18 bowls were fitted into these holes. The bowls were all of the same diameter and depth, 0.5m and 0.3m respectively arranged in 2 rows (Plate 10). The distance from one bowl to the other was 1.5m. All the bowls were filled to 1/5th of its capacity with soil from an original breeding site of *Anopheles* mosquito larvae and also with larval habitat water to 3/4th of the bowls capacity (Plate 9). This was done to create biotic conditions in the field trial similar to that found in a naturally occurring breeding site.

Plate 9: Filling bowls with larval habitat water from an original breeding site of *Anopheles* larvae

The set up was left standing for 5days to allow for oviposition of female *Anopheles* mosquito. During the rainy season the period allowed for oviposition was from 23th June 2011 to 28th June 2011 and for the dry season 27th October 2011 to 30th October 2011. The eggs that had been laid by the female *Anopheles* mosquito were left unattended for 7days to allow them to develop into 3rd and 4th instar larvae. During the rainy season the time span for the development of larvae to the 3rd and 4th instar was from the 29th of June to the 6th of July 2011 and from the 1st of November to the 8th of November 2011 for the dry season.

The bowls designated for the treatments had their respective concentrations added on 7th July 2011 for the rainy season and on 9th November 2011 for the dry season. Six bowls were used as control, six for the treatment concentration 0.5mg/l and six for the second treatment concentration 1.0mg/l. The treatment concentrations used were calculated based on similar work conducted in Tanzania (Ragoonanansingh *et al*., 1992) and on reports by Becker and Rettich (1994) that states that the LC_{95} value obtained from the bioassay is increased several times under field conditions to obtain

sufficient larval control. The control bowls were randomly chosen using the web based randomization formula (www.randomization.com).

The efficacy and residual activity of the biolarvicide at different dosages were determined from the post-treatment counts of live larvae and pupae in treated and control bowls compared with the pre-treatment counts and the control.

The 1st and 2nd larval instars were grouped, counted and recorded as early instars while the 3rd and 4th instar larvae were grouped as late instars. The counting of the larvae and the pupae were done using the dipping method where 10 dips (using 500ml dipper) were taking from different positions and the centre of each bowl. From every bowl the number of larvae per dip was recorded daily until the 12th day after which the counting and recording was made every 2days till the end of the 22nd day. Counting was stopped after the 22nd day because the number of late instar larvae in the treated habitat reached numbers similar to or greater than that of the control (Figure 7). This counting procedure was used for both the rainy and dry seasons.

The experiment was conducted during the rainy season and repeated during the dry season.

Plate 10: Bowls arranged for the controlled field trials

3.8 Data analysis

All recorded mortality during the bioassay were analyzed using the Simple Logistic Regression Model, looking for the probability of success or the chance of 50% dying, that is the LC_{50} and the chance of 95% dying, LC_{95}.

For the controlled field trials, mean number of larvae and pupae collected per dip and the percentage reduction on each day of observation for each replicate in treatment and control were calculated using Microsoft Excel 2007 and the unpaired *t* test. The unpaired *t* test was carried out using the GraphPad Prism 5.00 for Windows (GraphPad software San Diego California USA). In all statistical test a value of $p<0.05$ was considered significant.

The percentage reductions for the mean numbers were also calculated using the formula of Mulla *et al*. (1971): % Reduction = 100 - (C1/T1× T2/C2) ×100,

C1and C2 described the mean number of larvae in the control bowls pre and post-treatment respectively and T1 and T2 described the mean number of larvae in the treated tubs pre- and post-treatment respectively.

CHAPTER FOUR

4.0 RESULTS

4.1 Laboratory Studies

4.1.1 Rearing of Mosquito Larvae

Out of a total of 2795 mosquito larvae sampled from the identified breeding sites and bred from August 2010 to July 2011, 2482 (89%) were *Anopheles* and 313 (11%) were *Culex*. A total of 198 (8%) of the *Anopheles* and 72 (23%) *Culex* died during sampling and rearing (Table 1).

All *Anopheles* larvae sampled were identified as *Anopheles gambiae*.

Table 1: Number of mosquito larvae sampled from the field and reared at the KCCR insectary

GENERA OF MOSQUITO	SAMPLED	DIED DURING BREEDING
Anopheles	2482 (88.8%)	198 (7.1%)
Culex	313 (11.2%)	72 (2.6%)
TOTAL	**2795**	**270 (9.7%)**

4.1.2 Bioassays

From the bioassays conducted using the *Bs* formulation on the F1 larvae of *Anopheles gambiae*, LC_{50} and LC_{95} were recorded at concentrations of 0.0027mg/l and 0.0086mg/l after 24 hours of exposure (Figure 6).

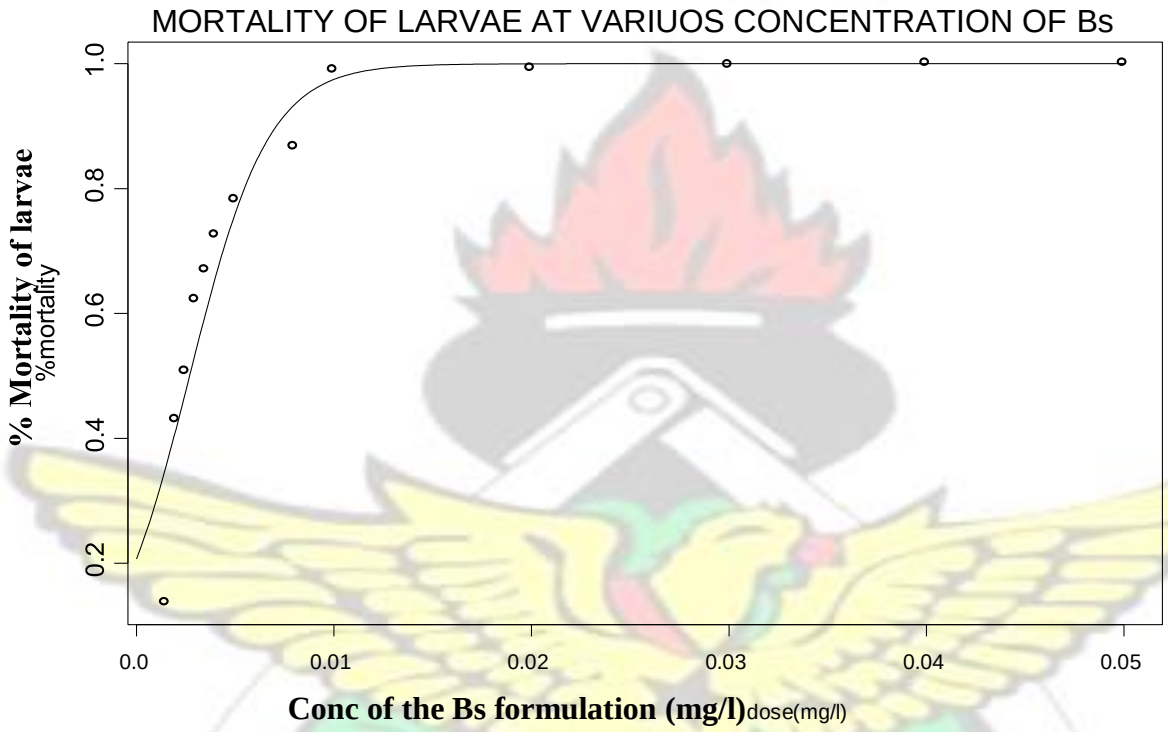

Figure 6: Percentage mortality of F1 *Anopheles* larvae at various concentrations of *Bs* formulation after 24hours (p<0.0001)

4.2 Controlled Field Trials

4.2.1 Rainy season

4.2.1.1 *Larval mortality*

The average numbers of *Anopheles* larvae per dip before the *Bs* formulations were added (pretreatment) were between 3.9 to 4.7 in all the bowls at day 0. Using the *Bs* formulation at 0.5mg/l concentration, after 24 hours, average number of larva per dip was 0.7 (79.5% die off) and

zeroed out (100% die off) afterwards till on the 7th day when the average number per dip recorded was 1 (66.9%) in the early instar. It zeroed out again in both the early and late instar till day 12 when 1.2 larva per dip was recorded. However, using the *Bs* formulation at 1.0mg/l average number of larva per dip after 24 hour was 0 (100% die off) (Figure 7 and Figure 8). Based on the perceived residual effect of the *Bs* formulation, percentage reductions were calculated to be 56.3% for the 0.5mg/l concentration and 84.9% for the 1.0mg/l on the12th day (Figure 8). At the end of the 22nd day 0.5mg/l and 1.0mg/l treatment recorded no percentage reductions (Figure 8). Larvae could again be counted in the bowls on the 1st, 7th and 12th day because fresh eggs that may have been laid in the treatment bowls had hatched into larvae. The 1st and 7th day early instar larvae that were counted however died the next day because of the residual effect of the *Bs* formulation. At the end of the 18day, average larval numbers increased slightly to 3.7 for the 0.5mg/l treatment and 2.2 for the 1.0mg/l treatment and thereafter decreased gradually until the 22nd day (Figure 7). In the control bowls, the average number of larvae per dip rather decreased from 4.7 to 1 at the end of the 22nd day (Figure 7).

There was statistically ($p=0.86$) no significant difference between the 0.5mg/l and 1.0mg/l treatment concentrations.

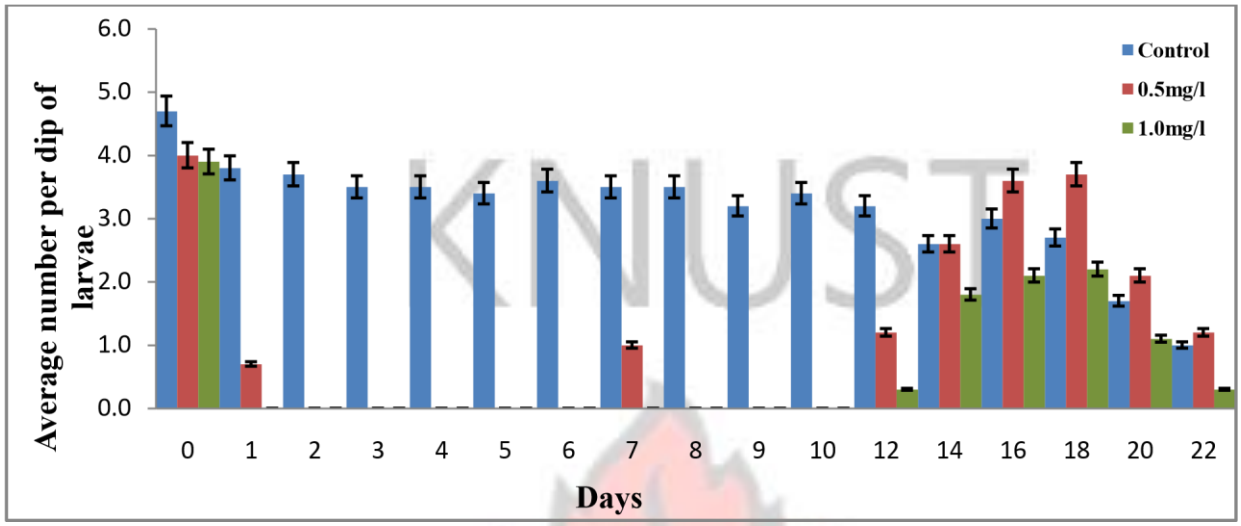

Figure 7: Average larval numbers exposed to different concentrations of *Bs* formulation in a controlled field trial (Rainy Season, 2011)

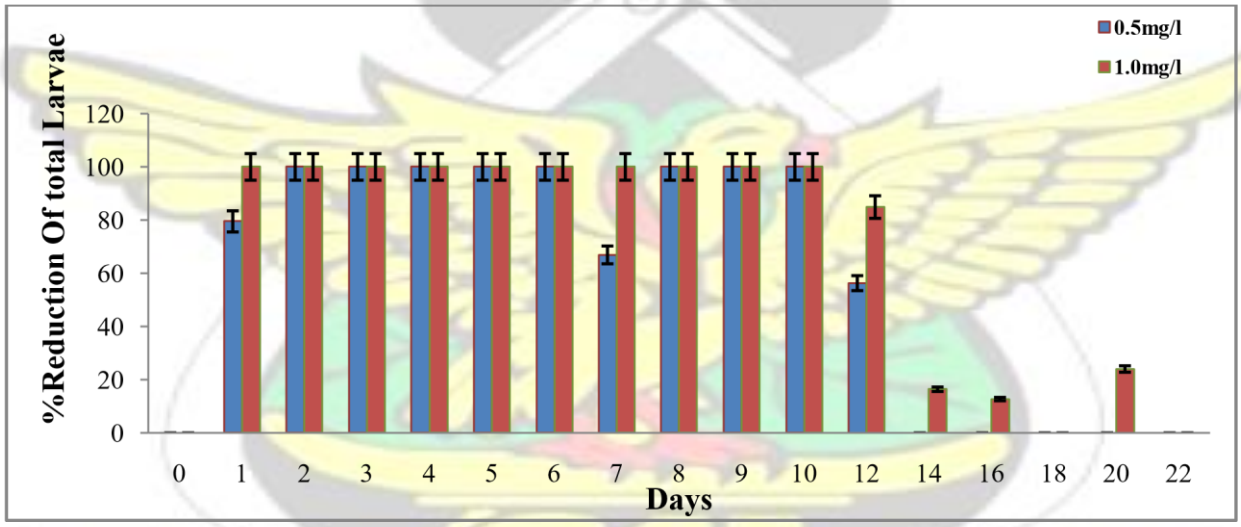

Figure 8: Percentage reductions in larval numbers after treatment with *Bs* formulation (Rainy Season, 2011)

4.2.1.2 *Pupation*

The average numbers of *Anopheles* pupae per dip before the *Bs* formulation was added (pretreatment) were 0 in all the treatment bowls and 0-1 in the control bowls at day 0. After the addition of the *Bs* formulation at 0.5mg/l concentration, there were no pupae till the 14th day when

small numbers of pupae were counted (0-1). The 1.0mg/l treatment concentration also recorded no pupae per dip till day 12 when 0-3 number of pupae were counted. In the control bowls pupae numbers increased from 0 to a range of 0-10 pupae by the 7th day then decreased gradually till the 22nd day (Figure 9).

There was statistically (p=0.95) no significant difference between the 0.5mg/l and 1.0mg/l treatment concentrations.

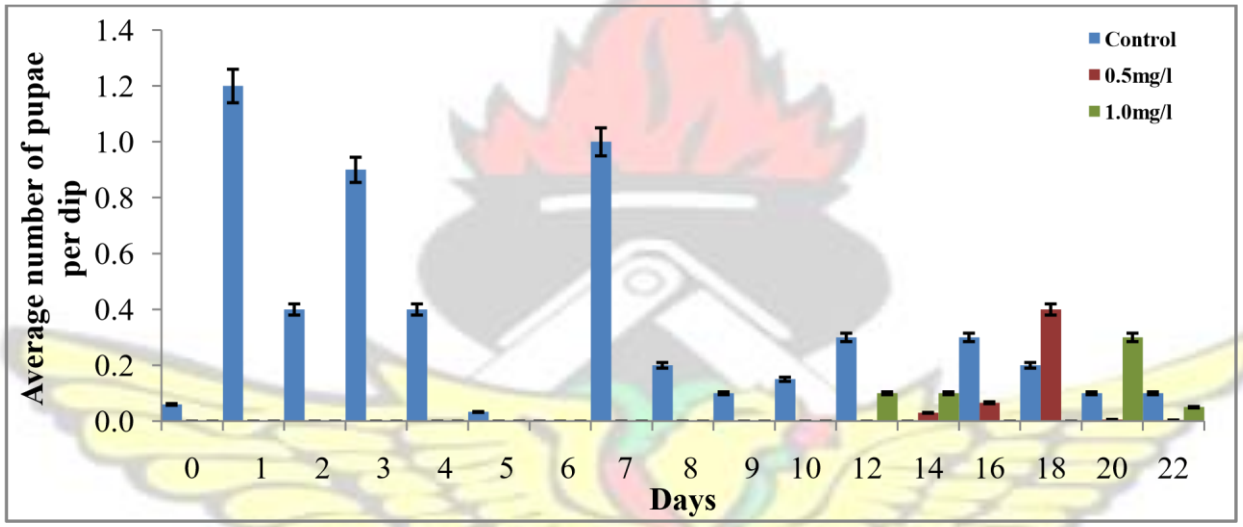

Figure 9: Average number of pupae exposed to different concentrations of *Bs* formulation in a controlled field trial (Rainy Season, 2011).

4.2.2 Dry season

4.2.2.1 *Larval mortality*

The average numbers of *Anopheles* larvae per dip before the *Bs* formulation was added (pretreatment) were between 4 and 6.9 in all the bowls at day 0 whereas the control bowls had an average of 2.8 larvae per dip. Using the *Bs* formulation at 0.5mg/l concentration, after 24 hours, average number of larvae per dip was 0 (100% die off). Similarly, at 1.0mg/l there was 100% die off. An average of 2 and 1.5 larvae per dip were counted in the 0.5mg/l and 1.0mg/l treatment

bowls respectively on the 10th day. These numbers could be counted because fresh eggs that were laid in the treatment bowls had hatched into larvae. Based on the perceived residual effect of the *Bs* formulation, percentage reductions were calculated to be 70.2% for the 0.5mg/l concentration and 87% for the 1.0mg/l at the end of the 10th day (Figure 11). At the end of the 12th day, average larval numbers increased slightly to 2.5 for 0.5mg/l and 2.3 for 1.0mg/l treatment and thereafter decreased gradually until the 22nd day (Figure 10). However in the control bowls the average number of larvae per dip decreased to 0.4 at the end of the 22nd day (Figure 10). At the end of the 22nd day 0.5mg/l treatment recorded no percentage reduction but the 1.0mg/l had 55.4% (Figure 11).

There was statistically (p=0.28) no significant differences between the 0.5mg/l and 1.0mg/l treatment concentrations.

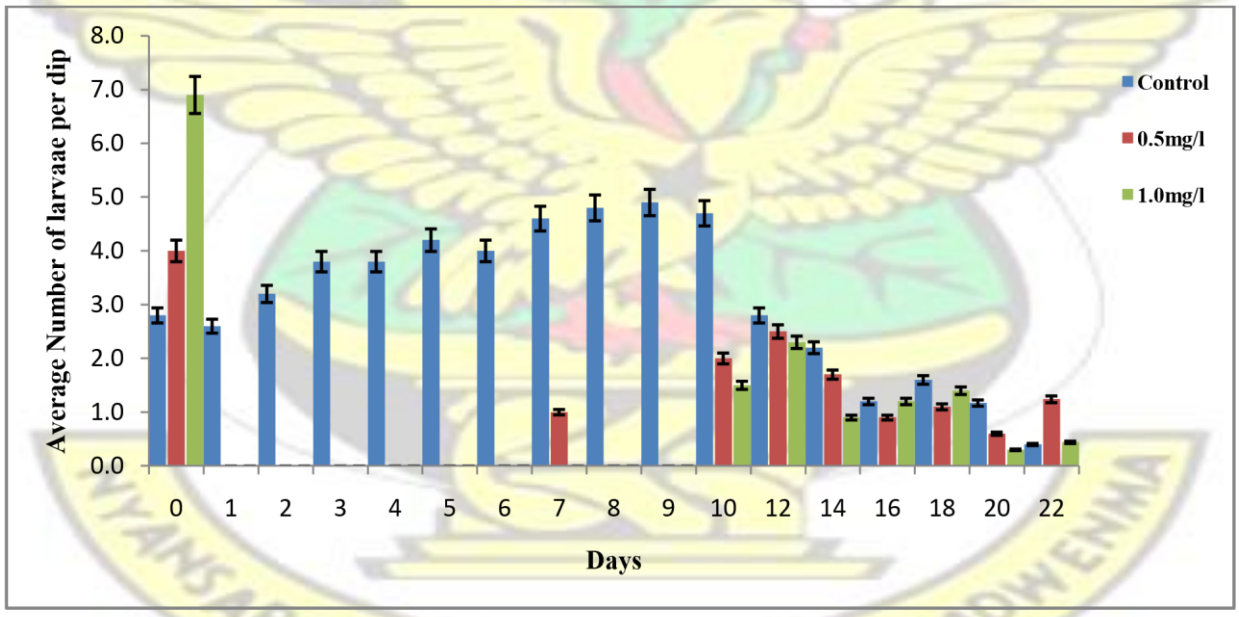

Figure 10: Average larval numbers exposed to different concentrations of *Bs* formulation in a controlled field trial (Dry Season, 2011)

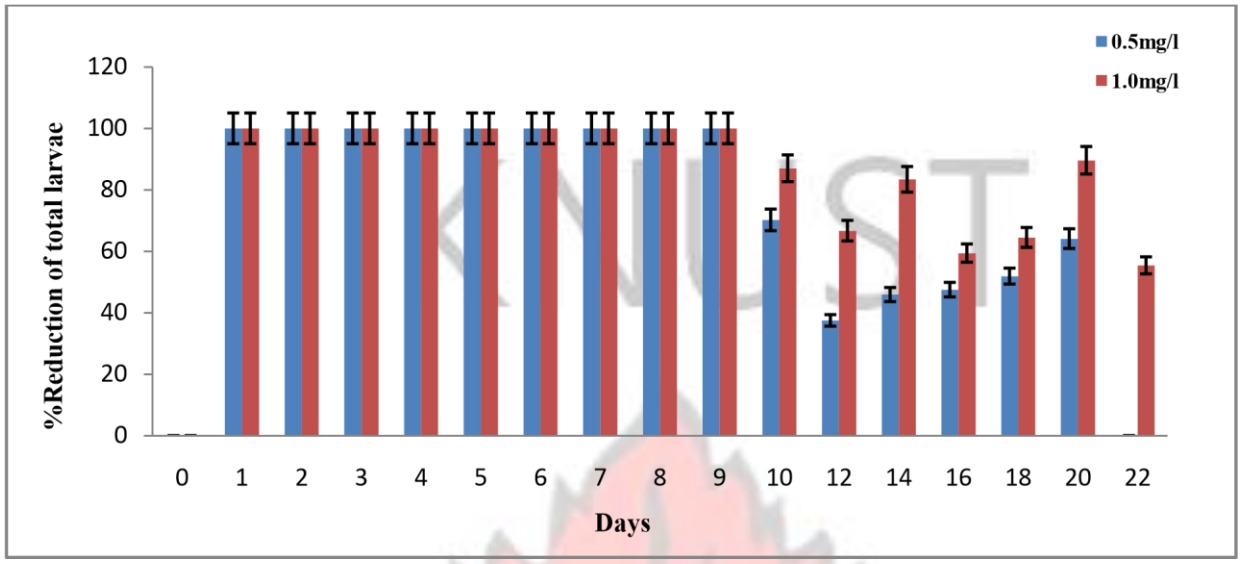

Figure 11: Percentage reductions in larval numbers after treatment with *Bs* formulation (Dry Season, 2011)

4.2.2.2 *Pupation*

There was no pupa before the *Bs* formulation was added (pre-treatment) in the control and 1.0mg/l bowls at day 0. The 0.5mg/l treatment bowls however had 0-1 number of pupae at day 0. On addition of the *Bs* formulation at 0.5mg/l concentration, there was no pupae till the 16th day when 0-1 number of pupae was counted. Similarly, at 1.0mg/l average number of pupa per dip was 0 till day 14 when 0-2 pupae were counted. Pupae in the control bowls on the other hand had the number of pupae increasing from 0 to a range of 0-12 by the 7th day then decreased gradually till the 22nd day (Figure 12).

There was statistically ($p=0.28$) no significant differences between the 0.5mg/l and 1.0mg/l treatment concentrations.

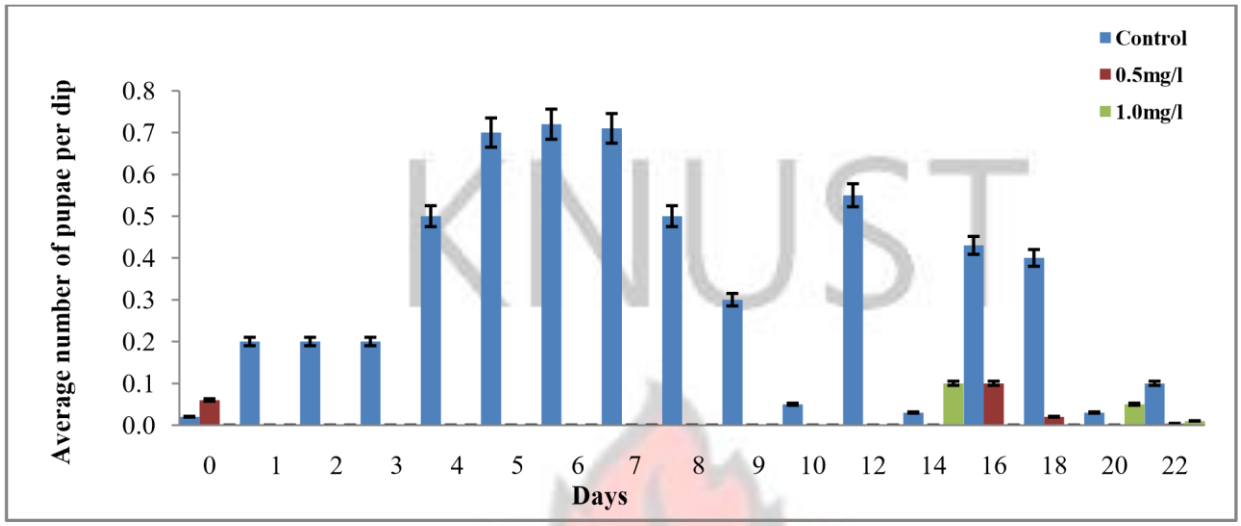

Figure 12: Average number of pupae exposed to different concentrations of *Bs* formulation in a controlled field trial (Dry Season, 2011)

Comparing the controlled field trial experiments conducted during the dry and rainy seasons, there was statistically no significant differences between the dry and rainy seasons results at both the 0.5mg/l concentrations (p=0.52) and at the 1.0 mg/l (p=0.71) (Table 2).

Table 2: Comparing the controlled field trial experiments conducted in the dry and rainy seasons, at both 0.5mg/l and 1.0mg/l concentrations.

DAYS	0.5mg/l		1.0mg/l	
	DRY SEASON	RAINY SEASON	DRY SEASON	RAINY SEASON
0	4	4	6.9	3.9
1	0	0.7	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0

5	0	0	0	0		
6	0	0	0	0		
7	0	1	0	0		
8	0	0	0	0		
9	0	0	0	0		
10	2	0	1.5	0		
12	2.5			1.2	2.3	0.3
14	1.7			2.6	0.9	1.8
16	0.9			3.6	1.2	2.1
18	1.1			3.7	1.4	2.2
20	0.6			2.1	0.3	1.1
22	1.2			1.2	0.4	0.3

CHAPTER FIVE

5.0 DISCUSSION

The results of the study showed that *Bacillus sphaericus* formulation is an effective and environmentally safe biological insecticide for controlling the larvae of the vector of malaria in Kumasi.

This study recorded LC_{50} and LC_{95} as 0.0027mg/l and 0.0086mg/l for the laboratory assay conducted with *Anopheles* larvae, similar to 0.004mg/l (LC_{50}) and 0.023mg/l (LC_{95}) in Gambia (Majambere *et al*., 2007), in Ethiopia with LC_{50} of 0.001mg/l (Seyoum and Abate, 1997) and in Tanzania with LC_{50} and LC_{95} of 0.002mg/l and 0.025mg/l (Ragoonanasingh *et al*., 1992) but differs

from studies in Kenya where the LC_{50} and LC_{95} were 0.004mg/l and 0.038mg/l (Fillinger *et al*., 2003) and in Burkina Faso, who had LC_{50} and LC_{95} as 0.022mg/l and 0.13mg/l (Majori *et al*., 1987). The similarities in vulnerability of the *Anopheles* larvae to the *Bs* in studies conducted in East Africa (Seyoum and Abate, 1997; Ragoonanasingh *et al*., 1992) and West Africa (Majambere *et al*., 2007) confirms that biolarvicidal activity is intrinsic to the mosquito but not ecologically determined (Charles *et al*., 1996).

Although the LC_{50} recorded in Kenya, 0.004mg/l (Fillinger *et al*., 2003) was similar to the LC_{50}, 0.0027mg/l of this study, that in Kenya however had a much higher concentration, 0.038mg/l as its LC_{95}. However the concentration, 0.0086mg/l obtained in this study as the LC_{95} depicts that *Bs* formulation is highly effective against *Anopheles* larvae, in Kumasi metropolis. The higher concentration observed in Kenya (Fillinger *et al*., 2003) was probably due to the fact that the (first generation) F1 *Anopheles* larvae used by Fillinger *et al*. (2003) were reared in natural habitat water of the wild larvae instead of tap water which was used in this study whilst in Burkina Faso (Majori *et al*., 1987), field larvae were used instead of laboratory reared F1 larvae and these alterations in conditions most likely decreased susceptibility of the larvae to the *Bs* formulation.

The F1 larvae used in this study came with its own setbacks such as the available and appropriate source of blood meal for the *Anopheles. Anopheles* is naturally anthropomorphic and feeding it with blood meal from guinea pigs rather took a while before the adult female mosquito adjusted to it. Since blood meal is the only source of nutrient for the female *Anopheles* to lay its eggs, it is expedient that further studies are done to develop special containers in which blood can be stored and fed to mosquitoes in its fresh state so that eggs are laid and hatched for F1 larvae.

The controlled field experiments during both the rainy season and the dry season had the residual effect, 10 to 12days and this compares favourably to studies carried out in several areas, in West Africa (Nicholas *et al*., 1987), New Jersey (Sutherland *et al*., 1989), Cuba (Lago *et al*., 1991), Thailand (Mulla *et al*., 1999), Kenya (Fillinger *et al*., 2003) and Gambia (Majambere *et al*., 2007). The residual effect was as a result of the recycling activity of *Bs* in dead mosquito larvae and the persistence of its spores in the soil (Becker *et al*., 1995). This characteristic of *Bs* is very important and will be of great value in any malaria vector control programme because several generations of mosquito larvae will be mortalised after the control programme has ended.

Since Fillinger and Lindsay (2006) and Karch *et al.* (1990) reports of a higher residual activity and reduction in resistance by the mosquito larvae to *Bs* by applying the formulation repeatedly, reapplication of the formulation in the natural breeding sites of mosquito larvae should be carried out every 10-12days during both the rainy and dry seasons as long as the control programme is in operation.

There have been other studies conducted using *Bs* in controlling larvae of *Culex quinquefasciatus*, *Culex pipiens*, *Anopheles stephensis* and *Anopheles gambiae* (Surendran and Vennison, 2011; Fillinger *et al*., 2003; Zahiri *et al*., 2004; Barbazan *et al*., 1997; Karch *et al*., 1992; Hougard, 1990 and Mulla *et al.*, 1985). These studies revealed that the mosquitoes were highly susceptible to *Bs* having their lethal concentrations as low as that obtained in this study with *Anopheles gambiae*. Although *Culex* and other mosquito larvae are not vectors of malaria, it is imperative that in controlling the *Anopheles*, the vector of *Plasmodium*, these mosquitoes are also controlled to curb nuisance biting and to reduce any psychological effects these bites might leave on the lay person who cannot differentiate between a vector of malaria and a non vector of malaria.

Such people might presume that the control intervention which did not target mosquitoes that were non malaria vectors were not effective.

Therefore *Bs* formulation when used as a control intervention or as part of an integrated vector management control programme should target all different types of mosquito breeding sites in the catchment area including all stagnant waters in car tires, tree holes, swamps, water tanks, septic tanks, road puddles, foot and hoof prints, man holes, cess pits and house drainages among others because different types of mosquitoes will normally breeding at different sites. The requirements of *Anopheles* larvae to breed and develop are mostly different from those of *Culex* larvae. *Anopheles* larvae breed in temporal, stagnant, well sunlit water bodies which are not too rich in nutrients (Kweka *et al.*, 2011) whiles *Culex* can breed and develop in temporaryand or permanent nutrient rich stagnant water found in shady areas (Bourget *et al*., 2004).

With the parameters for the *Anopheles* breeding site in mind sampling was done on a vegetable farm at KNUST campus because vegetable farms cultivated in an open field serve as a primary breeding site for *Anopheles* (Afrane *et al*., 2004). These differences in requirements for various types of mosquitoes to breed caused a large percentage of the sampled larvae for the bioassay to be *Anopheles* (89%) whiles a smaller percentage of the total larvae were *Culex* (11%). There must therefore be a training programme for personnel who will be engaged in a larviciding control programme. This will would enable them easily identify these types of breeding sites and handle appropriately all equipments needed to carry out the programme effectively.

During reapplication of the formulation the rainy seasons' should be intensive and more robust because *Anopheles* breeds mostly during the rainy season than in the dry season (Appawu *et al*.,

1994) which leads to higher incidence of malaria in Ghana in the rainy season (Binka *et al*., 1994 and Afari *et al*., 1993).

The number of larvae that were recorded in the bowls during the controlled field trial in the rainy season was more than that of the dry season supporting the seasonal oviposition preference of the *Anopheles*. Early instars of the *Anopheles* larvae appeared on the 1st, 7th and 12th day post application of the *Bs* formulation during the rainy season because extension in mortality of the larval population was not due to the absence of oviposition by the female *Anopheles* mosquito but to the biolarvicidal activity of the *Bs* formulation.

The larvae that appeared on the 1st and 7th day were first instars yet to feed or yet to digest its food, implying that the *Bs* formulation which lyses the cells of the lining of the midgut of the larvae had not yet began its activity (Charles *et al*., 1996). These larvae however died the next day by which time biolarvicidal activity had taken place in the mosquito. By day 12 of the post application of the *Bs* formulation the area that was cleared for the controlled field trial had a considerable amount of weeds growing on and around it. Rojas *et al*.(2001) reported that vegetation reduces biolarvicidal activity therefore after 12days biolarvicidal activity had worn out as a result and larval numbers started increasing (Figure 7 and Figure 10). As much as possible all vegetation in and around natural breeding site of the mosquito larvae where a biolarvicidal programme is being carried out should be cleared to obtain the maximum effect of the larvicidal activity of *Bs* formulation.

Heavy rainfall on the 19th day post-treatment was part of the reason that caused decline in the larval population from the 20th day post application during the rainy season (Figure 4). It caused

damaging effect on the larvae (Tuno *et al.*, 2005) and also caused flushing where the larvae were swept out of the basin by the rain water which killed them as a result (Paaijmans *et al*., 2007).

The larvae could have been flushed out also by the action of the strong winds during the rain. It rained 12 times more in the rainy season than in the dry season. The rains decreased the temperatures of the water in the treatment and control bowls whiles the dry season had increasing temperatures and since high temperature reduce biolarvicidal activity of *Bs* formulation (Rojas *et al*., 2001), the residual effect during the dry season were 2days shorter than that of the rainy season. *Bs* showed residual effect in both seasons because of the recycling activity of *Bs* dead larvae (Becker *et al*., 1995).

Another reason that caused the decline of larval population from the 20th day post application during the two seasons was ageing of the water body. As the water body increased in age predator population increased with an occurrence of algal bloom in some of the bowls. Predators that occur naturally have been shown to be an ecological factor that reduces *Anopheles gambiae* (Blaustein and Chase, 2007). During this study period the dominant predator was the tadpole and as their numbers increased the number of larvae decreased. However there was difficulty in quantify and identifying the exact impact of tadpoles on the population of mosquito larvae (Ohba *et al*., 2010). The predator prey association could not be specifically determined just by counting larvae alive at any point in time to the number of tadpoles present because the larvae might have died as a result of other external influences other than predation.

Molecular assay can be used effectively to detect the DNA of larvae immediately after ingestion of larvae by the predator. The level of detection of larval DNA however decreases over time, from the time of ingestion to the time of digestion (Schielke *et al.*, 2007). The tadpoles were dominant

predators because the area where the experiment was conducted was a wetland and tadpoles are the dominant predators of mosquito larvae in such areas (Kweka *et al*., 2011).

Interestingly it has been reported that, *Anopheles gambiae* typically breeds in temporal habitat where predators are not present or their presence is relatively low (Carlson *et al*., 2004) therefore as the water habitat age and predator population increased *Anopheles* gradually stopped ovipositing in these habitat. Ageing also comes about as a result of increasing algal content, larvae are not able to penetrate the algal mat and breathe and so adult mosquitoes would not prefer ovipositing on such sites (Shililu *et al*., 2003).

The number of pupae in the treatment bowls post application was considered the most important parameter in determining the effectiveness of a larval control (Tianyun and Mulla, 1999). This is so because the population of the pupae determines to a large extent the number of adult mosquitoes that will emerge. Although it has been reported that rainfall can decrease the population of pupae when hit directly by raindrops to cause hydrostatic balance to be loss (Romoser *et al*., 1994), the difference in the pupal numbers in the dry and rainy season in this study was not statistically significant.

The sampled *Anopheles* species were identified as *Anopheles gambiae*, consistent with previous work by Agyepong (2008) but in contrast with Coleman (2009) who found *Anopheles funestus* and *Anopheles ziemanni* in addition to *Anopheles gambiae* on KNUST campus. This study found only *Anopheles gambiae* because each species require different abiotic conditions (Keating *et al*., 2003). *Anopheles gambiae* breeds mostly in temporal pools, found during the rainy season whereas *Anopheles funestus* typically breeds in more permanent water which is mostly overgrown with vegetation (Appawu *et al*., 1994). *Anopheles ziemanni* however prefer animals to humans, they

are zoophilic (Gillies and De Meillon, 1968) therefore they mostly breed at areas that are close to where animals are reared, but the sample sites were not close to any animal farm.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Anopheles gambiae was identified as the most predominant malaria vector on Kwame Nkrumah University of Science and Technology campus. It was also found to be susceptible to *Bacillus sphaericus* WDG formulation (Vectolex®). A concentration of 0.0086mg/l of the *Bs* formulation was the minimum effective dosage against first generation *Anopheles* larvae during the laboratory studies. However, the optimum effective dosage from the controlled field trials was 0.5mg/l. *Bs* formulation is a good biolarvicide for the control of *Anopheles* species. The formulation can successfully be applied on all *Anopheles* breeding sites in the Kumasi Metropolis.

The residual activity during the Rainy Season (12days) was 2days more than the Dry Season (10days).

There was no observable harm to tadpoles, natural predators of the *Anopheles,* by the *Bs* formulation.

6.2 Recommendation

This work should be extended to the whole Kumasi metropolis on a large scale field application, to help curb *Anopheles* population and by extension control malaria in the metropolis.

The criteria used for identifying the *Anopheles* sampling sites is an important basic information for identifying and mapping all other breeding sites in the Kumasi metropolis to aid the large scale field application of *Bs* formulation for larval control. The progress of the control intervention can be monitored regularly using the GIS (Geographic Information System). The GIS database offers the ability to process large quantities of data that the manual systems can not process. Data can be

stored in a structured digital format, which would permit rapid retrieval and use. A hand held GPS (Global Positioning System) can be used to capture spatial co-ordinates to geo-reference features, households and breeding sites during the large scale field application.

The sustenance of the control intervention must be a priority so that the population of the *Anopheles* larvae is prevented from resistance development. There should be a reapplication programme designed to ensure that sustenance is maintained and prevention of resistance in the larvae to the *Bs* formulation is ensured. Different time interval for reapplication of the *Bs* formulation should be instituted for the rainy and dry seasons because they had different days of residual activity. Due to the 10 days residual activity of the formulation in the controlled field experiments during the dry season, reapplication should be carried out weekly and fortnightly during the rainy season because its residual activity days were 12. Vigilance for detection of resistance development should be practiced.

In eradicating malaria it is important to have a varied control programme, used in combination, to produce a synergistic effect. Since the laboratory assay and the controlled field trials have proved to be effective and efficient for malaria vector control, *Bs* formulation can be incorporated in an IVM (Integrated Vector Management) programme to control malaria in the metropolis and the nation as a whole. IVM approach can utilize environmentally friendly control measure, *Bs* formulation, Insecticide Treated Nets (ITNs) and Indoor Residual Spraying (IRS).

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APPENDIX

DATA RECORDING FORM FOR WILD MOSQUITO LARVAE

Date	No Of Larvae/ Pupae Sampled	Genus and specie of mosquito	No of dead larvae	Sampling site

DATA RECORDING FORMS FOR BIOASSAY

Experiment No: 1 **Investigator**: Sandra

Location: KCCR/ EntomologyLaboratory **Treatment Date**: 03/03/11

Material: *Bacillus sphaericus* (Vectolex WDG) **Formulation**: 1% stock

Temperature: 25± 1 **Lighting**: 24L

Species: *Anopheles* **Larval Instar**: 3rd and 4th instar.

Larvae/Cup or Vessel: 25 **Water**: Deionised

Volume of Water: 100 ml **Food**: Fish flakes

Date Stock Solution Made: 03/03/11

Experiment No: 2 **Investigator**: Sandra

Location: KCCR/ Entomology Laboratory **Treatment Date**: 15/04/11

Material: *Bacillus sphaericus* (Vectolex WDG) **Formulation**: 1%

Temperature: 25± 1 **Lighting**: 24L

Species: *Anopheles* **Larval Instar**: 3rd and 4th instar.

Larvae/Cup or Vessel: 25 **Water**: Deionised

Volume of Water: 100ml **Food**: Fish flakes

Date Stock Solution Made: 15/04/11

Experiment No: 3 **Investigator**: Sandra

Location: KCCR/ Entomology Laboratory **Treatment Date**: 02/06/11

Material: *Bacillus sphaericus* (Vectolex WDG) **Formulation**: 1%

Temperature: 25± 1 **Lighting**: 24L

Species: *Anopheles* **Larval Instar**: 3rd and 4th instar.

Larvae/Cup or Vessel: 25 **Water**: Deionised

Volume of Water: 100 ml **Food**: Fish flakes

Date Stock Solution Made: 02/06/11

Table 3: Number of dead *Anopheles* larvae at various concentrations of *Bs* formulation after 24hours (first occasion)

DAY 1		**24 hr(mg/l)**												
Date	**Replicate**	**0.05**	**0.04**	**0.03**	**0.02**	**0.01**	**0.008**	**0.005**	**0.004**	**0.0035**	**0.003**	**0 .0025**	**0.002**	**0.0015**	**0**
03/03/11	A	25	25	24	25	25	21	15	15	16	11	11	10	0	0
	1.	25	25	25	25	25	19	21	0	12	14	14	11	5	0
	2	25	25	25	25	25	24	16	10	16	15	12	9	9	0
	3	25	25	25	24	25	15	17	25	19	18	13	8	5	0
	4	25	25	25	24	24	20	19	24	13	12	9	10	2	0
	Total	125	125	124	123	124	99	88	74	76	70	59	48	21	0
	Average	25	25	24.8	24.6	24.8	19.8	17.6	14.8	15.2	14	11.8	9.6	4.2	0
	%mortality	100	100	99.2	98.4	99.2	79.2	70.4	59.2	60.8	56	47.2	38.4	16.8	0
LC_{50}															
LC_{95}															
Slope															

DAY 2 **24 hr(mg/l)**

Table 4: Number of dead *Anopheles* larvae at various concentrations of *Bs* formulation after 24hours (second occasion)

Date	Replicate	0.05	0.04	0.03	0.02	0.01	0.008	0.005	0.004	0.0035	0.003	0 .0025	0.002	0.015	0.0
15/04/11	A	25	25	25	25	25	21	20	15	16	18	10	10	0	0
	1	25	25	25	25	25	21	17	21	18	20	14	6	6	0
	2	25	25	25	25	24	24	20	15	16	16	12	11	9	0
	3	25	25	25	25	24	24	21	25	20	19	13	8	4	0
	4	25	25	25	24	24	20	20	18	21	20	11	17	7	0
	Total	125	125	125	124	122	110	98	94	91	93	60	52	26	0
	Average	25	25	25	24.8	24.4	22	19.6	18.8	18.2	18.6	12	10.4	5.2	0
	%mortality	100	100	100	99.2	97.6	88	78.4	75.2	72.8	74.4	48	41.6	20.8	0
LC_{50}															
LC_{95}															
Slope															

Table 5: Number of dead *Anopheles* larvae at various concentrations of *Bs* formulation after 24hours (third occasion)

DAY 3		**24hrs(mg/l)**													
Date	**Replicate**	**0.05**	**0.04**	**0.03**	**0.02**	**0.01**	**0.008**	**0.005**	**0.004**	**0.0035**	**0.003**	**0 .0025**	**0.002**	**0.0015**	**0.0**
02/06/11	A	25	25	25	25	25	21	24	25	11	11	12	19	3	0
	1	25	25	25	25	25	22	21	20	18	14	14	15	5	0
		25	25	25	25	25	24	24	10	19	15	13	10	10	0
	3	25	25	25	25	25	25	22	25	16	18	13	11	5	0
	4	25	25	25	25	25	24	16	24	20	12	19	6	2	0
	Total	125	125	125	125	125	116	107	104	84	70	71	61	4	0
	Average	25	25	25	25	25	23.2	21.4	20.8	16.8	14	14.2	12.2	5	0
	%mortality	100	100	100	100	100	92.8	85.6	83.2	67.2	56	56.8	48.8	20	0
LC_{50}															
LC_{95}															
Slope															

Table 6: Number of dead *Anopheles* larvae at various concentrations of *Bs* formulation after 24hours (all occasion)

DAY 3	24 hr(mg/l)													
Date	**Replicate**	**0.05**	**0.04**	**0.03**	**0.02**	**0.01**	**0.008**	**0.005**	**0.004**	**0.0035**	**0.003**	**0 .0025**	**0.002**	**0.0015**	**0.0**
03/03/11		25	25	24.8	24.6	24.8	19.8	17.6	14.8	15.2	14	11.8	9.6	4.2	0
15/04/11		25	25	25	24.8	24.4	22	19.6	18.8	18.2	18.6	12	10.4	5.2	0
02/06/11		25	25	25	25	25	23.2	21.4	20.8	16.8	14	14.2	12.2	5	0
	Total	75	75	74.8	74.4	74.2	65	58.6	54.4	50.2	46.6	38	32.2	4	0
	Average	25	25	24.9	24.8	24.7	21.7	19.5	18.1	16.7	15.5	12.7	10.7	4.8	0
	%mortality	100	100	99.7	99.2	98.9	86.7	78.1	72.5	66.9	62.1	50.7	42.9	19.2	0
LC_{50}															
LC_{95}															
Slope															

Table 7: Analysis of parameters obtained for the bioassay

Analysis Of Parameter Estimates						
Parameter	**Degree Of Freedom(D.F)**	**Estimate**	**Standard Error**	**Wald 95%**	**Confidence Limits**	**Pr > ChiSq**
Intercept	1	-1.1869	0.1330	-1.4476	-0.9262	<.0001
dose	1	378.4349	31.8470	316.0159	440.8538	<.0001

FORMULAR FOR LC50 and LC95

FIRST REPLICA : LC_{50}

$$\log\frac{\pi(x)}{1-\pi(x)} = -1.1869 + 378.4349dose$$

$$\log\frac{0.50}{0.50} = -1.1869 + 378.4349dose$$

Dose = 0.003136

LC_{50} = 0.0032

LC_{95}

$$\log\frac{\pi(x)}{1-\pi(x)} = -1.1869 + 378.4349dose$$

$$\log\frac{0.95}{0.05} = -1.1869 + 378.4349dose$$

Dose = 0.01091

LC_{95} = 0.01091

SECOND REPLICA : LC_{50}

$$\log\frac{\pi(x)}{1-\pi(x)} = -1.1718 + 491.7975dose$$

$$\log\frac{0.50}{0.50} = -1.1718 + 491.7975dose$$

Dose = 0.0024

LC_{50} = 0.0024

LC_{95}

$$\log\frac{\pi(x)}{1-\pi(x)} = -1.1718 + 491.7975dose$$

$$\log\frac{0.95}{0.05} = -1.1718 + 491.7975dose$$

Dose = 0.00837

LC_{95} = 0.00837

THIRD REPLICA : LC_{50}

$$\log\frac{\pi(x)}{1-\pi(x)} = -2.1491 + 804.5865dose$$

$$\log\frac{0.50}{0.50} = -2.1491 + 804.5865dose$$

Dose = 0.00267

LC_{50} = 0.00267

LC_{95}

$$\log\frac{\pi(x)}{1-\pi(x)} = -2.1491 + 804.5865dose$$

$$\log\frac{0.95}{0.05} = -2.1491 + 804.5865dose$$

Dose = 0.0063

LC_{95} = 0.0063

TOTALING ALL REPLICA : LC_{50}

$$\log\frac{\pi(x)}{1-\pi(x)} = -2.1491 + 604.59dose$$

$$\log\frac{0.50}{0.50} = -2.1491 + 604.59dose$$

Dose = 0.002688

LC_{50} = 0.002688

LC_{95}

$$\log\frac{\pi(x)}{1-\pi(x)} = -2.1491 + 604.59dose$$

$$\log\frac{0.95}{0.05} = -2.1491 + 604.59dose$$

Dose = 0.008573

LC_{95} = 0.008573

Table 8: Average mosquito larval numbers exposed to different concentrations of *Bs* formulation in a controlled field trials (Rainy Season, 2011)

RAINY SEASON (JULY)

Day	AVERAGE NUMBER PER DIP									PERCENTAGE REDUCTION					
	Total instars			Late instars			Early instars			Total instars		Late instars		Early instars	
	Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l	0.5mg/l	1.0mg/l	0.5mg/l	1.0mg/l	0.5mg/l	1.0mg/l
0*	4.7	4.0	3.9	3.1	2.7	2.9	1.6	1.3	1.0	-	-	-	-	-	-
1	3.8	0.7	0.0	1.8	0.0	0.0	2.0	0.7	0.0	79.5	100.0	100.0	100.0	60.0	100.0
2	3.7	0.0	0.0	1.9	0.0	0.0	1.9	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
3	3.5	0.0	0.0	1.7	0.0	0.0	1.8	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
4	3.5	0.0	0.0	2.0	0.0	0.0	1.5	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
5	3.4	0.0	0.0	2.1	0.0	0.0	1.3	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
6	3.6	0.0	0.0	2.1	0.0	0.0	1.5	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
7	3.5	1.0	0.0	1.6	0.0	0.0	1.9	1.0	0.0	66.9	100.0	100.0	100.0	38.1	100.0
8	3.5	0.0	0.0	1.5	0.0	0.0	2.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
9	3.2	0.0	0.0	1.4	0.0	0.0	1.8	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
10	3.4	0.0	0.0	1.4	0.0	0.0	2.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0

12.0	3.2	1.2	0.3	1.4	0.0	0.0	1.8	1.2	0.3	56.3	88.7	100.0	100.0	100.0	73.3
		2.6	1.8	1.4	1.4	1.4	1.2	1.2	0.4	-	16.4	-	-	-	46.7
		3.6	2.1	1.6	2.4	1.6	1.4	1.2	0.5	-	12.7	-	-	-	36.5
		3.7	2.2	1.3	1.9	1.9	1.4	1.8	0.4	-	-	-	-	-	57.2
		2.1	1.1	1.2	1.6	0.8	0.5	0.5	0.3	-	24.0	-	33.0	-	-
		1.2	0.3	1.0	1.0	0.1	0.0	0.2	0.2	-	-	-	-	-	-
14	2.6														
16	3.0														
18	2.7														
20	1.7														

DRY SEASON (NOVEMBER)

Day	AVERAGE NUMBER PER DIP									PERCENTAGE REDUCTION					
	Total instars			Late instars			Early instars			Total instars		Late instars		Early instars	
	Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l	0.5mg/l	1.0mg/l	0.5mg/l	1.0mg/l	0.5mg/l	1.0mg/l
0*	2.8	4.0	6.9	1.6	3.2	4.5	1.2	0.8	2.4	-	-	-	-	-	-
1	2.6	0.0	0.0	1.6	0.0	0.0	1.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
2	3.2	0.0	0.0	1.0	0.0	0.0	2.2	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
3	3.8	0.0	0.0	1.5	0.0	0.0	2.3	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0

22	1.0																			

Table 9: Average mosquito larval numbers exposed to different concentrations of *Bs* formulation in a controlled field trials (Dry Season, 2011)

4	3.8	0.0	0.0	0.8	0.0	0.0	3.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0					
5	4.2	0.0	0.0	1.0	0.0	0.0	3.2	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0					
6		4.0	0.0	0.0	1.0	0.0	0.0	3.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0				
7	4.6	1.0	0.0	2.0	0.0	0.0	2.6	1.0	0.0	84.8	100.0	100.0	100.0	42.3	100.0					
8	4.8	0.0	0.0	2.8	0.0	0.0	2.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0					
9	4.9	0.0	0.0	2.8	0.0	0.0	2.1	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0					
10	4.7	2.0	1.5	2.2	0.0	0.0	2.5	2.0	1.5	70.2	87.0	100.0	100.0	100.0	70.0					
12	2.8	2.5	2.3	1.3	1.3	1.6	1.5	1.2	0.7	37.5	66.7	50.0	56.2	-	76.7					
14	2.2	1.7	0.9	0.9	0.7	0.4	1.3	1.0	0.5	45.9	83.4	61.1	84.2	-	80.8					
16	1.2	0.9	1.2	0.3	0.3	0.4	0.9	0.6	0.8	47.5	59.4	50.0	52.6	0.0	55.6					
18	1.6	1.1	1.4	0.4	0.4	0.5	1.2	0.7	0.9	51.9	64.5	50.0	55.6	12.5	62.5					
20	1.2	0.6	0.3	0.7	0.2	0.1	0.5	0.4	0.2	64.1	89.6	85.7	94.9	-	78.7					
22	0.4	1.2	0.4	0.3	1.0	0.1	0.1	0.2	0.3	-	55.4	-	83.4	-	-					

				0.0		0.0	0.0			0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			

Table 10: Average larval numbers per breeding bowl exposed to different concentrations of *Bs* formulation in a controlled field trial (Rainy Season, 2011)

RAINY SEASON (JULY)

Day		AVERAGE NUMBER PER LARVAE PER DIP PER BOWL								
	BOWLS	Total instars			Late instars			Early instars		
		Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l
0	B1	22.0	38.0	40.0	15.0	34.0	38.0	7.0	4.0	2.0
	B2	27.0	12.0	21.0	27.0	9.0	15.0	0.0	3.0	6.0
	B3	13.0	22.0	12.0	12.0	7.0	6.0	1.0	15.0	6.0
	B4	27.0	17.0	10.0	18.0	8.0	5.0	9.0	9.0	5.0
	B5	22.0	16.0	22.0	6.0	10.0	19.0	16.0	6.0	3.0
	B6	31.0	9.0	11.0	16.0	6.0	4.0	15.0	3.0	7.0
1	B1	14.0	0.0	0.0	8.0	0.0	0.0	6.0	0.0	0.0
	B2	20.0	0.0	0.0	20.0	0.0	0.0	0.0	0.0	0.0
	B3	11.0	15.0	0.0	6.0	0.0	0.0	5.0	15.0	0.0
	B4	23.0	0.0	0.0	7.0	0.0	0.0	16.0	0.0	0.0
	B5	19.0	0.0	0.0	4.0	0.0	0.0	15.0	0.0	0.0
	B6	28.0	5.0	0.0	10.0	0.0	0.0	18.0	5.0	0.0
				0.0			0.0		0.0	0.0

2	B1	21.0	0.0	0.0	9.0	0.0	0.0	12.0	0.0	0.0
	B2	21.0	0.0	0.0	20.0	0.0	0.0	1.0	0.0	0.0
	B3	15.0	0.0	0.0	6.0	0.0	0.0	9.0	0.0	0.0
	B4	14.0	0.0	0.0	7.0	0.0	0.0	7.0	0.0	0.0
	B5	12.0	0.0	0.0	4.0	0.0	0.0	8.0	0.0	0.0
	B6	29.0	0.0	0.0	10.0	0.0	0.0	19.0	0.0	0.0
3	B1	19.0	0.0	0.0	8.0	0.0	0.0	11.0	0.0	0.0
	B2	24.0	0.0		19.0			5.0	0.0	
	B3	11.0	0.0		4.0			7.0	0.0	
	B4	16.0	0.0	0.0	6.0			10.0	0.0	0.0
	B5	11.0	0.0	0.0	3.0	0.0	0.0	8.0	0.0	0.0
	B6	26.0	0.0	0.0	12.0	0.0	0.0	14.0	0.0	0.0
4	B1	19.0	0.0	0.0	10.0	0.0	0.0	9.0	0.0	0.0
	B2	27.0	0.0	0.0	24.0	0.0	0.0	3.0	0.0	0.0
	B3	11.0	0.0	0.0	4.0	0.0	0.0	7.0	0.0	0.0
	B4	14.0	0.0	0.0	6.0	0.0	0.0	8.0	0.0	0.0
	B5	9.0	0.0	0.0	4.0	0.0	0.0	5.0	0.0	0.0
	B6	25.0	0.0	0.0	12.0	0.0	0.0	13.0	0.0	0.0
5	B1	19.0	0.0	0.0	10.0	0.0	0.0	9.0	0.0	0.0
	B2	20.0	0.0	0.0	20.0	0.0	0.0	0.0	0.0	0.0
	B3	13.0	0.0	0.0	7.0	0.0	0.0	6.0	0.0	0.0
	B4	14.0	0.0	0.0	7.0	0.0	0.0	7.0	0.0	0.0
	B5	9.0	0.0	0.0	5.0	0.0	0.0	4.0	0.0	0.0
	B6	17.0	0.0	0.0	4.0	0.0	0.0	13.0	0.0	0.0

				0.0		0.0	0.0			0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
6	B1	18.0	0.0	0.0	9.0	0.0	0.0	9.0	0.0	0.0
	B2	22.0	0.0	0.0	22.0	0.0	0.0	0.0	0.0	0.0
	B3	17.0	0.0	0.0	10.0	0.0	0.0	7.0	0.0	0.0
	B4	14.0	0.0	0.0	6.0	0.0	0.0	8.0	0.0	0.0
	B5	11.0	0.0	0.0	4.0	0.0	0.0	7.0	0.0	0.0
	B6	26.0	0.0	0.0	12.0	0.0	0.0	14.0	0.0	0.0
7	B1	19.0	0.0	0.0	7.0	0.0	0.0	12.0	0.0	0.0
	B2	18.0	0.0		18.0	0.0		0.0		
	B3	16.0	12.0	0.0	7.0	0.0	0.0	9.0	12.0	0.0
	B4	14.0	0.0	0.0	5.0	0.0	0.0	9.0	0.0	0.0
	B5	11.0	0.0	0.0	3.0	0.0	0.0	8.0	0.0	0.0
	B6	26.0	18.0	0.0	8.0	0.0	0.0	18.0	18.0	0.0
8	B1	17.0	0.0	0.0	7.0	0.0	0.0	10.0	0.0	0.0
	B2	15.0	0.0	0.0	15.0	0.0	0.0	0.0	0.0	0.0
	B3	15.0	0.0	0.0	6.0	0.0	0.0	9.0	0.0	0.0
	B4	17.0	0.0	0.0	5.0	0.0	0.0	12.0	0.0	0.0
	B5	15.0	0.0	0.0	4.0	0.0	0.0	11.0	0.0	0.0
	B6	26.0	0.0	0.0	8.0	0.0	0.0	18.0	0.0	0.0
9	B1	15.0	0.0	0.0	5.0	0.0	0.0	10.0	0.0	0.0
				0.0			0.0		0.0	0.0

	B2	16.0	0.0	0.0	16.0	0.0	0.0	0.0	0.0	0.0
	B3	14.0	0.0	0.0	5.0	0.0	0.0	9.0	0.0	0.0
	B4	18.0	0.0	0.0	6.0	0.0	0.0	12.0	0.0	0.0
	B5	10.0	0.0	0.0	2.0	0.0	0.0	8.0	0.0	0.0
	B6	23.0	0.0	0.0	8.0	0.0	0.0	15.0	0.0	0.0
10	B1	14.0	0.0	0.0	5.0	0.0	0.0	9.0	0.0	0.0
	B2	19.0	0.0	0.0	19.0	0.0	0.0	0.0	0.0	0.0
	B3	9.0	0.0	0.0	2.0	0.0	0.0	7.0	0.0	0.0
	B4	19.0	0.0	0.0	3.0	0.0	0.0	16.0	0.0	0.0
	B5	16.0	0.0	0.0	7.0	0.0	0.0	9.0	0.0	0.0
	B6	25.0	0.0	0.0	6.0	0.0	0.0	19.0	0.0	0.0
12	B1	14.0	10.0	8.0	7.0	0.0	0.0	7.0	10.0	8.0
	B2	19.0	0.0	4.0	19.0	0.0	0.0	0.0	0.0	4.0
	B3	10.0	8.0	0.0	2.0	0.0	0.0	8.0	8.0	0.0
	B4	15.0	17.0		1.0			14.0	17.0	
	B5	16.0	1.0		7.0			9.0	1.0	
	B6	23.0	0.0	0.0	7.0			16.0	0.0	0.0
14	B1	12.0	20.0	25.0	6.0	8.0	20.0	6.0	12.0	5.0
	B2	13.0	10.0	15.0	13.0	0.0	7.0	0.0	10.0	8.0
	B3	5.0	13.0	8.0	0.0	7.0	8.0	5.0	6.0	0.0
	B4	13.0	12.0	5.0	7.0	12.0	5.0	6.0	0.0	0.0
	B5	11.0	12.0	2.0	7.0	8.0	2.0	4.0	4.0	0.0
	B6	23.0	9.0	0.0	8.0	5.0	0.0	15.0	4.0	0.0
16	B1	12.0	48.0	27.0	6.0	30.0	20.0	6.0	18.0	7.0
	B2	15.0	23.0	6.0	15.0	16.0	4.0	0.0	7.0	2.0

				0.0		0.0	0.0			0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
	B3	4.0	15.0	0.0	0.0	12.0	0.0	4.0	3.0	0.0
	B4	16.0	2.0	12.0	4.0	0.0	12.0	12.0	2.0	0.0
	B5	13.0	8.0	11.0	8.0	8.0	8.0	5.0	0.0	3.0
	B6	21.0	11.0	8.0	7.0	5.0	4.0	14.0	6.0	4.0
18	B1	6.0	31.0	10.0	4.0	10.0	9.0	2.0	21.0	1.0
	B2	9.0	13.0	19.0	9.0	9.0	15.0	0.0	4.0	4.0
	B3	8.0	14.0	15.0	4.0	5.0	12.0	4.0	9.0	3.0
	B4	27.0	17.0	0.0	5.0	7.0	0.0	22.0	10.0	0.0
	B5	7.0	0.0	7.0	7.0	0.0	7.0	0.0	0.0	0.0
	B6	25.0	27.0	13.0	10.0	16.0	11.0	15.0	11.0	2.0
20	B1	4.0	21.0	6.0	4.0	18.0	5.0	0.0	3.0	1.0
	B2	9.0	11.0	11.0	9.0	3.0	10.0	0.0	8.0	1.0
	B3	5.0	8.0	11.0	4.0	7.0	5.0	1.0	1.0	6.0
	B4	19.0	7.0		5.0	7.0		14.0		
	B5	5.0	2.0	1.0	5.0	0.0	1.0	0.0	2.0	0.0
	B6	10.0	24.0	4.0	10.0	13.0	3.0	0.0	11.0	1.0
22	B1	3.0	3.0	0.0	3.0	3.0	0.0	0.0	0.0	0.0
				0.0			0.0		0.0	0.0

	B2	6.0	0.0	0.0	6.0	0.0	1.0	2.0	0.0	1.0
	B3	5.0	0.0	1.0	4.0	0.0	0.0	0.0	0.0	0.0
	B4	3.0	2.0	1.0	2.0	2.0	0.0	0.0	0.0	0.0
	B5	11.0	0.0	0.0	11.0	0.0	0.0	0.0	0.0	0.0
	B6	6.0	2.0	0.0	6.0	1.0	0.0	0.0	1.0	0.0

Table 11: Average larval nu nbers per breeding bowl ex osed to different c *Bs* formulation in controll d field trial (Dry Season, 2011) ncentrations of

DRY SEASON (NOVEMBER)

Day		AVERAGE NUMBER PER LARVAE PER DIP PER BOWL								
	BOWLS	Total instars			Late instars			Early instars		
		Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l	Control	0.5mg/l	1.0mg/l
0*	B1	12.0	27.0	24.0	8.0	21.0	18.0	4.0	6.0	6.0
	B2	11.0	17.0	35.0	1.0	12.0	20.0	10.0	5.0	15.0
	B3	17.0	45.0	50.0	12.0	35.0	39.0	5.0	10.0	11.0
	B4	10.0	3.0	37.0	0.0	3.0	27.0	10.0	0.0	10.0
	B5	26.0	2.0	24.0	20.0	2.0	15.0	6.0	0.0	9.0
	B6	8.0	26.0	37.0	7.0	23.0	16.0	1.0	3.0	21.0
1	B1	13.0	0.0	0.0	8.0	0.0	0.0	5.0	0.0	0.0

			0.0		0.0	0.0			0.0
			0.0		0.0	0.0			0.0
					0.0	0.0			
B2	15.0	0.0	0.0	5.0	0.0	0.0	10.0	0.0	0.0
B3	15.0	0.0	0.0	9.0	0.0	0.0	6.0	0.0	0.0
			0.0			0.0		0.0	0.0

			0.0	0.0		0.0	0.0		0.0	0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
						0.0	0.0			
						0.0	0.0			
	B4	10.0			3.0			7.0		
	B5	19.0	0.0		18.0			1.0	0.0	
	B6	6.0	0.0	0.0	5.0			1.0	0.0	0.0
2	B1	5.0	0.0	0.0	2.0			3.0	0.0	0.0
	B2	13.0	0.0	0.0	5.0			8.0	0.0	0.0
	B3	15.0	0.0	0.0	0.0	0.0	0.0	15.0	0.0	0.0
	B4	25.0	0.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0
	B5	37.0	0.0	0.0	20.0	0.0	0.0	17.0	0.0	0.0
	B6	11.0	0.0	0.0	3.0	0.0	0.0	8.0	0.0	0.0
3	B1	13.0	0.0	0.0	8.0	0.0	0.0	5.0	0.0	0.0
	B2	15.0	0.0	0.0	9.0	0.0	0.0	6.0	0.0	0.0
	B3	19.0	0.0	0.0	7.0	0.0	0.0	12.0	0.0	0.0
	B4	25.0	0.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0
	B5	29.0	0.0	0.0	14.0	0.0	0.0	15.0	0.0	0.0
	B6	13.0	0.0	0.0	7.0	0.0	0.0	6.0	0.0	0.0
4	B1	11.0	0.0	0.0	5.0	0.0	0.0	6.0	0.0	0.0
	B2	9.0	0.0	0.0	3.0	0.0	0.0	6.0	0.0	0.0
			0.0	0.0		0.0	0.0		0.0	0.0

			0.0	0.0		0.0	0.0		0.0	0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
						0.0	0.0			
						0.0	0.0			
	B3	19.0	0.0	0.0	4.0	0.0	0.0	15.0	0.0	0.0
	B4	8.0	0.0	0.0	0.0	0.0	0.0	8.0	0.0	0.0
	B5	29.0	0.0	0.0	10.0	0.0	0.0	19.0	0.0	0.0
	B6	8.0	0.0	0.0	2.0	0.0	0.0	6.0	0.0	0.0
5	B1	7.0	0.0	0.0	3.0	0.0	0.0	4.0	0.0	0.0
	B2	19.0	0.0	0.0	8.0	0.0	0.0	11.0	0.0	0.0
	B3	13.0	0.0	0.0	1.0	0.0	0.0	12.0	0.0	0.0
	B4	32.0			0.0			32.0		
	B5	40.0			13.0			27.0		
	B6	15.0	0.0		5.0			10.0	0.0	
6	B1	16.0	0.0	0.0	7.0			9.0	0.0	0.0
	B2	20.0	0.0	0.0	12.0			8.0	0.0	0.0
	B3	15.0	0.0	0.0	0.0			15.0	0.0	0.0
	B4	31.0	0.0	0.0	1.0	0.0	0.0	30.0	0.0	0.0
	B5	29.0	0.0	0.0	10.0	0.0	0.0	19.0	0.0	0.0
	B6	9.0	0.0	0.0	0.0	0.0	0.0	9.0	0.0	0.0
7	B1	19.0	0.0	0.0	12.0	0.0	0.0	7.0	0.0	0.0
			0.0	0.0		0.0	0.0		0.0	0.0

			0.0	0.0		0.0	0.0		0.0	0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
						0.0	0.0			
						0.0	0.0			
	B2	22.0	0.0	0.0	16.0	0.0	0.0	6.0	0.0	0.0
	B3	11.0	0.0	0.0	2.0	0.0	0.0	9.0	0.0	0.0
	B4	41.0	0.0	0.0	9.0	0.0	0.0	32.0	0.0	0.0
	B5	33.0	0.0	0.0	18.0	0.0	0.0	15.0	0.0	0.0
	B6	12.0	0.0	0.0	3.0	0.0	0.0	9.0	0.0	0.0
8	B1	22.0	0.0	0.0	10.0	0.0	0.0	12.0	0.0	0.0
	B2	28.0	0.0	0.0	22.0	0.0	0.0	6.0	0.0	0.0
	B3	8.0	0.0	0.0	5.0	0.0	0.0	3.0	0.0	0.0
	B4	43.0	0.0	0.0	19.0	0.0	0.0	24.0	0.0	0.0
	B5	27.0	0.0	0.0	17.0	0.0	0.0	10.0	0.0	0.0
	B6	16.0	0.0	0.0	11.0	0.0	0.0	5.0	0.0	0.0
9	B1	20.0	0.0	0.0	8.0	0.0	0.0	12.0	0.0	0.0
	B2	29.0	0.0	0.0	20.0	0.0	0.0	9.0	0.0	0.0
	B3	12.0	0.0	0.0	8.0	0.0	0.0	4.0	0.0	0.0
	B4	47.0	0.0	0.0	27.0	0.0	0.0	20.0	0.0	0.0
	B5	27.0			13.0			14.0		
	B6	12.0			8.0			4.0		
10	B1	31.0	27.0		12.0			19.0	27.0	
			0.0	0.0		0.0	0.0		0.0	0.0

			0.0	0.0		0.0	0.0		0.0	0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
						0.0	0.0			
						0.0	0.0			
	B2	25.0	4.0	5.0	14.0			11.0	4.0	5.0
	B3	10.0	15.0	16.0	6.0			4.0	15.0	16.0
	B4	36.0	0.0	4.0	8.0			28.0	0.0	4.0
	B5	25.0	12.0	19.0	18.0	0.0	0.0	7.0	12.0	19.0
	B6	14.0	2.0	1.0	8.0	0.0	0.0	6.0	2.0	1.0
12	B1	14.0	27.0	0.0	6.0	15.0	0.0	8.0	12.0	0.0
	B2	12.0	0.0	3.0	5.0	0.0	2.0	7.0	0.0	1.0
	B3	2.0	18.0	29.0	0.0	11.0	18.0	2.0	7.0	11.0
	B4	38.0	9.0	2.0	22.0	0.0	0.0	16.0	9.0	2.0
	B5	12.0	16.0	35.0	3.0	8.0	28.0	9.0	8.0	7.0
	B6	6.0	5.0	0.0	3.0	5.0	0.0	3.0	0.0	0.0
14	B1	16.0	16.0	0.0	7.0	7.0	0.0	9.0	9.0	0.0
	B2	13.0	0.0	3.0	7.0	0.0	0.0	6.0	0.0	3.0
	B3	4.0	7.0	1.0	0.0	1.0	1.0	4.0	6.0	0.0
	B4	22.0	18.0	15.0	9.0	8.0	7.0	13.0	10.0	8.0
	B5	9.0	10.0	7.0	4.0	5.0	3.0	5.0	5.0	4.0
	B6	2.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0
			0.0	0.0		0.0	0.0		0.0	0.0

			0.0	0.0		0.0	0.0		0.0	0.0
				0.0		0.0	0.0			0.0
						0.0	0.0			
						0.0	0.0			
						0.0	0.0			
16	B1	4.0	10.0	0.0	1.0	3.0	0.0	3.0	7.0	0.0
	B2	9.0	1.0	1.0	2.0	1.0	1.0	7.0	0.0	0.0
	B3	0.0	2.0	2.0	0.0	0.0	0.0	0.0	2.0	2.0
	B4	20.0	9.0	18.0	6.0	4.0	8.0	14.0	5.0	10.0
	B5	1.0	5.0	15.0	0.0	1.0	3.0	1.0	4.0	12.0
	B6	2.0			0.0			2.0		
			0.0	0.0		0.0	0.0		0.0	0.0

				0.0		0.0			0.0
				0.0		0.0			0.0
						0.0			
18	B1	7.0	39.0		0.0	5.0	7.0	34.0	
	B2	8.0	0.0		2.0	0.0	6.0	0.0	
	B3	4.0	0.0	5.0	1.0	0.0	3.0	0.0	5.0
	B4	25.0	19.0	21.0	7.0	4.0	6.0	18.0	15.0	15.0
	B5	2.0	5.0	15.0	0.0	3.0	8.0	2.0	2.0	7.0
	B6	2.0	0.0	1.0	2.0	0.0	1.0	0.0	0.0	0.0
20	B1	7.0	7.0	3.0	3.0	2.0	0.0	4.0	5.0	3.0
	B2	7.0	0.0	0.0	4.0	0.0	0.0	3.0	0.0	0.0
	B3	4.0	1.0	0.0	1.0	1.0	0.0	3.0	0.0	0.0
	B4	17.0	9.0	4.0	12.0	3.0	2.0	5.0	6.0	2.0
	B5	1.0	1.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0
	B6	0.0	0.0	2.0	0.0	0.0	1.0	0.0	0.0	1.0
22	B1	3.0	9.0	3.0	3.0	9.0	1.0	0.0	0.0	2.0
	B2	3.0	0.0	3.0	1.0	0.0	0.0	2.0	0.0	3.0
	B3	1.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0
	B4	4.0	21.0	4.0	4.0	17.0	1.0	0.0	4.0	3.0

PUPAE PER DIP

	Control	0.5mg/l	1.0mg/l
0	0.06	0.00	0.00

B5	1.0	6.0	0.0	0.0	4.0	0.0	1.0	2.0	0.0	B6	0.0	0.0
			2.0	0.0	0.0	1.0	0.0	0.0	1.0			

Table 12: Average number of pupae exposed to different concentrations of *Bs* formulation in a controlled field experiment (Rainy Season, 2011)

1	1.20			0.00		0.00
2	0.40	0.00	0.00			
3	0.90	0.00	0.00			
4	0.40	0.00	0.00			
5	0.03	0.00	0.00			
6	0.00	0.00	0.00			
7	1.00	0.00	0.00			
8	0.20	0.00	0.00			
9	0.10	0.00	0.00			
10	0.15	0.00	0.00			
12	0.30			0.00		0.07
14	0.00			0.01		0.10
16	0.30			0.07		0.00
18	0.20			0.40		0.00
20	0.10			0.01		0.30
22	0.10			0.00		0.05

Table 13: Average number of pupae exposed to different concentrations of *Bs* formulation in a controlled field experiment (Dry Season, 2011)

	PUPAE PER DIP		
	Control	0.5mg/l	1.0mg/l
0	0.02	0.06	0.00
1	0.2	0.00	0.00
2	0.2	0.00	0.00
3	0.2	0.00	0.00
4	0.5	0.00	0.00
5	0.7	0.00	0.00
6	0.72	0.00	0.00
7	0.71	0.00	0.00
8	0.5	0.00	0.00
9	0.3	0.00	0.00
10	0.05	0.00	0.00
12	0.55	0.30	0.4
14	0.03	0.00	0.01
16	0.43	0.07	0.00
18	0.40	0.02	0.00
20	0.03	0.00	0.05
22	0.1	0.00	0.01

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Table 14: Average pupal numbers per breeding bowl exposed to different concentrations of *Bs* formulation in a controlled field experiment (Rainy Season, 2011)

		RAINY SEASON (JULY)		
Days	Bowls	Control	0.5mg/l	1.0mg/l
0*	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	0	0

					0	0
					0	0
					0	0
					0	0
					0	0
	B5		1			
	B6		0			
1	B1	1				
	B2		0			
	B3		2			
	B4		0		0	0
	B5		3		0	0
	B6		0		0	0
2	B1	1	0	0		
	B2		1		0	0
	B3		0		0	0
	B4		4		0	0
	B5		0		0	0
	B6		0		0	0
3	B1	0	0	0		
	B2		3		0	0
	B3		0		0	0
	B4		1		0	0
						0
						0

					0	0
					0	0
					0	0
					0	0
					0	0
	B5			2	0	0
	B6			0	0	0
4	B1	5	0	0		
	B2			3	0	0
	B3			2	0	0
	B4			0	0	
	B5			3	0	
	B6			2		
5	B1	3				
	B2			4		
	B3			4		
	B4			0		
	B5			5	0	0
	B6			5	0	0
6	B1	3	0	0		
	B2			0	0	0
	B3			7	0	0
	B4			5	0	0
						0
						0

					0	0
					0	0
					0	0
					0	0
					0	0
	B5			4	0	0
	B6			2	0	0
7	B1	7	0	0		
	B2			0	0	0
	B3			4	0	0
	B4			10	0	0
	B5			3	0	0
	B6			0	0	0
8	B1	3	0	0		
	B2			1	0	0
	B3			0	0	0
	B4			0	0	0
	B5			5	0	
	B6			0	0	
9	B1	0				
	B2			1		
	B3			0		
	B4			0		
						0
						0

					0	0
					0	0
					0	0
					0	0
					0	0
	B5			0		
	B6			1	0	0
10	B1	0	0	0		
	B2			7	0	0
	B3			1	0	0
	B4			5	0	0
	B5			2	0	0
	B6			1	0	0
12	B1			0	0	0
	B2			1	0	0
	B3			0	0	0
	B4			0	0	0
	B5			0	0	2
	B6			0	0	0
14	B1			1	0	0
	B2			1	0	1
	B3			6	1	3
	B4			0	0	0
						0
						0

			0	0
			0	0
			0	0
			0	0
			0	0
	B5	2	0	0
	B6	3	0	
16	B1	3	1	
				0
				0

			0	0
			0	0
				0
				0
				0
	B2	1		
	B3	1		
	B4	3	1	
	B5	0	0	
	B6	4	0	
18	B1	1	3	0
	B2	3	3	0
	B3	0	0	0
	B4	0	6	0
	B5	0	0	0
	B6	2	0	0
20	B1	2	1	0
	B2	0	0	0
	B3	0	0	0
	B4	0	0	5
	B5	0	0	3
	B6	1	0	1
22	B1	0	0	0
	B2	0	0	0
	B3	1	0	0
	B4	1	0	0
	B5	0	0	0

	B6	1	0	2

Table 15: Average number of pupae exposed to different concentrations of *Bs* formulation in a controlled field experiment (Dry Season, 2011)

DRY SEASON(NOVEMBER)

Days	Bowls	Control	0.5mg/l	1.0mg/l
0*	B1	0	1	0
	B2	1	0	0
	B3	0	0	0
	B4	0	0	0
	B5	1	0	0
	B6	0	1	0
1	B1	8	0	0
	B2	7	0	0
	B3	9	0	0
	B4	0	0	0
	B5	12	0	0
	B6	0	0	0
2	B1	0	0	0
	B2	4	0	0
	B3	1	0	0
	B4	3	0	0
	B5	2	0	0
	B6	2	0	0
3	B1	4	0	0

			0	0	
			0		
				0	
				0	
				0	
	B2		7	0	0
	B3		6	0	0
	B4		2	0	0
	B5				0
	B6		5		0
4	B1	4	0		
	B2		7		0
	B3		0		0
	B4		0	0	0
	B5		0	0	0
	B6		1	0	0
5	B1	0	0	0	
	B2		0	0	0
	B3		0	0	0
	B4		0	0	0
	B5		1	0	0
	B6		0	0	0
6	B1	0	0	0	
	B2		0	0	0
					0
					0

	B3	0	0	0
	B4	0	0	0
	B5	0	0	0
	B6	0	0	0
7	B1	0	0	0
	B2	12	0	0
	B3	2	0	0
	B4	7	0	
	B5	4	0	

				0	0	
					0	
					0	
					0	
					0	
		B6				0
8		B1	0	0		
		B2			3	0
		B3			0	0
		B4			0	0
		B5			0	0
		B6			4	0
9		B1	0	0	0	
		B2			0	0
		B3			0	0
		B4			0	0
		B5			2	0
		B6			1	0
10		B1	0	0	0	
		B2			3	0
		B3			0	0
		B4			0	0
		B5			0	0
						0
						0

			0	
			0	
			0	
			0	
			0	
	B6	2	0	0
12	B1	1	2	8
	B2	0	0	2
	B3	0	1	2
	B4	3	3	0
	B5	1	2	
	B6	4	1	
14	B1			1
	B2	0		0
	B3	0		0
	B4	0		0
	B5	0		0
	B6	0	0	0
16	B1	2	2	0
	B2	1	0	0
	B3	0	0	0
	B4	2	0	0
	B5	4	0	0
	B6	1	0	0
18	B1	0	8	0

		0	0	
			0	
			0	
			0	
			0	
	B2	4	2	0
	B3	1	0	0
	B4	0	1	0
	B5	0	1	0
	B6	1	0	0
20	B1	1	0	0
	B2	0	0	0
	B3	2	0	1
	B4	0	0	0
	B5	0	0	0
	B6	0	0	
22	B1	0	0	
	B2	1		1
	B3	0		0
	B4	1		0
	B5	0		0
	B6	1		0
				0
				0

0

0

0

0

0