KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI

COLLEGE OF SCIENCES

DEPARTMENT OF THEORITICAL AND APPLIED BIOLOGY

USE OF *Bacillus thuringiensis* var *israelensis* AS A VIABLE OPTION IN THE INTEGRATED VECTOR MANAGEMENT PROGRAMME OF MOSQUITOES IN THE KUMASI METROPOLIS.

BY

RITA NARTEY

(Bsc. Entomology and Wildlife)

JUNE, 2012

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A DEGREE OF MASTER OF PHILOSOPHY IN THE DEPARTMENT OF THEORITICAL AND APPLIED BIOLOGY, COLLEGE OF SCIENCE

BY

RITA NARTEY

JUNE, 2012

DECLARATION

I hereby declare that the experimental work described in this thesis is my own work towards the M.phil and to the best of my knowledge; it contains no material previously published by another person or material which has been submitted for any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to my family for their prayers, love and support.

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To God be the glory great things He has done. I acknowledge the sufficient grace of God that has sustained me through the difficult moments encountered during the course of this work.

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ABBREVIATION/ACRONYMS

Bs: *Bacillus sphaericus*

Bti: *Bacillus thuringiensis israelensis*

DDT: Dichloro Diphenyl-Trichloroethane

F1: First filial generation

GMT: Greenwich Meridian Time

GNA: Ghana News Agency

ITU: International Toxic Units

IVM: Integrated Vector Management

KCCR: Kumasi Center for Collaborative Research into Tropical Medicine

KMA: Kumasi Metropolitan Assembly

KNUST: Kwame Nkrumah University of Science & Technology

LC: Lethal Concentration

MoH: Ministry of Health

OPD: Out Patient Department

STD: Standardised Field Test

USA: United States America

UTM: Universal Transverse Mercator

WDG: Water-dispersible granular formulations

WG: Water Granular

WHO: World Health Organisation

ABSTRACT

Extensive use of chemical insecticides against vector mosquitoes in the control of malaria and other mosquito borne diseases have resulted in the development of resistance in vector mosquitoes and hazards to the environment. In addition to bed nets, residual indoor spraying and appropriate diagnosis and treatment of malaria parasites, use of microbial larvicide, *Bacillus thuringiensis* var *israelensis* (*Bti*) could be an effective and environmentally safe tool to combat malaria. The study investigated the contribution of *Bacillus thuringiensis* var *israeliensis* in the control of malaria by targeting the larvae, and also to map and document major breeding sites of mosquitoes in the Kumasi metropolis. Using a hand held GPS receiver unit, all breeding sites within the metropolis were mapped out during the larval survey. Mosquito larvae were also collected from the breeding sites of mosquitoes and reared in an insectory to obtain F1 generation for laboratory bioassays. The minimum effective dosage of *Bti* Water dispersable granular formulation (WDG) was determined by a series of bioassays. Based on the results obtained in the laboratory, the optimum effective dosage of *Bti* formulations against naturally occurring larvae of the indigenous mosquito species was determined through standardised field trials. A total of 33 breeding sites were identified and geo-referenced during the larval surveys with majority of the breeding sites located in the Asokwa sub-metropolis. A *Bti* (3,000 International Toxic Units (ITU)/mg) concentration of 0.026 mg/l resulted in 50% mortality whiles a concentration of 0.136mg/l resulted in 95% mortality. Results from the open field trials with *Bti* showed that a dosage of 0.2mg/l is as effective as 0.4mg/l in suppressing late instars and resulting pupae. This study reveals that *Bti* at a very low dosage of 0.2 mg/l is highly effective against mosquito larvae and therefore offer viable options for the management of vector mosquitoes. The study also presents the possibility of using GIS tools for health research, in Ghana and Kumasi in particular, where GIS application in the health sector has not been exploited extensively.

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CHAPTER ONE

INTRODUCTION

BACKGROUND

Malaria is an urgent threat to global health (WHO, 2005). Malaria-related death rates have doubled over the last thirty years and nearly 1.3 million people, mostly children under the age of five die from malaria every year (WHO, 2005). Over forty percent of the world's population is at risk of becoming infected and in many of the poorest tropical and subtropical regions of Africa, Asia, the Middle East, and Central and South America, malaria causes more than 500 million cases of clinical disease annually (WHO, Malaria Fact Sheet, 2007). Estimates showed that nearly 60% of clinical malaria and over 90% of deaths, around 1million (ranging deaths, from 700,000 to 1.3 million) is attributable to malaria and occur in Africa South of the Sahara (WHO/AFO, 2006). In general, it is estimated that malaria accounts for an average of 3% of the total global disease burden as a single disease in 1990 and in sub-Saharan Africa, 10.8% of all Disability– Adjusted Live Years (DALYs) were lost to malaria in the same year (Ankomah and Asenso-Okyere, 2003).

Malaria is hyper endemic in Ghana, with a crude parasite rate ranging from 10 – 70% with *Plasmodium falciparum* dominating (Ankomah and Asenso-Okyere, 2003). It is the number one cause of morbidity accounting for over 40 % of outpatient attendance in public health facilities in Ghana, with annual reported cases of about 2.2 million between 1995 and 2001, and with over 10 % ending up on admission (Ankomah and Asenso-Okyere, 2003).

In a report by UNDP, it is stated that malaria is a major killer in Ghana (UNDP, 2000). The disease accounts for an average of 13.2% of all mortality cases in Ghana and 22% of all

mortalities in children under 5 years (Antwi and Marfo, 1998). In pregnant women, out of the total number reporting at the health institutions, 13.8% suffer from malaria and 9.4% of all deaths in pregnant women is as a result of malaria (Antwi and Marfo, 1998). It is estimated that malaria prevalence (notified cases) is 15, 344 per 100, 000 with malaria death rate for all ages being 70 per 100, 000. In the case of the 0 – 4 years, it is 448 per 100, 000 reported for the year 2000 (United Nations, 2003).

The favourable climatic condition for the breeding of mosquitoes, poor environmental sanitation, weak health system, poor strategies development and inadequate funding for control programmes, all play their part in perpetuating the high incidence of the mortality of malaria in developing countries including Ghana (WHO 1998). According to Ahmed (1998), malaria is endemic throughout Ghana and distribution follows distinct climatic and ecological zones. The seasonal pattern of malaria differs throughout the year in various parts of the country as a result of the rainfall pattern, with two seasons in the coastal and forest ecological zones, as compared to one in the northern savanna (Ahmed, 1998).

According to the World Health Organisation (WHO) report on Malaria in Ghana, US$ 27 million and US$ 38 million was spent on Malaria in 2008 and 2009 respectively (WHO 2008). In spite of the huge amount of money invested, Ghana still recorded very worrying figures with 3,694,671 cases in 2009 compared to 3,200,147 in 2008 (WHO 2008). Admission to hospitals due to malaria also went up from 272,802 in 2008 to 277,047 in 2009 (WHO 2008).

Death due to malaria from the records of clinics and hospitals in Ghana stood at 3,378 in 2009 (WHO 2008). The disease has inflicted tattered penury on the already poor. It is against this backdrop that the fight against malaria should be treated with precision and a sense of urgency.

Despite the increased funds made available to fight malaria, millions of people are still reeling under the threat of the disease.

In 2006, the Global Fund alone distributed about 18 million insecticide treated bed nets and reached 5.3 million patients with artemisinin- based therapies (ACTs), (The Global Fund, 2008). The challenges of malaria are many and complex and addressing some but not others is an act of futility because the remaining constraints will exert a leveling effect so powerful that whatever gains have been made would eventually dissipate. The Roll Back Malaria Programme of the World Health Organisation has implemented measures aimed at controlling the incidence of malaria. The measures have included enhanced use of insecticides treated bed nets, indoor residual spraying and improved case management (The Global Fund, 2008).

The most unfortunate development in the fight against malaria and its associated morbidity and mortality is the emergence of resistance to both pesticides and anti-malaria drugs (Etang and Fondjo, 2006). Biological control is however a successful but much neglected approach (Fillinger *et al.*, 2003). This involves steps in decimating malaria parasite right from the breeding ground. The most effective and environmentally sound vector control programs are usually based on a contribution of methods including source reduction, chemical and biological control.

A combination of these techniques into one thoughtful ecologically valid programme is referred to as Integrated Vector Management Program (IVMP). The standard chemical, biological and physical control used to kill mosquitoes and other insect vectors, as well as active and passive case-detection and treatment of human infection, have a long and proven track-record of saving lives.

However, the potential benefits of integrating vector control strategies into national health and community systems have not been fully realised, especially in sub-Saharan Africa. Integrated Vector Management (IVM) is not a new concept and the basic principles of IVM have been used over the past century in the USA for mosquito control (Beier, 1998). It is guided by some basic principles which are, to effectively reduce adult mosquito vector populations and transmission; that control strategies should not create adverse effects such as environmental contamination, or the development of resistance, nor should they have a negative impact on non-target organisms, including beneficial insects, humans, domestic animals and wildlife and that interventions should be ecologically acceptable (Beier, 1998). These measures are vital to the realisation of effective malaria control which is integral to the attainment of the economic, social and health-care goals of Ghana.

Reductions of vector- mosquito population by means of larval biological control are gaining prominence as a viable alternative to the use of chemical insecticides in malaria control programmes. *Bacillus thuringiensis israeliensis* (*Bti*), a microbial control agent is known to effectively control the larval stages of mosquitoes and has been used successfully in Integrated Vector control programmes.

Excellent results in suppressing mosquito populations have been obtained by the application of microbial control agents in Europe, Australia and the Americas without harming the environment (Becker, 2003). Larval control using *Bti* has major advantages over adult control.

In addition to the relative ease with which *Bti* can be mass-produced, this bacterial control agent is highly efficient, environmentally safe, easy to handle, stable when stored, cost effective, and suitable for Integrated Control Programmes based on community participation.

Furthermore, the cost for development and registration of *Bti* (about $500,000) are many times lower than those for a conventional chemical insecticide (about $20,000,000) (Becker, 2003). The risk of resistance when *Bti* is used is much lower than when conventional insecticides are used (Wirth, 2010; Walker, 2003). Integrated biological control of mosquito larvae by means of microbial control agents and alteration of breeding sites, with the reduced need for chemical insecticide application, will result in considerable public health and economic benefit.

1.1 RATIONALE OF STUDY

The distribution pattern of malaria in Ghana follows ecological zones, incidence being highest in the forest zone, followed by the coastal zone and then the northern savannah (Afari *et al*., 1995). The Ashanti region, which falls within the forest zones of Ghana, in the year 2000, was reported as the region with the greatest number of reported cases of malaria and the highest prevalence of malarial parasitaemia (Browne *et al*., 2000).

A survey conducted in Kumasi between April and May 2005, found *Plasmodium falciparum* prevalence rate of 37.8% among 296 children in 184 households from Moshie Zongo a suburb of Kumasi (Ronald *et al*., 2006). It is worth emphasizing that, due to its central location all road networks linking the northern sector and the southern sector of Ghana pass through Kumasi, resulting in a high daily influx of traders and civil workers. The complex mix of people also serves as a likely malaria threat to inhabitants since migration has been tagged as a probable precipitating cause of the most serious malaria problems in Africa (WHO, 1996).

Extensive use of chemical insecticides against vector mosquitoes, for the control of malaria and other mosquito borne diseases, for about four decades, have caused development of resistance in vector mosquitoes to these insecticides and hazards to the environment (Mittal, 2003). In spite of

the sustained and prolonged use of chemical insecticides, these diseases are not only still prevalent but also cause epidemics (Mittal, 2003). Research undertaken to reduce malaria incidence in Ghana and Kumasi in particular, have over the years focused only on the adult vectors. Therefore, to minimise the dependency on chemical insecticides, there is an urgent need to explore alternative measures for the control of vector mosquitoes. To date, no study on the contribution of *Bacillus thuringiensis* var *israelensis* has been carried out in the Kumasi metropolis despite the importance of its inclusion in malaria control programmes.

The advantages of *Bti* in comparison to chemical control is its effectiveness at relatively low doses, safety to humans and non-target wildlife, low cost of production in some cases and lower risk of resistance development (Yap, 1985). Furthermore, the larvae are concentrated in predictable sites that can be easily accessed, treated or manipulated with no chance of larvae escaping. It is the first study to be carried out on *Bacillus thuringiensis* var *israelensis (Bti)* in Kumasi. It focuses on the contribution of microbial agents in Integrated Vector Control Programmes with emphasis on *Bti* and exploits the application of Geographical Information Systems (GIS) Technology in mapping breeding areas. The result of this work should serve as reference on malaria risk areas in Kumasi and a guideline for future research on the contribution of microbial agents and the importance of its inclusion in the implementation of future malaria control programmes.

1.2 GENERAL OBJECTIVE

The main objective of this study is to investigate the contribution of *Bacillus thuringiensis* var *israliensis*, a microbial larvicide, in the control of malaria targeted at the mosquito larvae.

1.3 SPECIFIC OBJECTIVES

- To identify and map out malaria vector breeding sites in the Kumasi metropolis using geographic information systems (GIS) and global positioning systems (GPS).
- To collect and identify *Anopheles* larvae
- To breed *Anopheles* mosquito larvae in insectory in order to obtain F1 generation 3rd and 4th instar larvae for laboratory assays
- To determine the minimum effective dosage of *Bti* formulations through a series of laboratory assays
- To determine the optimum effective dosage for field application against mosquito larvae in their natural breeding site based on the results obtained in the laboratory
- To evaluate the effectiveness of the use of *Bti* as a biolarvicide in the biological control of vector-mosquito population

CHAPTER TWO

LITERATURE REVIEW

2.1 MALARIA

Malaria is a life threatening disease caused by parasites that are transmitted to people through the bites of infected mosquitoes. A child dies of malaria every 30 seconds. There were 247 million cases of malaria in 2006, causing nearly one million deaths, mostly among African children. Malaria is preventable and curable. Approximately half of the world's population is at risk of malaria, particularly those living in lower income countries (WHO 1999).

2.1.1 Prevalence of malaria

Malaria is among one of the oldest diseases of mankind, with human-adapted species appearing to have evolved along with us (Bourgon *et al.,* 2004). In past years, seasonal fevers have been associated with people living close to marshy areas, hence the name malaria, meaning 'bad air' (Coluzzi and Corbellini, 1995). Human malaria, a parasitic disease transmitted mainly by bites of infected female mosquitoes of the genus *Anopheles*, is one of the world's most serious diseases (White, 2003). An estimate by the World Health Organisation showed that in Africa, malaria mortality in young children almost doubled from the 1980s to the 1990s (WHO, 2003). Malaria causes about 3000 deaths each day and imposes huge losses in economic productivity (Sachs and Malaney, 2002). Ten to thirty thousand clinical cases of malaria are diagnosed throughout the world each year among travelers from non-endemic countries to endemic countries, resulting in outbreaks, including some deaths, and the risk of establishing malaria endemicity in new areas (Kain and Keystone, 1998).

2.1.2 Malaria Risk Area

About half the world's population (3.3 billion) live in areas that have some risk of malaria transmission and one fifth (1.2 billion) live in areas with a high risk of malaria (more than 1 reported case per 1000 population per year (WHO, 2008). Although low-risk areas cover a large number of people living across a wide geographical area, they produce a relatively small number of malaria cases each year (less than 2 million) and account for less than 3% of cases reported by countries in 2006 (WHO, 2008). The largest populations at any risk of malaria are found in the South-East Asia and Western Pacific regions. Africa has the largest number of people living in areas with a high risk of malaria, followed by the South-East Asia region (WHO, 2008). There were an estimated 247 million malaria cases worldwide in 2006, of which 91% or 230 million were due to *Plasmodium falciparum* (WHO, 2008). The vast majority of cases (86%) were in the African Region, followed by the South-East Asia (9%) and Eastern Mediterranean regions (3%) (WHO, 2008). Figure 1 shows countries with endemic malaria in 2008.

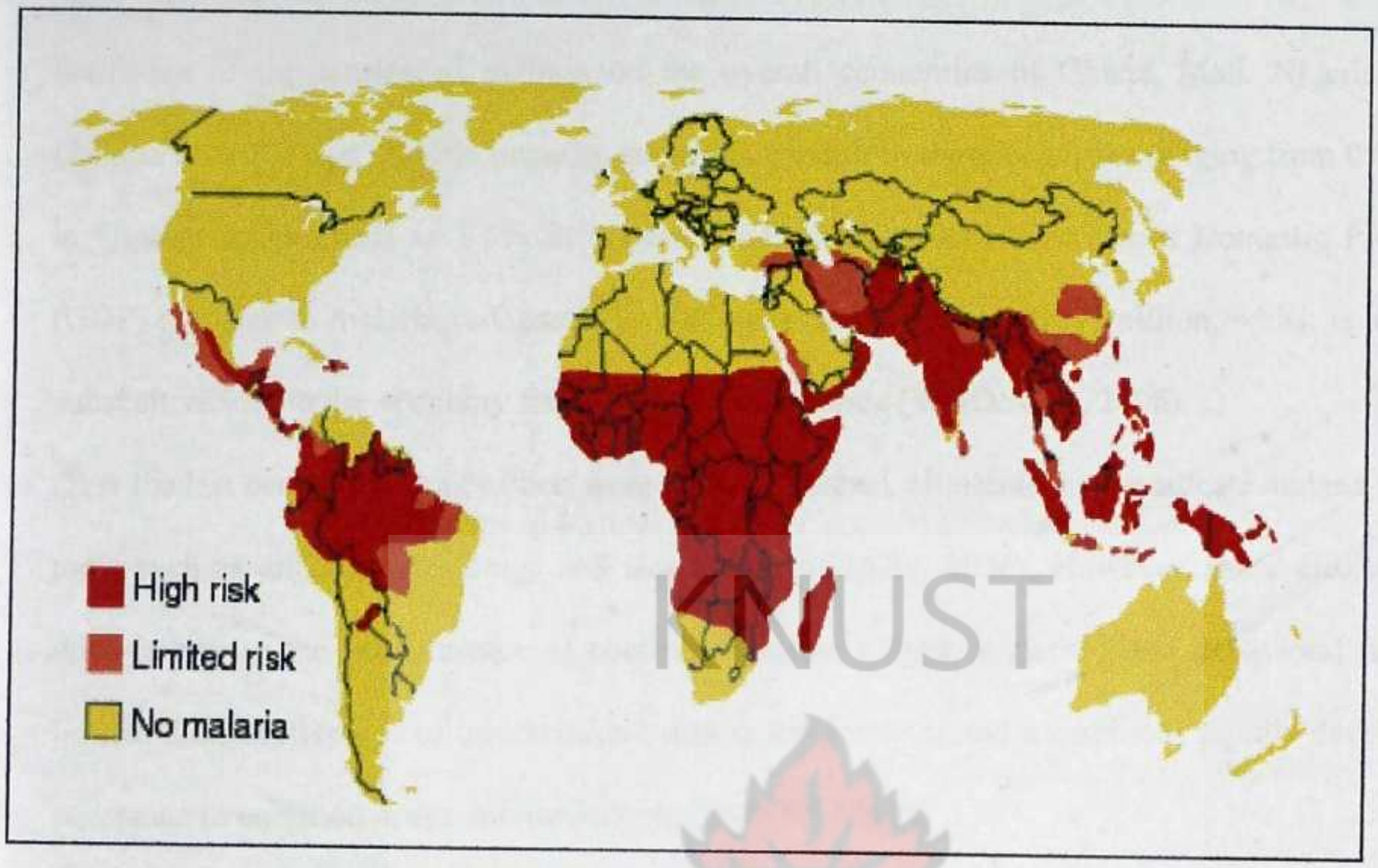


Figure 1: Malaria endemic countries, 2008

Source: http://www.traveldoctor.info/diseases/html. Accessed on 24th October, 2010)

The economic impact of malaria includes costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (Greenwood *et al.*, 2005). In some countries with heavy malaria burden, the disease may account for as much as 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits (WHO/AFO, 2006).

Recent studies showed that, the direct cost of a single episode of malaria to a household was US$ 6.87 in Ghana, US$ 4.8 in Uganda and US$ 4.5 in Mali. In Nigeria, it costs about US$ 1 to treat a malaria episode by self medication, and about US$ 10 to treat it by the use of orthodox health care provider when admission is not involved (WHO/AFO, 2006).

Estimates of the burden of malaria on the overall economies of Ghana, Mali, Nigeria, and Uganda revealed that malaria impedes economic growth in these countries ranging from 0.067% in Uganda to as much as 3.8% in Nigeria (WHO/AFO 2006). The Gross Domestic Product (GDP) loss due to malaria in Uganda in 2003 was equivalent to US$11million, which is a very substantive loss to the economy for a country like Uganda (WHO/AFO, 2006).

Over the last century, valiant efforts were made to control, eliminate, and eradicate malaria using tools such as anti malarial drugs and insecticides (NIAID, 2008). However, these efforts met with failure as the maintenance of control programs waned; logistical and behavioral factors limited the effective use of interventions, and as the parasites and mosquitoes rapidly developed resistance to common drugs and insecticides (NIAID, 2008).

2.1.3 Development of Malaria Vaccine

Vaccines for malaria are under development, with no completely effective vaccine yet available. The first promising studies demonstrating the potential for a malaria vaccine were performed in 1967 by immunising mice with live, radiation-attenuated sporozoites, providing protection to about 60% of the mice upon subsequent injection with normal, viable sporozoites (Nussenzweig *et al.*, 1967). Since the 1970s, there has been a considerable effort to develop similar vaccination strategies within humans (Hoffman *et al.*, 2002). It was determined that an individual can be protected from a *Plasmodium falciparum* infection if they receive over 1000 bites from infected, irradiated mosquitoes (Hoffman *et al.*, 2002). An effective malaria vaccine would be a huge step forward in preventing malaria cases worldwide (Postnote, 2007).

However, due to the difficulty in identifying appropriate vaccine targets and a lack of understanding about the types of immune response involved in protection, only one vaccine (developed by GlaxoSmithKline) has made significant progress to date. This vaccine (RTS,

S/AS02A) is currently in mid-late stage clinical trials (Postnote, 2007). It reduces the risk of clinical disease by 35% and has 50% efficacy against severe malaria in children under four (Postnote, 2007). However it would not be a substitute for other control methods and would need to be deployed in combination with other preventative measures (Postnote, 2007). Another area of ongoing research involves genetically modifying mosquitoes so that they are unable to transmit malaria (NAS, 2007).

2.2 ENTOMOLOGICAL STUDIES

2.2.1 Studies on mosquitoe larvae

The larval aquatic habitat is an important part of the mosquito life cycle and strongly influences the distribution and abundance of malaria vectors. Although a range of mosquito habitats exist, the larval stage is mainly confined to stagnant water pools (Pfaehler *et al.*, 2006). The proximity of houses to locations with suitable topography for mosquito breeding may be an important determinant of malaria risk (Carter *et al.*, 2000). *Anopheles* breeding sites may occur where water collects and forms a pool for a period of time sufficient to permit larval development and adult emergence (Minakawa *et al.*, 1999). It is believed that topography has an effect on breeding selection, since small temporary pools and larger more permanent aquatic habitats are more likely to exist in flat, relatively low-lying regions (Carter *et al.*, 2000). Variable characteristics of *Anopheles* larval habitats have been identified; a common characteristic is a shallow larval habitat with the presence of algae, although such a correlation is not systematic (Gimnig *et al.*, 2002).

Other habitats identified consist of animal hoof or human foot prints, or small ponds of still temporary water created by irrigation projects or rainfall. However the characteristics of the larval habitat that are adequate for a given species are still unclear (Pfaehler *et al*, 2006).

In a study by Matthys *et al.*, (2006) on urban agricultural land use and characterisation of mosquito larval habitats in a medium-sized town of Côte d'Ivoire, it assessed risk factors for productive *Anopheles* breeding sites. Typical *Anopheles* larval habitats were characterised by the presence of algae, and the absence of floating vegetation.

The highest *Anopheles* larval productivity was observed in rice paddies, agricultural trenches between vegetable patches, and irrigation wells. The study established an indirect link between the occurrence of productive *Anopheles* breeding sites and agricultural land use through specific man-made habitats, in particular agricultural trenches, irrigation wells, and rice paddies. A study on regulatory factors affecting larval mosquito populations in container and pool habitats by Washburn (1995) revealed that physical and biological features have significant implications for successful implementation of biological control agents. Knowledge gained from larval studies has important implications in developing control strategies.

2.2.2 Biological profile of *Bti*

For the past six decades, humans have been almost completely dependent upon synthetic organic insecticides for agriculture, forestry and vector control purposes (Kunz and Kemp, 1994). However, the properties that made these chemicals useful long residual action and toxicity for a wide spectrum of organisms have brought about serious environmental problems and many concerns in the population (Kunz and Kemp, 1994).

The emergence and spread of insecticide resistance in many species of vectors, the concern with environmental pollution, and the high cost of the new chemical insecticides made it apparent that insect pest control could no longer be safely dependent upon the utilisation of chemicals (Mario, 2005). Different formulations of *Bti* can be used to control larvae in these various breeding sites.

The type of formulations and dosages must be adjusted to the types of sites encountered (Mario, 2005).

2.2.3 Spectrum of Activity of *Bti*

Since its discovery, *Bti* has been found to be toxic for practically all filter-feeding mosquito and black fly larvae tested. References have been reviewed by Lacey *et al* (1984) for mosquitoes and Molloy (1990) and MacFarlane (1992) for black flies. *Bti* proved to be effective against at least 72 species of mosquitoes from 11 different genera *Anopheles, Aedes, Culex, Culiseta, Limatus, Uranotaenia, Psorophora, Mansonia, Armigeres, Trichoprospon* and *Coquillettidia* (Margalit and Dean, 1985). Toxicity of Bti was also demonstrated for at least 22 species of black fly larvae from 7 different genera *Simulium, Cnephia, Prosimulium, Austrosimulium, Eusimulium, Odogmia* and *Stegoptera* (Margalit and Dean, 1985). Insects most susceptible to *Bti* crystals are mainly in genera within the same family, presumably with a common ancestor (MacFarlane, 1992).

The spectrum of activity of *Bti* is mostly restricted to the members of the suborder Nematocera within the order *Diptera* (MacFarlane, 1992). However, the greatest degrees of susceptibility are found within a few families the *Culicidae* (mosquitoes), the *Simuliidae* (black flies) and the *Chironomidae* (midges) with mosquitoes and black flies being the most susceptible (MacFarlane, 1992).

2.2.4 Studies on the safety of *Bti* to the environment

In United States and Canada, bio-pesticides must be registered before their utilisation. In both countries, criteria for the acceptance of the products are very stringent (infectivity, pathogenicity, toxicity, laboratory and field experiments) and when a product is finally accepted, one can be

sure that the product is safe for the humans and the environment and that it has proven its efficacy toward the target insects (Mario, 2005). In Canada, *Bti* has undergone a full health impact assessment which shows that it poses no risk to mammals, including humans (Mario, 2005). Based on the lack of human health risk and long history of safe use associated with *Bti*, the Canadian Pest Management Regulatory Agency (PMRA) has no human health and safety concerns with the application of registered products containing *Bti* to bodies of water that will be used for human consumption (PMRA, 2001).

In 1999, conclusions and recommendations of a task group on environmental health gathering people from the United Nations Environment Programmes, the International Labour Organisation and the World Health Organisation stated that *Bti* is safe for use in aquatic environments including drinking-water reservoirs for the control of mosquito, black fly and nuisance insect larvae (WHO, 1999).

The United States Environmental Protection Agency (USEPA) categorises the risk posed by *Bti* strains to non-target organisms as minimal to non-existent. So, the weight of scientific evidence indicates that *Bti* is non-infectious and non-toxic to humans and other mammals and poses little risk at dosage levels permitted in insect control programs (PMRA, 2001).

2.3 ROLE OF GEOGRAPHICAL INFORMATION SYSTEM IN MALARIA CONTROL

Geographic information systems (GIS), global positioning systems (GPS) and remote sensing have been widely applied in public health settings since the 1990s (Kaiser *et al.*, 2003). It is a useful tool for malaria research and control in most Sub-Saharan African countries (Hightower *et al.*, 1998).

The Spatial modelling capacity offered by GIS is directly applicable to understanding the spatial variation of the disease, and its relationship to environmental factors and the health care system (Tanser and le Sueur, 2002) and may also help to identify high-risk diseases areas, sources of diseases and high-risk populations (Gatrell and Bailey, 1996).

Environmental factors such as topography, temperature, rainfall, land use, population movements, and extent of deforestation are believed to have a profound influence on the temporal and spatial distribution of malaria vectors and malaria (Brêtas, 1996).

GIS can be used to investigate associations between such environmental variables and the distribution of the different species responsible for malaria transmission (Sweeney, 1998). For example, during the last 15 years entomological teams from the Australian Army Malaria Institute, operating from vehicles and helicopters, have collected more than 30,000 *Anopheles* specimens from over 1,000 localities in northern Australia and Papua New Guinea (Sweeney, 1998). These statistical techniques are based on case events and count data, where known geographic locations (x-y coordinates) of disease cases are commonly represented as points (Lawson and Denison, 2002).

Despite its importance, the study of environmental determinants of malaria have been hindered by the difficulties related to collecting and analysing environmental data over large areas, and to the rate of change in the malaria spatial-epidemiological situation (Brêtas, 1996).

GIS has great potential in health care systems of Africa since health is largely determined by environmental factors (including the socio-cultural and physical environment) which vary greatly in ~~space~~ (Tanser and le Sueur, 2002).

2.3.1 Use of GIS for Malarial Research

GIS holds considerable promise for health research and development in Africa (Kaiser *et al.*, 2003). The 'mapping malaria risk in Africa' (MARA) research collaboration is an African research endeavour that makes extensive use of GIS technology. MARA has been highly successful in collating malaria data from around the continent (Tanser and le Sueur, 2002). The ability to map spatial and temporal variation in disease risk areas is critical, given the ever-increasing disease burden especially of malaria in Africa (MARA, 1998). MARA employs the use of GIS and other remote sensing techniques to establish a continental data base of the spatial distribution of the disease (MARA, 1998).

GIS also allows the planning of control strategies and the delivering of interventions where the need is greatest and sustainable success is most likely, for example, in four districts of Kenya, GIS is being used to capture and model both population's access and utilisation of health services with a view to increase the effectiveness of malaria treatment coverage (Kaiser *et al.*, 2003).

GIS presents wide-ranging possibilities over which it can contribute to malaria control programmes therefore the use of GIS as a malaria research tool is a viable and relevant objective (Sweeney, 1998). Application of GIS as an operational planning aid is an extension of geographical reconnaissance to promote better programme management. Its use as an evaluation tool provides an additional means of spatially analysing outputs generated by health information systems in graphic visual formats which can be readily understood by field workers and programme managers (Sweeney, 1998). The use of GIS as a malaria research tool is a worthy objective of academic research institutions at national or international levels, however, the

application of GIS must be commensurate with the existing infrastructure within malarious countries (Sweeney, 1998)..

2.4 BIOLOGY OF THE MOSQUITO

Mosquitoes are insects with long slender bodies, narrow wings with a fringe of scales on the hind margins and along the veins, and long, very, thin legs. In females, the elongated proboscis is firm and usually adopted for piercing and sucking blood, the males do not suck blood, but both sexes feed on nectar of various plants (FDACS, 1994).

There are four life stages: Egg, Larva, Pupa and winged adult. Eggs may be laid singly or in rafts, deposited in water, on the side of containers where water will soon cover them, or on damp soils where they must undergo a maturing process before they can hatch when flooded by rainfall or high tides. After the tiny eggs hatch, the larvae (commonly called wigglers) begin to feed on very small plant and animal particles, going through four growth stages (called instars) before becoming pupae (FDACS, 1994).

Most larvae except in the genera *Mansonia* and *Coquillettidia* must breathe at the surface of the water. The two named genera have a sharp pointed siphon with which to pierce the roots and stems of aquatic plants and get their oxygen from the plants. The pupal stage of the mosquito is comparatively brief. The pupa does not feed and is active generally only if disturbed. When it has matured, the pupa remains on the surface, the chitinous pupal skin splits, the emerging young one briefly dries its wings and flies away. Only female mosquitoes bite, using blood protein for the development of their eggs. The flight range of mosquitoes varies greatly, from several hundred feet in some species to more than 20 miles in others (excerpted from Public Health Pest Control Applicator Training Manual, FDACS, 1994).

2.4.1 The Malaria Vector

Diversity among the mosquito vectors that harbor and transmit malaria to humans contributes significantly to the complexity of disease control (NIAID, 2008). The *Anopheles* genus encompasses approximately 400 species distributed across all continents except Antarctica; 30-50 of those species have been identified as vectors of the *Plasmodium* parasite species that cause human malarial disease (Bruce-Chwatt, 1985).

Several physiological, behavioral, and ecological characteristics determine the effectiveness of various *Anopheles* species as malaria vectors including: susceptibility to *Plasmodium* parasite infection, geographic distribution, preference for feeding on humans versus animals, tendency to enter houses, tendency to feed or rest indoors versus outdoors, ability of populations to rebound following dry seasons, longevity of individual mosquitoes, and susceptibility to climatic factors and control measures such as insecticides (NIAID, 2008). Malaria transmission is driven by the mosquito vector system, which in most of Sub-Saharan Africa consists of three primary species, namely *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* (Cohuet *et al.*, 2004).

Figure 2: The Anopheline mosquitoe (Source: CDC, 2008)

2.5 THE MALARIA PARASITE AND THE DISEASE

In humans, malaria is caused by four species of the plasmodium protozoa (single celled parasites) – *P. falciparum, P. vivax, P. ovale* and *P. malariae*. Of these species *P. falciparum* accounts for the majority of infections and is the most lethal (CDC, 2008).

The parasite in the mosquitoe host (Vector phase)

Anopheline mosquitoes feeding on infected hosts (Figure 2) ingest sexual forms developing in red blood cells. The female macrogametocytes and male microgametocytes mature in the mosquito's stomach and combine forming a zygote that undergoes mitosis (Bruce-Chwatt, 1985).

The products of mitosis are ookinetes, which force themselves between the epithelial cells to the outer surface of the stomach, and form small spheres called oocysts. The oocysts enlarge as the nucleus divides, eventually rupturing and releasing thousands of motile sporozoites into the body cavity. The sporozoites migrate to the salivary glands, making the female mosquito infective (Bruce-Chwatt, 1985). The vector phase of the life cycle, (Plate 1) called sporogony, is complete in 8 to 35 days depending on species and environmental conditions (Gilles, 1993).

The parasite in the vertebrate host

Sporozoites which are uninucleate and approximately 11 microns long circulate in the blood stream for a short time before entering hepatocytes (Parry *et al.,* 2004). Here, they replicate rapidly by asexual division before the bursting out of the hepatocytes, releasing merozoites into the bloodstream. The length of this hepatic phase varies with species and in *Plasmodium falciparum* it is typically about 6 days, by which time the single sporozoite has divided to form a multinucleate schizont with up to 30 000 daughter merozoites packing the hepatocytes (Parry *et*

al., 2004). A proportion of the sporozoites of *Plasmodium vivax* and *Plasmodium ovale* may not develop immediately into hepatic schizonts but enter into a dormant phase, known as hypnozoite which may go on to form schizonts at intervals many months later (Parry *et al.*, 2004).

These later infections are known as relapses. *Plasmodium falciparum* and *Plasmodium malariae* do not have a dormant liver stage, though if not adequately treated, the blood stage may persist at undetectable levels for long period. The subsequent reappearance of parasites in the blood is known as recrudescence (Parry *et al.*, 2004).

The Erythrocyte (Blood) Stage

Merozoites released from the hepatic schizonts have only a short life before being either cleared or enter host cells. Once inside the red cell, the parasite again undergoes a process of asexual division to form multinucleate schizonts, which then bursts, releasing daughter merozoites which attach to and enter new red cells and so repeat the cycle (Parry *et al.*, 2004).

The whole red cell cycle takes around 48 hours in *Plasmodium falciparum* (Parry *et al.*, 2004). These repeated cycles of asexual division lead to rapid exponential growth of the numbers of infected red cells and it is during this period that the characteristics symptoms of malaria appear (Aravind *et al.*, 2003).

The mosquito becomes infectious to its next blood meal donor approximately two weeks after ingesting gametocytes, a time frame that is influenced by the external temperature (Aravind *et al.*, 2003). Development of *Plasmodium vivax* within the mosquito can occur at a lower environmental temperature than that required for the development of *Plasmodium falciparum*, explaining the preponderance of *Plasmodium vivax* infections outside tropical and subtropical regions (Aravind *et al.*, 2003).

The time from infection to the appearances of parasites detectable on a blood film is known as the pre patent period and is 9 -10 days for *Plasmodium falciparum* (Brian *et al.*, 2008). The time from infection to reappearance of symptoms is the incubation period, which is typically around 12 days (Brian *et al.*, 2008). Red cells containing older parasites undergo specific attachment to the endothelial lining of the small blood vessels, a process known as cytocoherence (Brian *et al.*, 2008).

This withdrawal of mature forms of the parasite in peripheral circulation is known as sequestration (Brian *et al.*, 2008). This is characteristic of *Plasmodium falciparum* and does not occur to any extent with the other malaria parasites that infect humans (Brian *et al.*, 2008).

At some part in the erythrocyte cycle a proportion of the merozoites entering red cells follow a different developmental path and rather than dividing to form yet another schizont, develop instead into the sexual stage of the parasite, forming either a male or female gametocyte (Parry *et al.*, 2004).

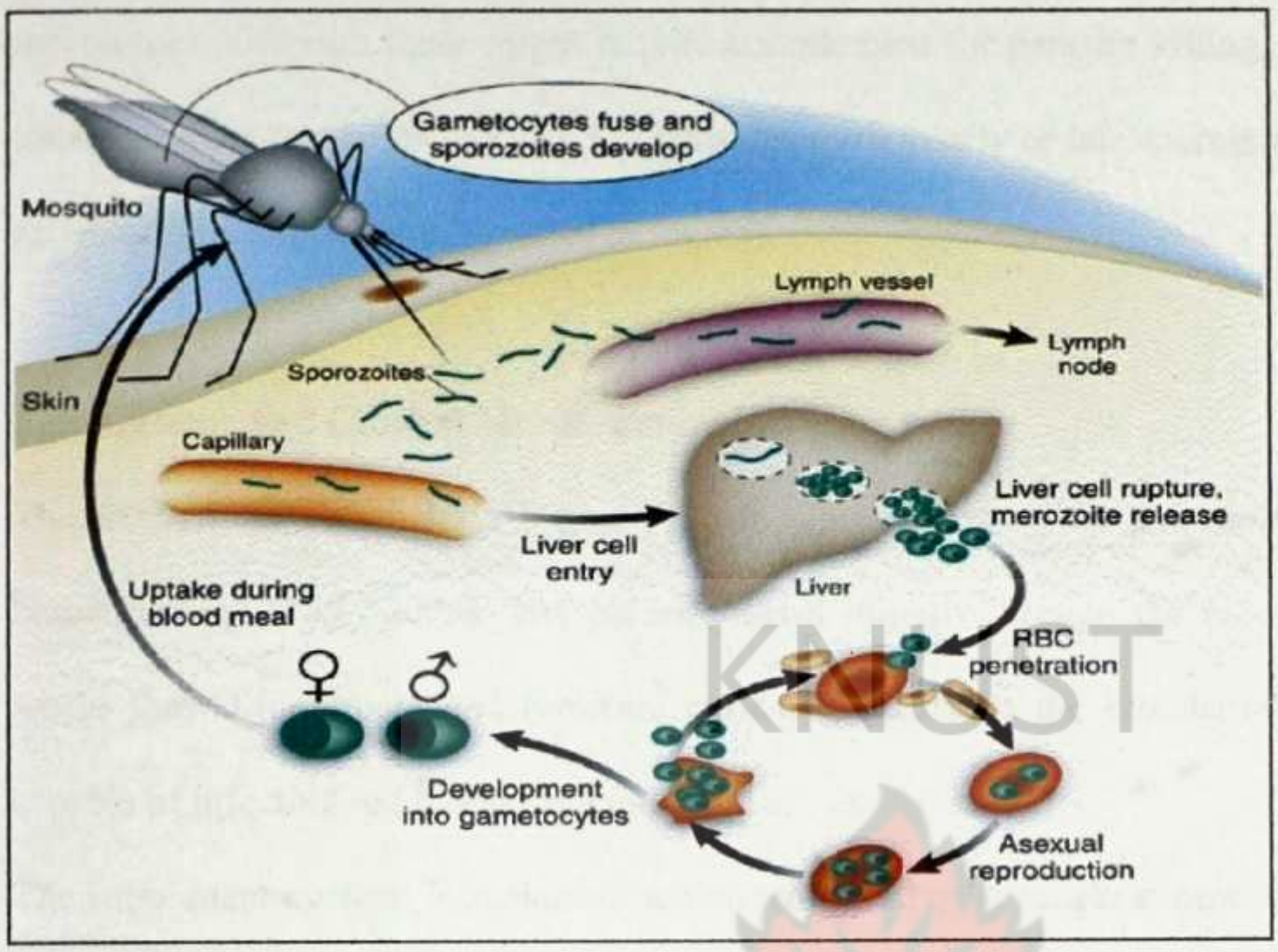


Plate 1: Cycle of development of malaria parasites in the *Anopheles* vector and in the human host

Source: Sorphorn, 2010.

Upon inoculation by an *Anopheles* mosquito into the human dermis, elongated motile sporozoites must evade antibodies to access blood vessels in the skin and then transit through liver macrophages and hepatocytes to initiate liver stage infection (Snow *et al.*, 2005). Intra hepatocytic parasites are susceptible to cytotoxic T lymphocytes (Snow *et al.*, 2005).

After approximately one week, infected hepatocytes rupture and release merozoites as aggregates called merosomes that might allow merozoites to evade antibodies and invade erythrocytes. Intraerythrocytic parasites are susceptible to opsonising antibodies and macrophages, and cytokine responses have been related to both protection and disease during this stage of infection (Snow *et al.*, 2005).

Antibodies that block binding of *Plasmodium falciparum* infected erythrocytes to endothelium might prevent disease and control parasitaemia. Human antibodies specific for sexual stage parasites are taken up by mosquitoes during the blood meal and can block transmission to

mosquitoes, although these might require complement for parasite killing. *Anopheles* mosquito innate immune responses can also kill parasites during early or late sporogonic stages and lead to refractoriness to infection (Snow *et al.*, 2005).

Pathogenesis and Clinical Manifestation of Malaria

The transmission of malaria begins with the inoculation of the *Plasmodium* parasites into a human (Snow *et al.*, 2005). The parasite travel initially through the bloodstream to the liver, where they differentiate and replicate prior to re-entering the bloodstream in a form that is capable of infecting red blood cells (Snow *et al.*, 2005).

The subsequent cyclical infection of human red blood cells causes symptomatic malarial illness, which manifests as recurring episodes of chills, fever, and sweating, headache, malaise, fatigue, and body aches can last for weeks (Snow *et al.*, 2005).

In some cases of *Plasmodium falciparum* infection, "uncomplicated malaria" can progress to "severe malaria," which manifests as coma and seizures (cerebral malaria), severe anemia, respiratory distress, kidney and liver failure, and cardiovascular collapse (NIAID, 2008).

Typical symptoms of severe malaria includes acidosis, severe anaemia, renal failure, pulmonary oedema, convulsions, splenomegaly, respiratory distress, impaired consciousness, hypoglycemia and jaundice often leading to death with the four last symptoms being the best prognostic indicators (Marsh *et al.*,1995).

Pregnant women are particularly susceptible to placental infection by *Plasmodium falciparum,* which can result in severe forms of malaria that contribute to premature delivery and low-birth-weight infants (White, 2003).

Overall, hospitalised children and adults with severe forms of malaria have a 20 percent chance of dying, and many others experience varying levels of short and long-term physical and cognitive disabilities, creating significant socioeconomic burdens on families and communities (NIAID, 2008). Parasite-host interactions play a common and particularly important role in the pathogenesis of malaria. For example, the binding of *Plasmodium falciparum*-infected red blood cells to the inner surfaces of blood vessels initiates complex inflammatory processes that cause fever and other symptoms of malaria (NIAID, 2008).

MALARIA CONTROL

Malaria control aims at the long-term reduction of the disease to a level at which it ceases to be a major public health problem (WHO 2003). In 1977 the World Health Assembly adopted the concept of "Health for All by the Year 2000" aiming at the attainment by all people of the world of an improved and equable level of health (WHO, 1978). The way to the achievement of this goal was stated by the International Conference on Primary Health Care held in 1978 (WHO, 1978).

The Conference described the principles of primary health care and stressed the need for integration of some anti-malaria activities within the armbit of primary health care. This demands the use of health technology adapted to local needs, scientifically sound, acceptable, and usable by the people concerned, affordable by the community involved in its own health and socioeconomic future (WHO, 1978)

Objectives of any malaria control program have been classified as ultimate, intermediate, and immediate (Bruce-Chwatt, 1984). Although the ultimate objective is that of elimination of malaria from a given country or area, the intermediate objective is a reduction of the prevalence

of the disease to levels that are compatible with the health situation or with socio-economic development. However, immediate objectives of malaria control, in endemic areas, aim at the reduction of morbidity and mortality, followed by a decrease of the number of transmission (Bruce-Chwatt, 1985).

2.5.1 Strategy and Tactics of Malaria Control

Generally, measures for the prevention of malaria in individuals and for large scale control of the disease are designed to interfere with the cycle of the parasite within its human and insect hosts (Bruce-Chwatt, 1985).

The measures are divided into five groups aimed at the following objectives:

1. Prevention of mosquitoes from feeding on man.
2. Reduction of mosquito breeding sites by eliminating collections of water or by altering the environment.
3. Reduction of mosquito larvae, by their destruction.
4. Reduction of adult mosquitoes, by insecticides.
5. Elimination of malaria parasites in the human host.

2.5.2 Prevention of mosquitoes

Protecting people from bites of *Anopheles* through screening of dwellings, wearing of appropriate clothing after dusk, and the use of treated bed nets should be practiced whenever possible. These methods of personal protection are of great value when properly employed. Indoor spraying of pyrethrum solutions or other insecticides, as well as the use of mosquito's

repellant compounds or fumigant coils is of value (Bruce-Chwatt, 1985). The effectiveness of these methods depends not only on their regular application but also on the biting habits of the vector; because if the transmission of malaria is largely due to exophillic *Anopheles* mosquitoes, indoor protection is of limited value (Bruce-Chwatt, 1985).

Reduction of Mosquito Breeding Sites

Operations to reduce mosquito breeding sites, often called source reduction, include filling of unnecessary depressions in which water collects, regulation and improvement of natural water courses, water impoundment, drainage activities, weed control, flushing, and intermittent drying of rice-fields. Such mechanical or hydraulic methods were an outstanding success in the formerly malarious areas of the USA, Europe, North Africa, India, and a few countries of Southeast Asia (Bruce-Chwatt, 1985).

Destruction of Mosquito Larvae

Larvicidal methods by oiling the collections of standing water or dusting them with Paris green (*copper acetoarsenate*) were the mainstay of malaria control for half a century (Bruce-Chwatt, 1985). The success of biological control, through the introduction of larvivorous fish and changing the ecology of actual or potential environmental breeding sites, depends on the careful study of every situation and on the sound knowledge of the local *Anopheles* species (Bruce-Chwatt, 1985). Nearly all these methods of malaria control were eclipsed at the end of World War 2 by the possibility of large scale attack on the adult *Anopheles* through the application of residual spraying of DDT and other insecticides (Bruce-Chwatt, 1985).

However, with the increase in insecticide resistance, the older ways of mosquito control have now become more promising. Some larvicides such as temephos (Abate) combine long effect

with low toxicity to animals and humans, and are more widely used. Various larvivorous fish and particularly *Gambusia affinis* have often been used with good results. Other biological methods, such as use of predators of mosquitoes and mosquito pathogens have undergone field trials (Bruce-Chwatt, 1985).

Fungi *(Coelomomyces)*, nematode worms (*Romanomermis*), and bacteria have been used, but greater success was obtained with the use of *Bacillus thuringiensis*, a spore –forming bacterium that produces crystallized toxic protein. When *Anopheles* larvae ingest this isolated bacterial protein, their intestine is paralysed, causing death. Mass production of these and other bacteria (*B. sphericus*) has been developed and commercial formulations of such larvicides are available (Bruce-Chwatt, 1985).

Reduction of Numbers of Adult Mosquitoes (Adulticiding)

Treatment of adult mosquitoes is the most visible practice exercised by mosquito control operations. Although killing of adult *Anopheles* by indoor spraying of houses with pyrethrum solution achieved in the 1930s some degree of malaria control in South Africa, the introduction of *dichloro-diphenyl-trichloroethane*, or DDT, in 1945 was a milestone in the history of malaria control (Bruce-Chwatt, 1985). In contrast to the short knock-down effect of pyrethrum, mosquito that rest on a surface coated with minute crystals of DDT or other residual insecticide will be continually poisoned and die within a few hours. This toxic effect on mosquitoes may persist for up to six months or longer after one spraying with DDT of all indoor surfaces of a house or premise where *Anopheles* may find a shelter. Alternate compounds (dieldrin, malathion, carbamates) persist for a shorter period and are more expensive. These insecticides, however present great toxic hazards to humans and have been banned in most developed countries (Bruce-Chwatt, 1985).

Elimination of Malaria parasites in the human host

The uses of anti-malarial drugs are protective (prophylactic), curative (therapeutic) and prevent transmission. Anti-malarial drugs may be employed as an alternative or additional malaria control measure; their effects on transmission of malaria depend on the type of drug employed, degree of coverage of the community and the timing and regularity of drug distribution.

Moreover, the efficacy of the drug depends also on the strain of the parasite, the severity of the infection and the subject's immune status.

During the past years, the use of drugs for malaria control has gradually become more important. The drugs' main shortcoming is that, when they must be given at repeated short intervals to a large proportion of the population, their administration becomes complex and expensive in countries with rudimentary health services. Furthermore, resistance of plasmodia, especially that of *P. falciparum*, has developed in some parts of the tropical world, even to chloroquin, which was once the most dependable compound (Bruce-Chwatt, 1985).

2.6 INTERGRATED VECTOR CONTROL (IVC)

In order to accomplish long-range, intelligent and environmentally sound vector control, the management and manipulation of pests must be accomplished using not just one but all available vector control methods. Integrated vector management utilises all available environmentally friendly control measures (FDAS, 1994).

A typical mosquito control programme first determines the species list and abundance of mosquitoes through larval and adult surveys and then uses the most efficient and effective means of control. In some situations, water management programmes or sanitation programmes can be instituted to reduce breeding areas. When this approach is not practiced, then a larviciding

programme is used so that specific breeding areas can be treated. Where larviciding is not effective, adulticides are used. The choice of larvacides and adulticides used is based on the species targeted for control and on environmental restrictions. An important part of this programme is public education. Public participation can do much to reduce breeding sites of domestic mosquitoes. Public education can be most effective during disease epidemics to inform the public concerning mosquito habits and ways individuals can protect themselves from mosquito bites.

In this project, the contribution of *Bti* in reducing vector mosquitoe populations will be investigated and its input in the implementation of integrated vector control programmes assessed.

2.6.1 Larviciding Based on Microbial Control Agents

Larviciding is a general term used for the process of killing mosquitoes by applying natural agents or commercial products designed to control larvae and pupae (collectively called larvae) in aquatic habitats (Najera and Zaim, 2002). Larvicide treatments can be applied from either the ground or air.

Larviciding was implemented as a malaria control procedure in the early 1900's and over the years has become prominent (Florida Mosquito Control, 2009). *Bacillus thuringiensis israelensis (Bti)* and *Bacillus sphaericus* 2362 are environmentally friendly bio-control agents, which effectively control the larval stages of mosquitoes. They have minimal effect on non-target organisms and are safer to the user (Majambere *et al.*, 2007). The discovery of the gram-positive, endospore-forming soil bacterium *Bacillus thuringiensis* subsp. *israelensis* in the Negev desert of Israel in 1976 and of the potent strains of *B. sphaericus* in recent years have inaugurated a new

chapter in the control of mosquitoes and blackflies (Becker, 2003). The new subspecies of *B. thuringiensis* is highly toxic to larvae of most mosquito species and to blackfly larvae (Becker, 2003). New strains of *B .sphaericus*, such as strain 2362 isolated from an adult blackfly in Nigeria are much more potent than the first isolates and are particularly active against larvae of *Culex* species and *An. gambiae* (Mulla *et al.*, 2003).

2.6.2. Mode of Action of *Bacillus thuringiensis israelensis* (*Bti*)

This *Bacillus* produces protein toxins during sporulation that are concentrated in a parasporal body (PSB), called the protein crystal. These proteins are highly toxic to mosquito and blackfly larvae (Becker, 2003; 2006; 2011).

The selectivity of the *Bacillus* derives from a variety of factors:

1. The protein crystal (inactive protoxin) must be ingested by the target insect, and this depends on its feeding habits.
2. Proteases must then convert the protoxin into biologically active toxins in the alkaline midgut milieu of the target insect.
3. The toxins must then bind to a cell surface receptor (glycoprotein) of the midgut milieu of the target insect.

This disturbs the osmoregulatory mechanisms of the cell membrane, thereby swelling and bursting the midgut cells (Becker and Rettich, 1994). Non-target-organisms do not activate the protoxin into the toxin, or remain undamaged because of the lack of specific receptors on their intestinal cells (Becker *et al.*, 2010). The insecticidal effect of *Bti* emanates from the parasporal body (P.S.B), which contains 4 major protein toxins of different molecular weight, referred to as the ~~Cry4A~~(125kDa), Cry4B(135kDa), Cry4B(135kDa), Cry10A(58kDa) and Cry11A(68kDa) (Becker, 2011). These toxins bind to specific glycoprotein receptors on the larval mid gut brush

border. A 5th toxin, called the CytA protein (27kDa), binds to lipids and does not exhibit the specific binding mechanism, which the Cry proteins do (Becker, 2006; Becker *et al.*, 2010). Neither the spore nor the living bacilli appear to be involved in the insecticidal process. The high toxicity of the P.S.B to a great variety of mosquito and black fly species is the most remarkable property of *Bti*. Only at significantly higher dosages are certain other Nematocera species affected, but no other organisms are harmed (Becker, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The Ashanti Region is centrally located in the middle belt of Ghana. It lies between longitudes 0° 9'W and 2° 15'W, and latitudes 5° 30'N and 7° 27'N, and occupies a total land area of 24,389 km representing 10.2% of the total land area of Ghana (Ghana Statistical Service, 2000). The region has a population density of 148.1 persons per km, compared with a national average of about 80 persons per km (Ghana Statistical Service, 2000). The Kumasi metropolitan area which is the capital of the Ashanti Region of Ghana was the study area.

3.1.1 Location and size of study area

Kumasi is located 300 kilometers Northwest of Accra, and is the most populous district. It lies within a tropical rainforest belt between the northern and southern savannah zones of Ghana (Ghana Statistical Service, 2000). The metropolis covers a land area of 299 sq km. Kumasi has ten (10) sub-metros but in the health sector, it is divided into five sub metros as shown in Figure 4 and Figure 5 shows malaria cases reported in 2009 and 2010 in the five health sub-metros in the metropolis (KMA, 2011). Laboratory and standardised field trials were carried out at the Kumasi Center for Collaborative Research (KCCR) on the Kwame Nkrumah University of Science & Technology (KNUST) campus (Figure 6). KNUST is situated about 8 km away from the city center (Figure 3). It covers an area of about 18 km^2 of undulating land and lies between latitude 6°39' & 6°47' N and longitude 1°26' & 1°40' W (Facts and Figures of KNUST, 2008).

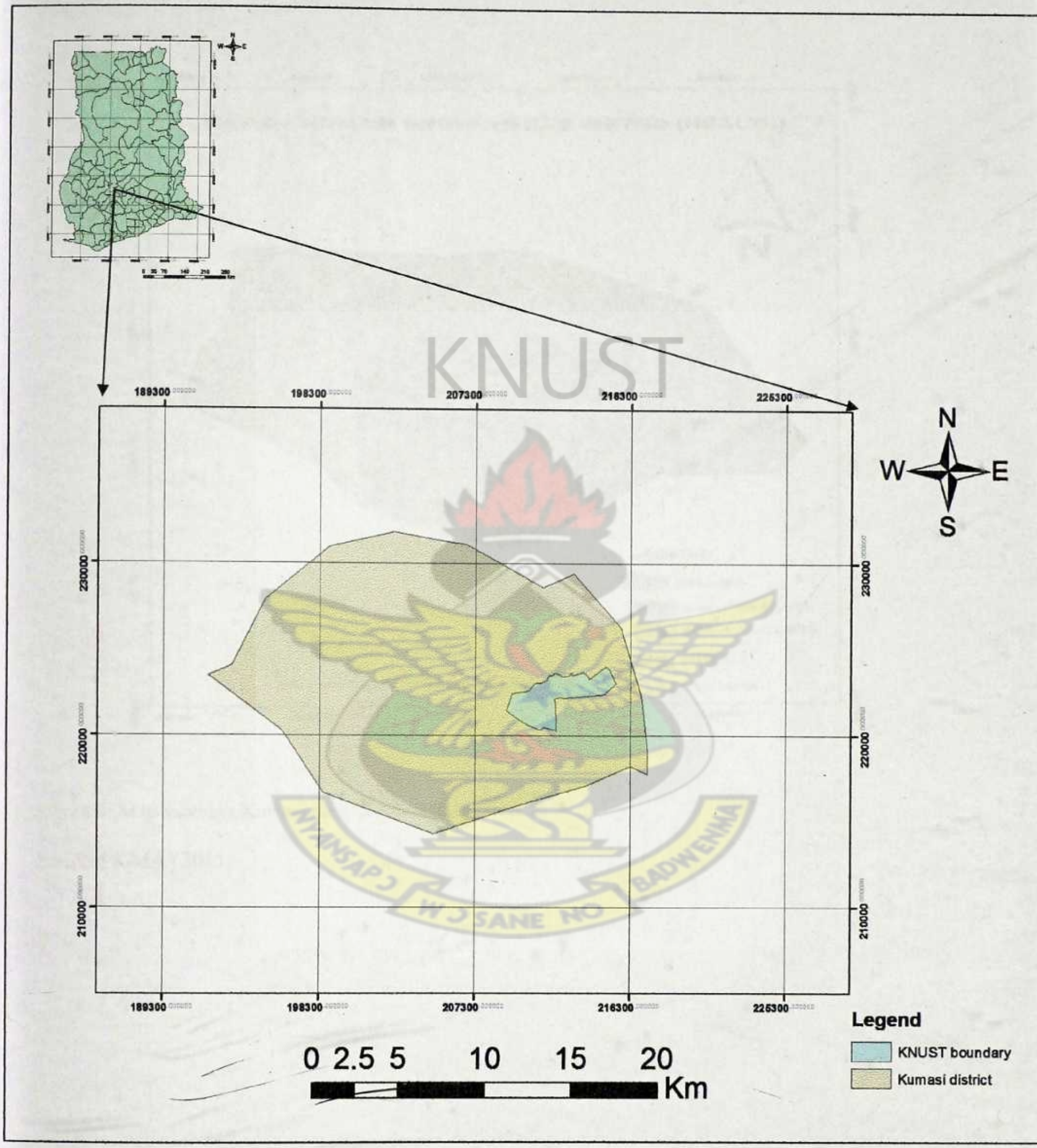


Figure 3: District map of Kumasi showing location of KNUST.

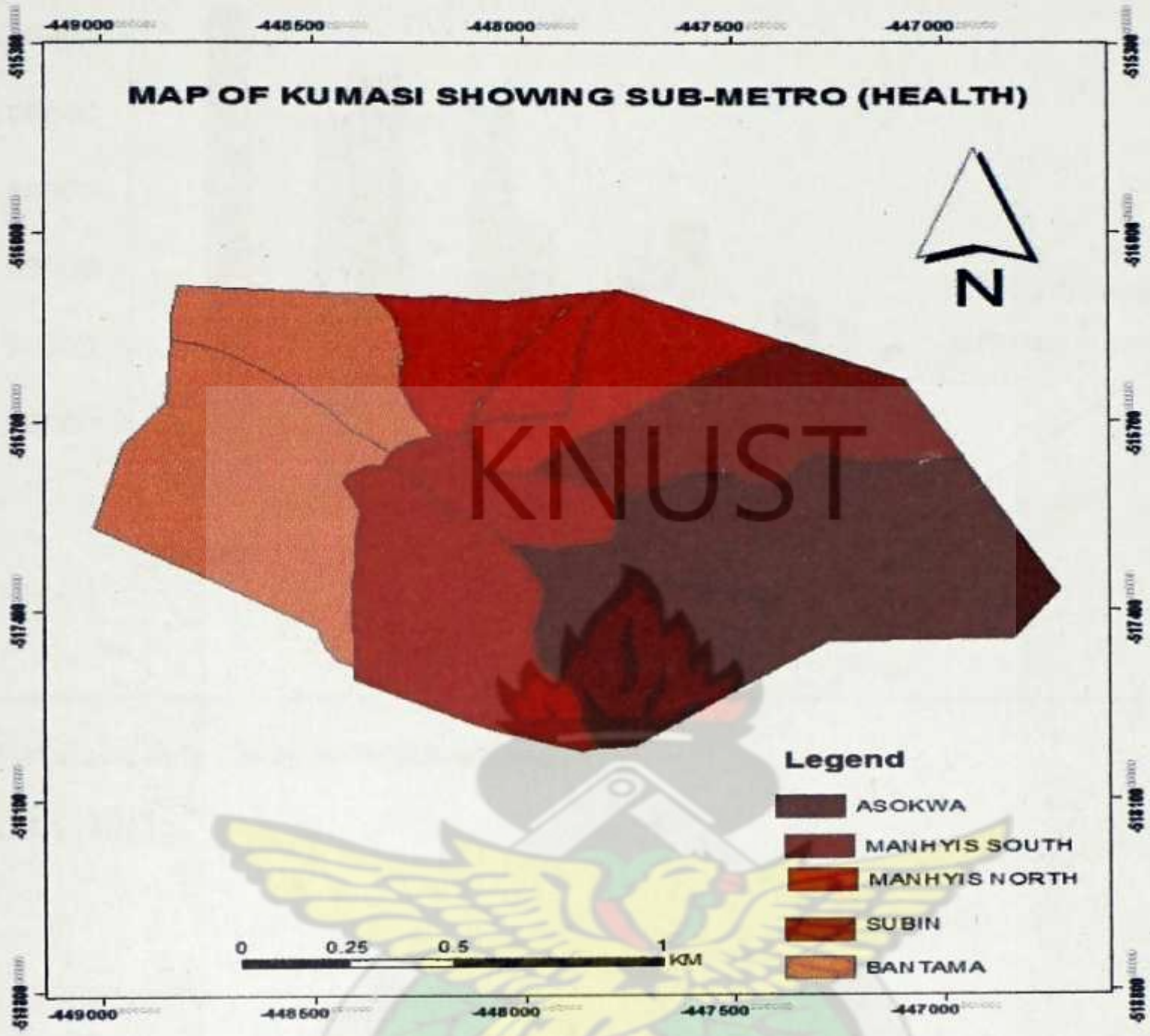


Figure 4: Map showing Kumasi district sub-metros (Health)

Source: KMA (2011)

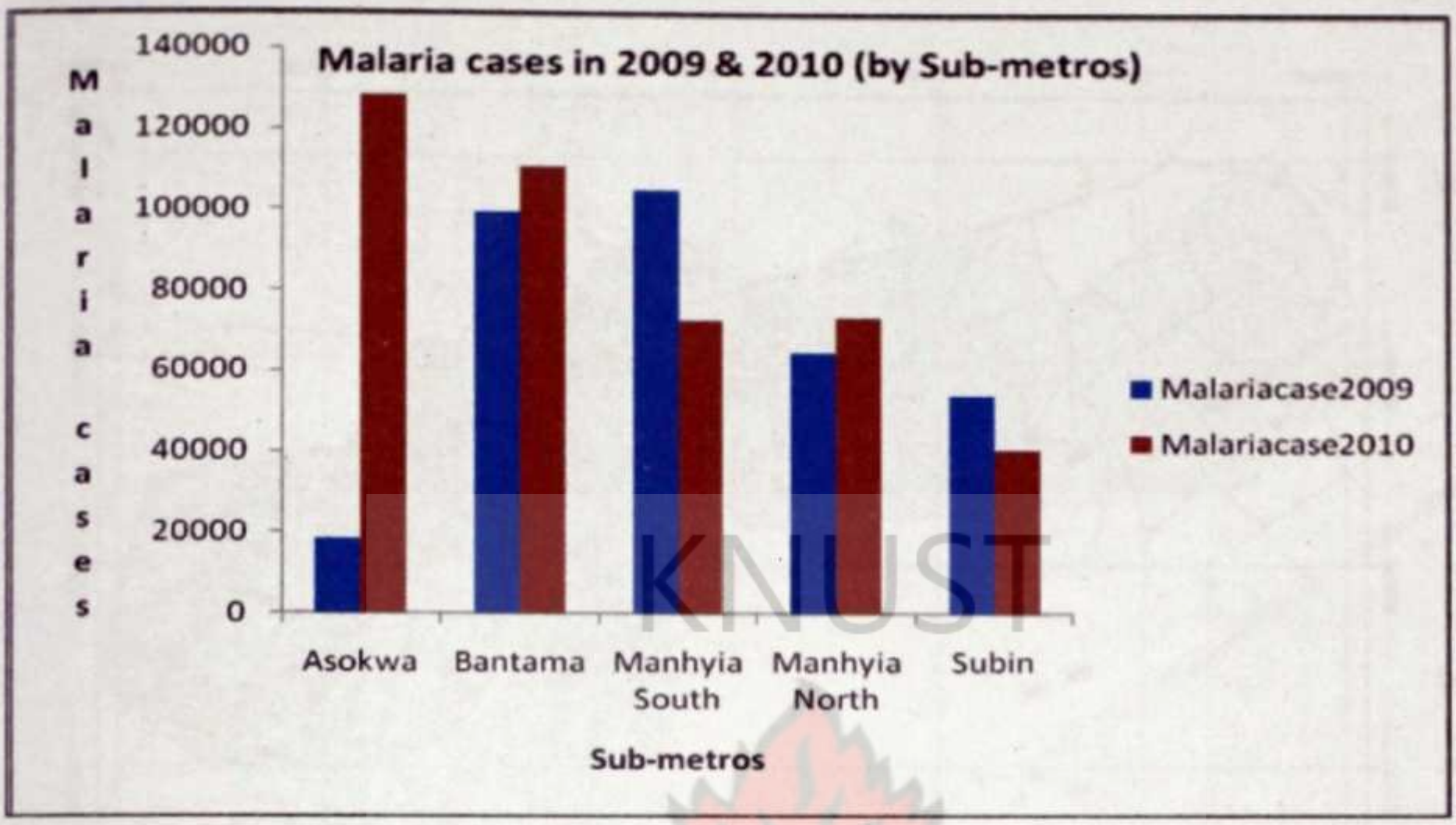


Figure 5: Malaria cases in 2009&2010 (Sub-metros)

Source: KMA (2011)

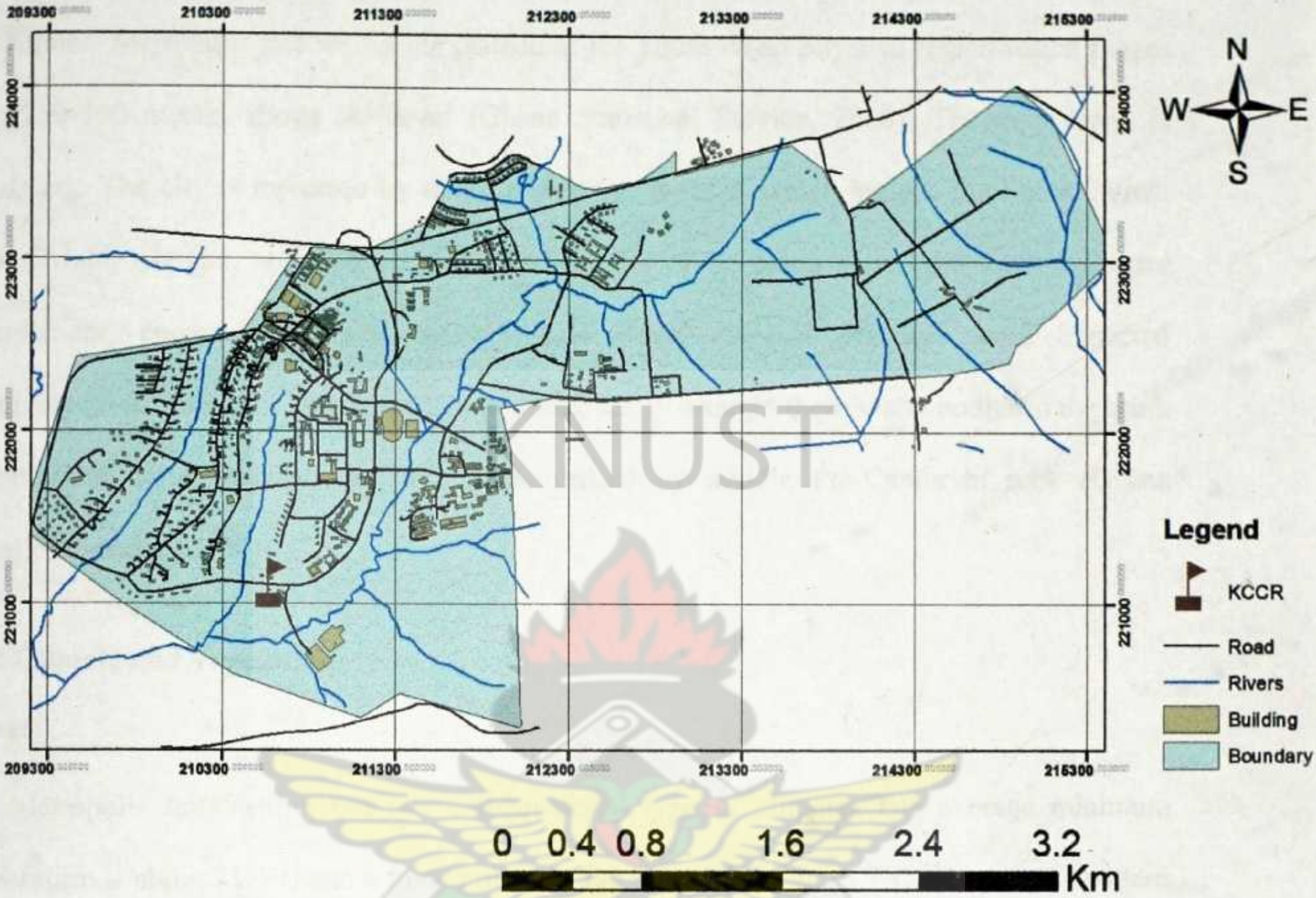


Figure 6: Map of KNUST showing location of KCCR

3.1.2 Relief and Drainage

The Kumasi Metropolis lies within the plateau of the South–West physical region which ranges from 250-300 meters above sea level (Ghana Statistical Service, 2000). The topography is undulating. The city is traversed by major rivers and streams, which include the Subin, Wiwi, Sisai, Owabi, Aboabo, and Nsuben among others. However, biotic activity in terms of estate development, encroachment and indiscriminate waste disposal practices have impacted negatively on the drainage system and have consequently brought these water bodies to the brink of extinction. The Metropolitan area is dominated by middle Pre-Cambrian rock (Ghana Statistical Service, 2000).

3.1.2 Climate and Vegetation

Climate

The Metropolis falls within the wet sub-equatorial type of climate. The average minimum temperature is about 21.5°C and a maximum average temperature of 30.7°C. The rainfall pattern is seasonal with an annual rainfall varying between 127.0 cm and 165.0 cm. The rainy (wet) season consists of two rainy periods, the major rains falling between late April and July with the peak in June while the minor rains fall between August and October. The dry season (harmattan) is from November to March. Kumasi has a semi humid tropical climate with humidity ranging between 53% and 93% and is 287.3 meters above sea level. The average humidity is about 84.16 per cent at 0900 GMT and 60 per cent at 1500 GMT

Vegetation

The city lies within the moist semi-deciduous South-East Ecological Zone. Predominant species of trees found are *Ceiba*, *Triplochlon*, and *Celtis* with exotic species. The rich soil has promoted agriculture in the periphery (Ghana Statistical Service, 2000). A patch of vegetation reserve within the city has led to the development of the Kumasi Zoological Gardens. Apart from the zoological gardens, there are other patches of vegetation cover scattered over the peri-urban areas of the metropolis. However, the rapid spate of urbanisation has caused the depletion of most of these nature reserves (Ghana Statistical Service, 2000).

3.1.3 Demographic Characteristics of Kumasi

The 2000 census recorded the region's population at about 3.5 million people, representing 19.1 per cent of the country's population. The urban population (51.3%) in the region exceeds that of the rural population (48.7%). The region is currently the second most urbanised in the country after Greater Accra (87.7%), the national capital. The housing stock in the region is 329,478, of which about 37% are in urban areas and 63% in rural areas (Ghana Statistical Service, 2000).

The Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, being the only Science and Technology University in Ghana attract scientists and technologists not only from Ghana but from other African countries as well. KNUST has a student population of about 32,000. It has 741 teaching staff and 2424 non teaching staff (Facts & Figures of KNUST, 2008).

3.2 Ethics

Ethical approval for this study was given by the Committee on Human Research Publications and Ethics (CHRPE) of Kwame Nkrumah University of Science & Technology, Kumasi, Ghana.

3.3 MAPPING OF BREEDING SITES

The usefulness of geographical information system for planning and managing control programmes depends on the availability of accurate and timely raw data on malaria cases. Kumasi has ten (10) sub-metros but in the health sector, it is divided into five sub metros as shown in Figure 4. Figure 5 shows the number of malaria cases reported in 2009 and 2010 in the five health sub-metros in the metropolis. Data on the malaria cases from the different sub-metros gave an idea about the number of breeding sites within the different sub-metros.

Monthly ground surveys were conducted in all the five sub-metros from October 2010 to February 2011 (dry season) and from April to July 2010 (rainy season). Aquatic habitats that posed as potential breeding places for mosquitoes in all five sub-metros were identified. Some of these habitats included; drainage ditches, stream edges, swamps, footprints and other depressions that had collected rain water. The surveys were conducted at every fortnight for the rainy and dry seasons.

A hand held GPS receiver unit, was used to capture spatial co-ordinates (i.e. longitude and latitude) to map breeding sites. The coordinates were transformed to local coordinate system that is Transverse Mercator (TM) Zone 30N and Legion datum map projection system. The point plotted on the map of the Kumasi metropolis shapefile that was developed using Arc Map 10.

3.4 STAGES OF *Bti* EVALUATION

Laboratory and standardised field tests were carried out at the Kumasi Center for Collaborative Research into Tropical Medicine Laboratories located on KNUST campus.

Table 1: Sequence of the stages of evaluation of microbial larvicide (*Bti*)

Phase	Type of study	Aim
Phase I	Laboratory studies	- Biopotency and activity - Diagnostic concentration
Phase II	Standardised field trials	- To identify optimum dosages of *Bti* under field conditions - Method and rate of application - Initial and residual activity

3.4.1 Phase I: Laboratory Studies

Laboratory assays were carried out with third instar larvae of insectary-reared *Anopheles gambiae* larvae which were originally collected from a lettuce farm on KNUST campus, about 3 km from KCCR in August 2010, and reared at the KCCR insectory.

Collection of mosquito larvae

A 250 ml blue plastic bowl was used to collect larvae from water samples in ditches, and at the edges of other large bodies of water and swamps within the study area. For small breeding sites, such as human footprints a ladle of about 12cm in diameter was used as the dipper. Water collected in either the plastic bowl was examined carefully and the larvae coming to the surface were collected by a pipette. Each aquatic habitat that contained anopheline larvae (identified by the position with respect to the water surface) was then geo-referenced using a hand-held GPS.

The larvae were transferred into large labelled collection bottles half filled with water from the breeding site. The larvae were then transported to the insectary and reared to adulthood in-order to obtain F1 generation *Anopheles* larvae for bioassays and for morphological identification using the keys of Gilies and Demeillon (1968) and Gilies and Coetzee (1987).

A. **Description of Selected Sites of Larvae Collection**

The anopheline larval habitat types identified were characterised by clear or turbid shallow and sunlit conditions. Some anopheline larvae were also found breeding in either puddles collected in human footprints or drying stream beds, conduits on some vegetable farms, rain-pools, or in swamps. In some breeding sites/ habitat tadpoles, dragonflies, and water bugs, were also found and most often very few mosquito larvae or no mosquito larvae were present where they were found.

Plate 2: Mosquito breeding site on a cabbage farm

Plate 3: Mosquito larvae in a plastic bowl

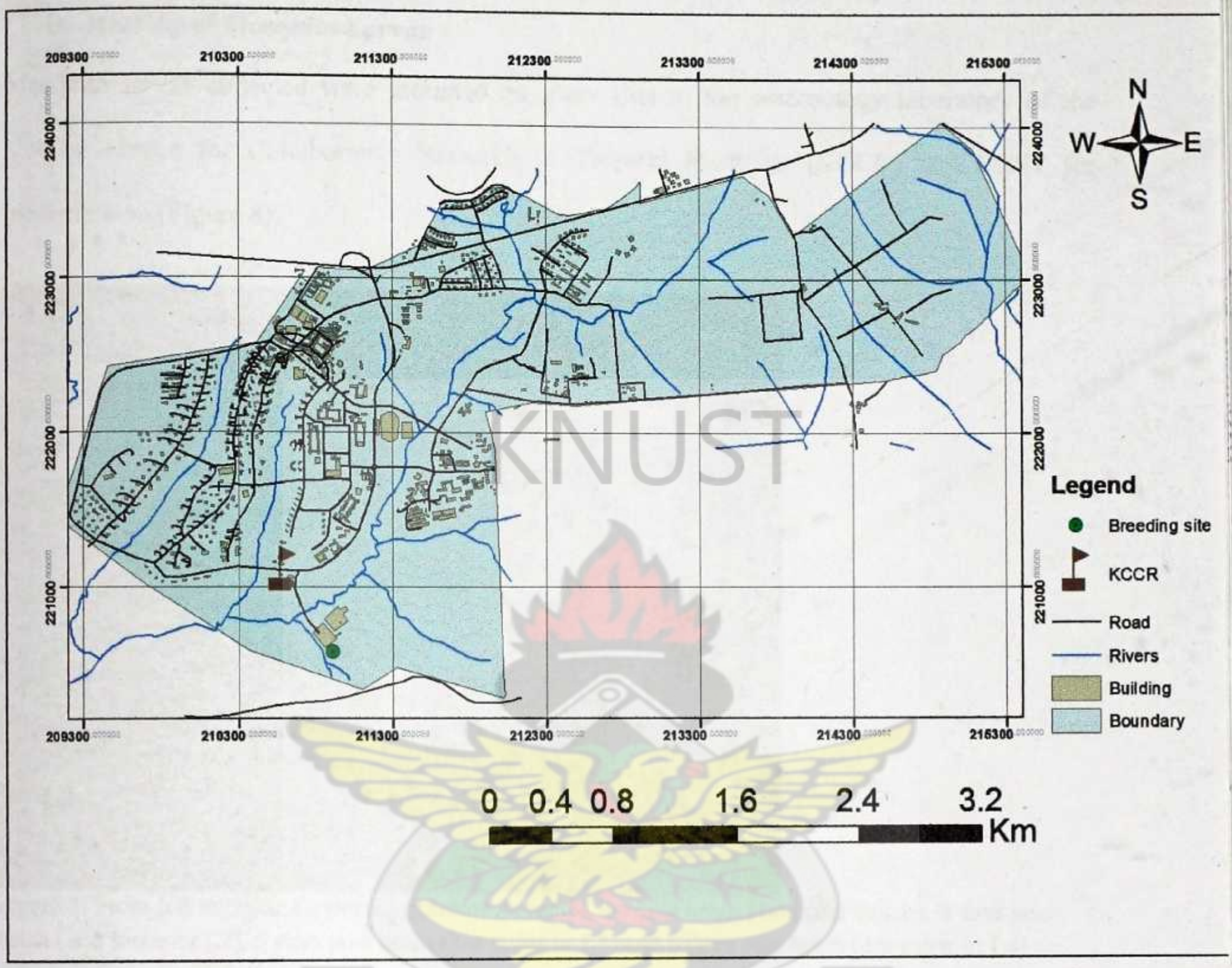


Figure 7: Map of KNUST showing location of KCCR and lettuce farm where *Anopheles* larvae were collected

B. **Rearing of Mosquito Larvae**

Mosquito larvae collected were mounted on glass slits in the entomology laboratory of the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) at KNUST for identification (Figure 8).

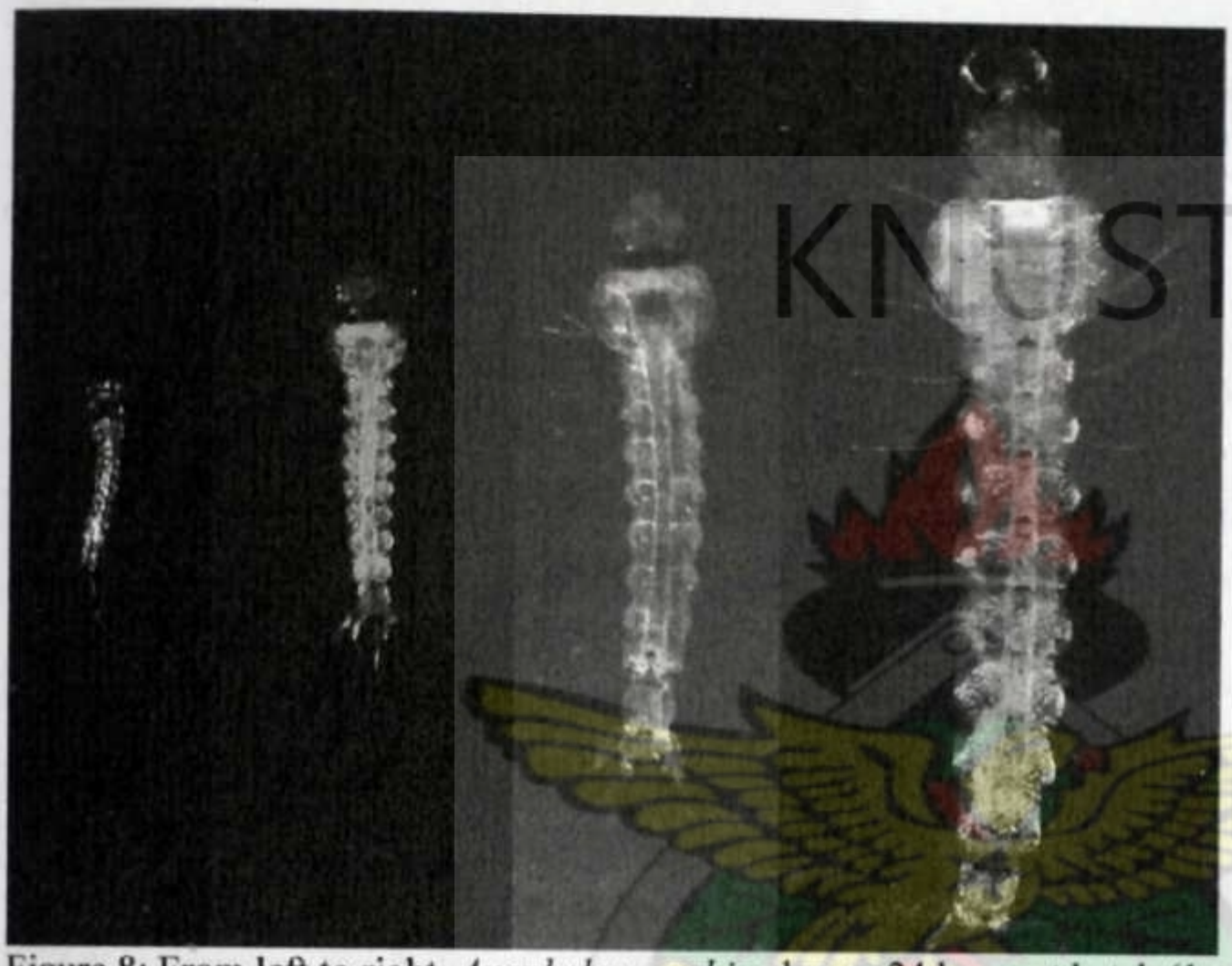


Figure 8: From left to right, *Anopheles gambiae* larvae 24 hrs post hatch (1st instar or L1|), 2 days post hatch (2nd Instar or L2), 5 days post hatch (3rd instar or L3) and 6 days post hatch (4th instar or L4)

Source METHODS IN ANOPHELES RESEARCH, 2007

Figure 9: Aedes head and mouthparts

Figure 10: *Anopheles* head and mouthparts

Figure 11: Culex head and mouthparts

Figure 12: *Anopheles gambiae* pupae

Source METHODS IN *ANOPHELES* RESEARCH, 2007

The larvae were first separated into anophelines and culicines and *Anopheles* larvae were reared to the adult stage following procedures in the Manual for *Anopheles* research (MR4 manual). Adult *Anopheles* mosquitoes were fed with 10% sugar solution. Blood meal was provided for ovary development of the female *Anopheles* mosquitoe by using guinea pigs that were reared on KCCR premises. The belly of the guinea pigs was shaved prior to feeding in order to make the skin easily accessible to the mosquitoes. Petri dish with a strip of filter paper soaked in water was introduced into the cages where the adult mosquitoes were kept and left open for oviposition. Eggs laid on the filter paper were transferred into larval rearing bowls and reared to the third instar stage. The third instar larvae were then used for the laboratory assays.

All mosquito larvae used in the laboratory experiments were reared at a room temperature of 28°C, 80% relative humidity and approximate 12 hour light: 12 hour dark cycle. Larvae were reared in 26 x 24 x 5 cm transparent, rectangular plastic containers filled with 300 ml tap water that had been left in the insectary for at least 48 hours to equilibrate. Larvae were fed by adding a

pinch of crushed Tetramin@ (Tetra, Germany) fish food spread evenly on the water surface twice daily.

3.4.2 Bacillus Formulation

Water-dispersible granular (WDG) formulations of the commercial strains of *Bti* (VectoBac@ strain AM65-52; Lot number 187-600-PG, 3000 ITU/mg; ValentBioScience Corporation, Illinois, USA,) were tested in the laboratory and under field conditions. This was done in a similar manner to that described in the WHO Guidelines for Laboratory and field testing of mosquito larvicides (WHO/CDS/WHOPES/GCDPP/2005.13). *Bti* WDG formulations were applied as liquid with 250 ml handheld sprayers. It was observed that *Bti* WDG formulation dispersed readily when mixed with water and remained like that for at least several minutes.

3.4.3 Preparation of *Bacillus* Formulation for bioassay.

Distilled water was used in the preparation of 1% stock solution. A stock suspension was prepared by weighing 1000 mg of the *Bti*, the 1000mg of the Bti was dissolved in 100ml of distilled water to yield 1% stock suspension. It was then shaken vigorously to facilitate dissolution of the Bti. From the 'stock suspension', all subsequent dilutions were prepared.

Plastic cups were filled with 100 ml distilled water. Twenty-five (25) late third or early fourth instar larvae of *A. gambiae* were added to each cup.

Using micropipettes, 0.01ul, 0.02ul, 0.04ul, 0.08ul, 0.16ul and 0.32ul of the 1% stock were added to the cups and the solutions mixed to produce final concentrations of 0.001 mg/l, 0.002 mg/l, 0.004 mg/l, 0.008 mg/l, 0.016 mg/l, and 0.032 mg/l respectively of the *Bti*. Four replicate cups were used for each concentration and the control, which was 100 ml distilled water.

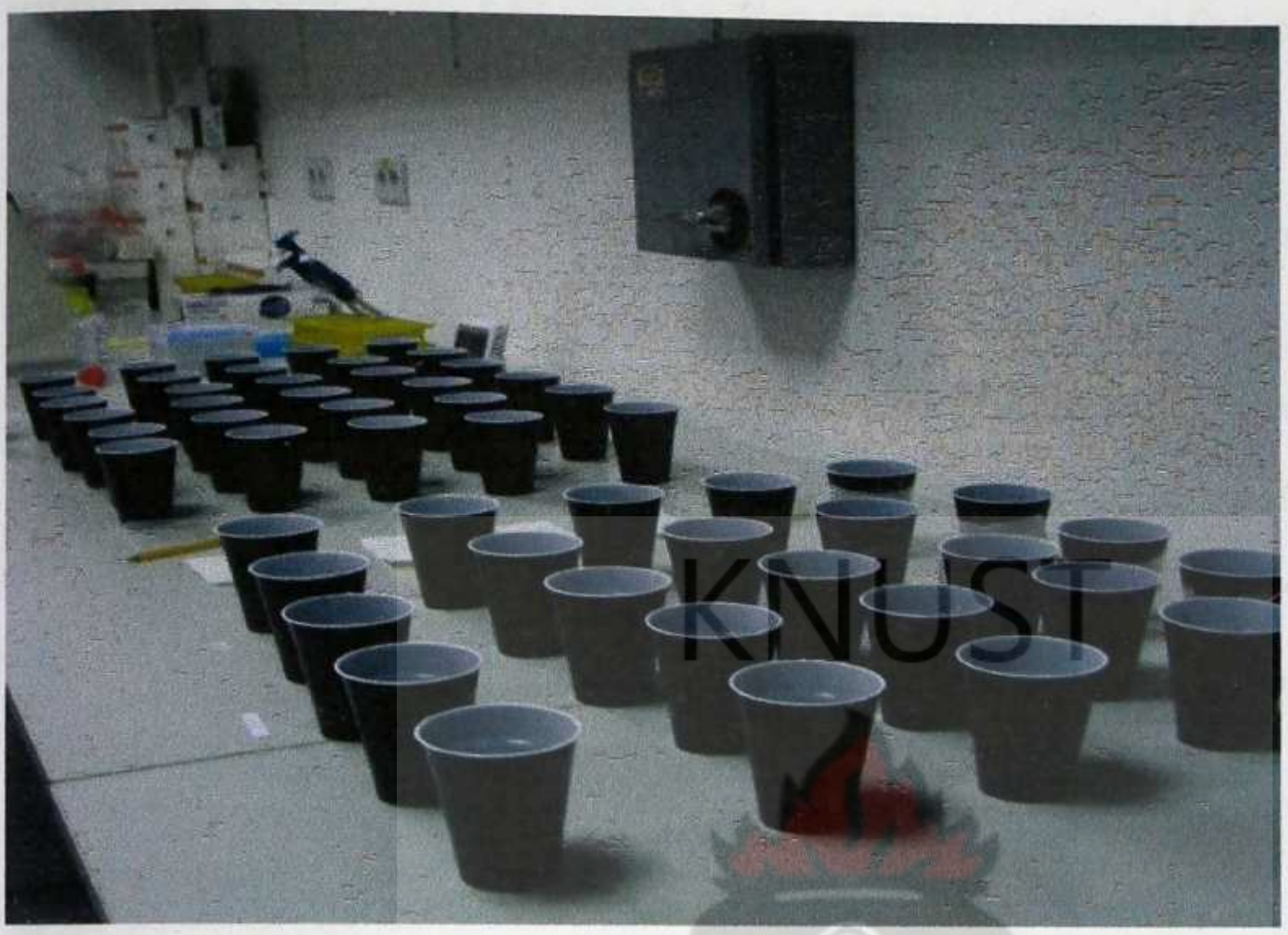


Plate 4: Set up for bioassay

3.5 BIOASSAYS

Laboratory assays were conducted using WDG formulation of VectoBac@ to determine the minimum effective dosages following the standard testing procedures for microbial test (WHO 1999). Twenty-five third instar larvae were randomly collected for the experiment from several containers to compensate for size difference and feeding history which are known to be influenced by larval density (Lyimo and Takken, 1993). They were then transferred to new disposable 300 ml plastic containers filled with 100 ml of distilled water.

On every test date, fresh stock solutions were prepared and test aliquots made with distilled water. After range finding test (10), six different test concentrations ranging between 0.001 and 0.032 mg/l were chosen. The bioassays were run in the six different concentrations plus controls

and replicates. Each experiment had a control. The experiments were run in four replicates at the same time and the entire experiments carried out on three different occasions

Results from all the replicates were pooled and analysed using the computer software (Raymond, 1985) for probit- regression analysis following method of Finney (1971). Larvae were not fed during the experiments and all tests were run at ambient temperature ranging between 21°C and 34°C. Larval mortality was determined by counting the live third instar larvae of *An. gambiae* remaining after 24 hours exposure to *Bti* WDG (VectorBac@, 3000 ITU/mg) and recorded (Table 9 in Appendix). Moribund larvae were counted and added to dead larvae in calculating the percentage mortality.

3.6 PHASE II: STANDARDISED FIELD TRIALS

Standardised field trials were conducted on a field behind KCCR on KNUST campus during the rainy (7th to 16th July 2011) and dry seasons (8th to 17th November 2011) to identify the optimum dosage of *Bti* required under field conditions and to evaluate the residual effect and re-treatment intervals for the test microbial.

Climate

Data on daily minimum and maximum temperatures and rainfall were obtained from the meteorological station near the Faculty of Agriculture about 1.3 km from KCCR on the KNUST campus.

3.6.1 Preparation of Field for Standardised Field Trials

An open sunlit area of about $28m^2$ was cleared of all vegetation. Artificial ponds were then created following the experimental design of Fillinger *et al.*, (2003). Eighteen 0.6 m diameter

plastic bowls were buried at a depth of about 0.2 m-0.3 m in two rows of nine containers each. Distances between the containers were 1.5 m.

Plate 5: Set up of standardised field trials behind KCCR

3.6.2 Field trials

Top soil and mud from the *Anopheles* breeding site at the cabbage farm was added to each of the plastic bowls (one-third of its volume) to provide the abiotic and biotic conditions suitable for mosquitoes. The containers were filled with tap water and maintained at a depth of 0.2-0.3 m. The habitats were then left open for mosquitoes oviposition. The trials were conducted with offsprings of wild *A. gambiae* females that oviposited in the experimental containers. Colonisation of experimental containers occurred within 4 days and sometimes included larvae of *Culex*.

Experiments were carried out nine days after the containers were set-up to allow third and fourth instar larvae to develop. Water temperatures during the experiments ranged between 23 °C and 40 °C. After colonisation of the containers, a completion of the larvae life cycle was found to take only 10 days, due to high water temperatures. In order to prevent the emergence of the malaria vector, all habitats were carefully screened for pupae twice daily (visually and with a dipper) and any mature pupae present were removed.

Out of the 18 artificial containers, six served as controls, whereas each half of the remaining 12 containers were treated with the test formulation. Containers were matched on the basis of larval density so that control and test treatment containers had similar densities at the start of the experiment. *Bti* concentrations were selected on the basis of laboratory results and studies reported elsewhere (Karch, *et al.*, 1992; Romi, *et al.*, 1993; Barbazan, *et al.*, 1997; Tianyun and Mulla, 1999).

Treatment concentrations were calculated on the basis of a standard water depth of 0.1 m and a fixed surface area using the methods of Schnetter *et al.*, (1981) and Ragonanansingh *et al.*, (1992) irrespective of the actual water depth and this was done to simulate operational procedures. Laboratory tests were conducted under standardised conditions without major abiotic and biotic influences, therefore the Lethal Concentration (LC) values represented minimum dosages only. Under field conditions application, rates normally have to be increased up to several times the LC_{95} to obtain sufficient larval control (Becker and Rettich 1994).Water-dispersible granular (WDG) formulations of *Bti* is not considered to show a long residual effect. From findings made elsewhere by Fillinger *et al.* (2003) and others; therefore, the search for the optimum effective dosage is the search for the minimum dosage for 100% larval mortality 48h

after application. The microbial larvicides, *Bti* WDG used in the study were at concentrations of 0.2 mg/l and 0.4 mg/l.

The liquid formulations of *Bti* were sprayed evenly over the entire water surface using a 250 ml handheld sprayer. Afterwards, all the containers were examined daily and the average number of larvae and pupae per dip (350 ml capacity dipper, Clarke Mosquito Control products, Illinois, USA) was determined by taking five dips from four different directions of each container close to the edge and one from the middle. Mosquito larvae were classified as *Anophelines* or *Culicines* and recorded as early (1^{st} and 2^{nd}) or late (3^{rd} and 4^{th}) instars. All larvae were counted, classified to genus and development stage and then returned to their respective sites. All pupae were removed.

3.7 ANALYSES

From the bioassays results, LC_{50} and LC_{95} values were determined using log-probit regression analysis in SPSS software version 16.0 (SPSS Inc., 2008). LC_{50} represents the probability of success or the chance of 50% of the larvae dying and LC_{95}, the chance of 95% dying. The percentage reduction in larval mosquito densities were calculated using the formula of Mulla *et al.*, (1971) which takes into account that natural changes (for instance through predation) in the mosquito larval populations are taking place at the same level and rate in both treated and untreated sites:

Percentage reduction = 100-(C1/T1 x T2/C2) x 100

where C1 and C2 describe the average number of larvae in the control containers pre- and post-treatment, like T1 (0.2mg/l) and T2 (0.4mg/l) for the containers treated with experimental formulations. Average number of larvae and pupae per dip in the control and treatment sites in

the field tests were compared using two-way ANOVA. The tests were implemented separately for each sampling day comparing average numbers of immature stages in the controls with treatments. All analyses were carried out using version 9.2 of SAS statistical software package (SAS Inc., 2012).

CHAPTER FOUR

RESULTS

4.1 MAPPING BREEDING SITES

4.1.1 Description of breeding sites

In all, 33 major mosquitoe breeding sites within the Kumasi metropolis were mapped out (Figure 13).

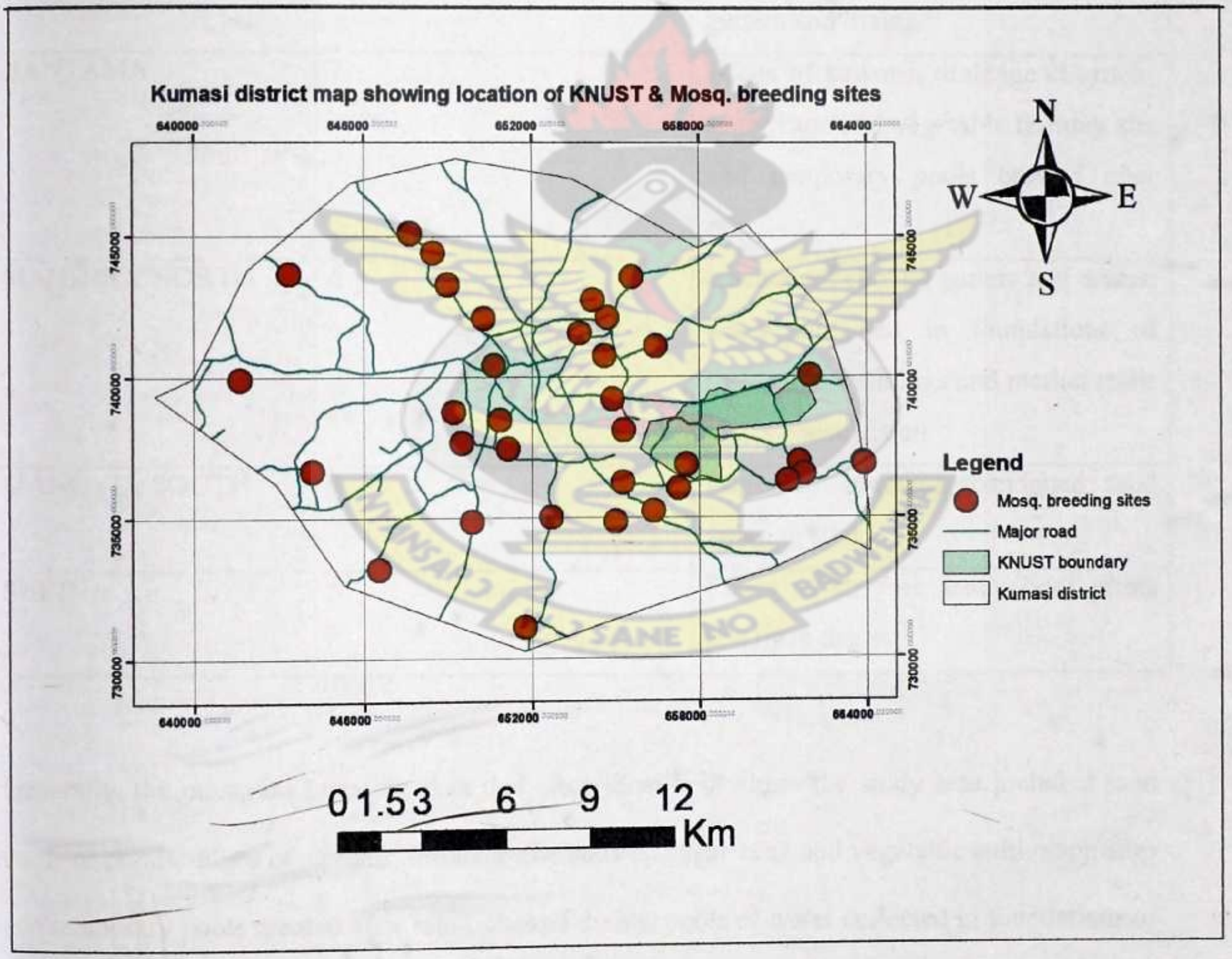


Figure 13: Map of Kumasi District showing map of KNUST and breeding sites

Of the five Ghana Health Service sub metropolitan divisions in the Kumasi metropolis, the Asokwa sub-metro had the highest number of breeding sites (9), followed by the Bantama sub-metro (7) whilst the Subin sub-metro recorded the least number (5) of breeding sites (Table 2).

Table 2: Characteristic of major Mosquito breeding sites in the five health sub-metropolitan areas in the Kumasi metropolis

SUB-METROPOLIS	NUMBER OF BREEDING SITES (%)	CHARACTERISTICS OF BREEDING SITES
ASOKWA	9	Vegetable farming, uncovered choked gutters and drains.
BANTAMA	7	Edges of streams, drainage channels, sugar cane and vegetable farming site and temporary pools created after rains.
MANHYIA NORTH	6	Uncovered choked gutters and drains, water collected in foundations of residential buildings and market stalls under construction
MANHYIA SOUTH	6	Edges of streams, abandoned sand winning sites.
SUBIN	5	Edges of streams, cattle hoof prints and open drains

Generally, the mosquito breeding sites that were identified within the study area included sand pools or ponds, edges of streams, drainage channels on sugar cane and vegetable cultivation sites and temporary pools created after rains, choked drains, pools of water collected in foundations of uncompleted buildings and abandoned sand winning sites (Table 2).

Out of the total of 33 breeding sites identified during the surveys, 3 were temporary breeding sites and 30 were permanent breeding sites. The breeding sites that had clear and shallow water produced higher densities of anopheline larvae whilst domestic waste water in chocked gutters were high with *Culex* but no *Anopheles* larvae.

4.2 LABORATORY ASSAYS

Of the various concentrations tested (0.00, 0.001, 0.002, 0.004, 0.008, 0.016 and 0.032), the *Bti* concentration of 0.026 mg/l resulted in 50% mortality (Table 9 in Appendix) while the 0.136mg/l concentration caused 95% mortality. Figure 14 shows percentage mortality at the various concentrations after 24 hr exposure of *Anopheles* larvae to the *Bti* formulation.

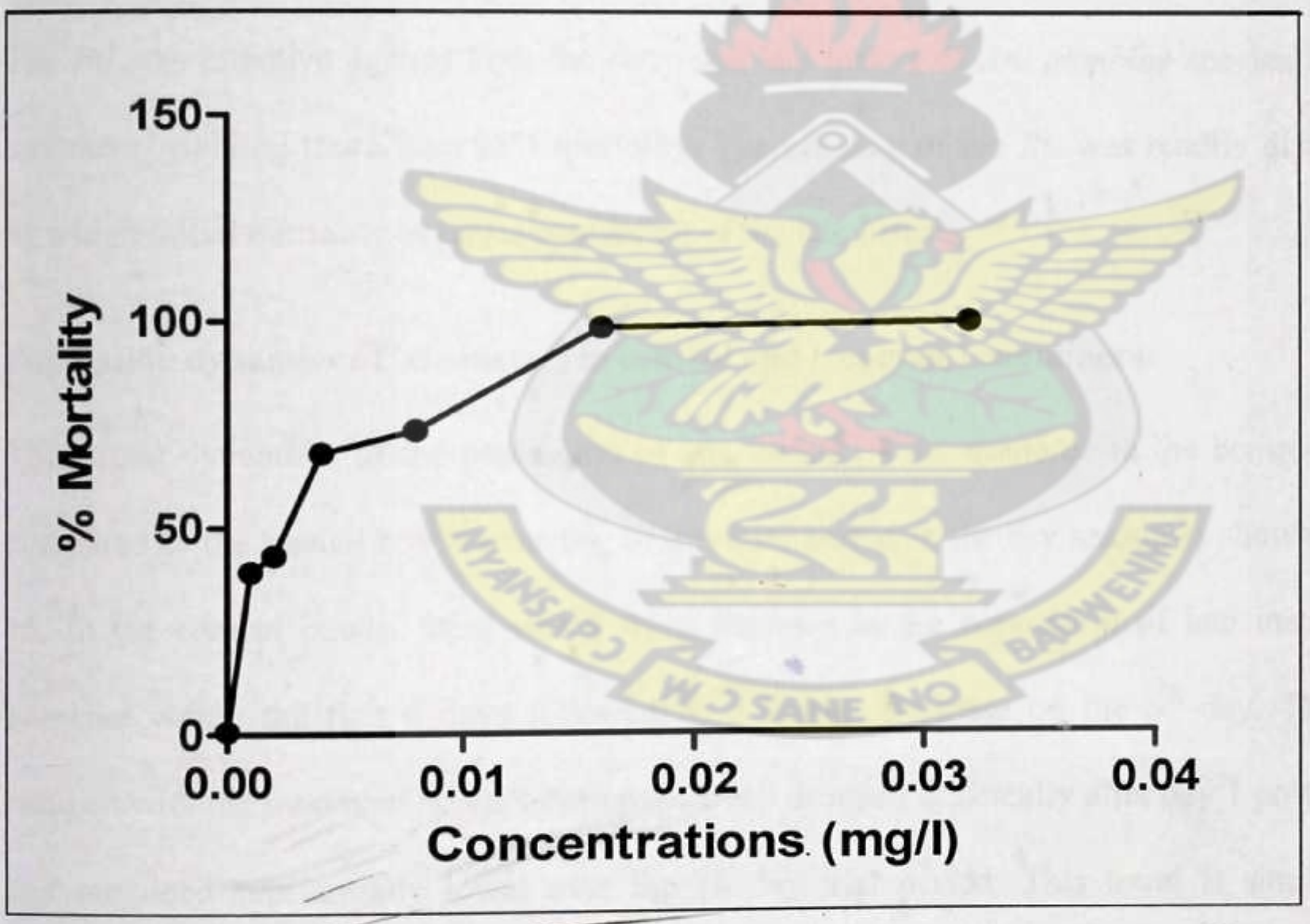


Figure 14: Graph showing the percentage mortality of *Anopheles* larvae at various concentrations after 24 hours exposure to the *Bti* formulation.

4.3 STANDARDISED FIELD TRIALS

Anopheline and Culicine mosquito larvae were detected 4 to 5 days after the artificial habitat was set-up. About 13% of the total larval population were *Culex* and since there is no significant difference in terms of the impact of the *Bti*; on *Anopheles* and *Culicines* larvae in the standardised field trials, results from both genera were pooled for all analyses. The early instars represented the first and second instar larvae whiles the third and fourth instar larvae were the late instar larvae. In both rainy and dry season, *Bti* WDG provided 100% mortality within 24 hours at all doses of application.

4.3.1 Field Efficacy of Bti.

The *Bti* was effective against both the early and late instars of *An. gambiae* species 24hrs post treatment, yielding more than 95% mortality. The efficacy of the *Bti* was readily demonstrated by a high initial mortality in larval population of the late instars.

Population dynamics of late instars in control and treatment containers.

The strong dynamism of the population of late instars of *An. gambiae* in the control bowls as compared to the treated bowls over the 10 day trial period in the dry season is shown in Figure 15. In the control bowls, there was a sharp increase in the population of late instars of *An. gambiae* within the first 4 days followed by a sudden decrease on the 5th day. This was in contrast with the treatment bowls where population dropped drastically after day 1 post treatment and remained substantially lower over the 10 day trial period. This trend is similar to that observed during the rainy season (Figure 15).

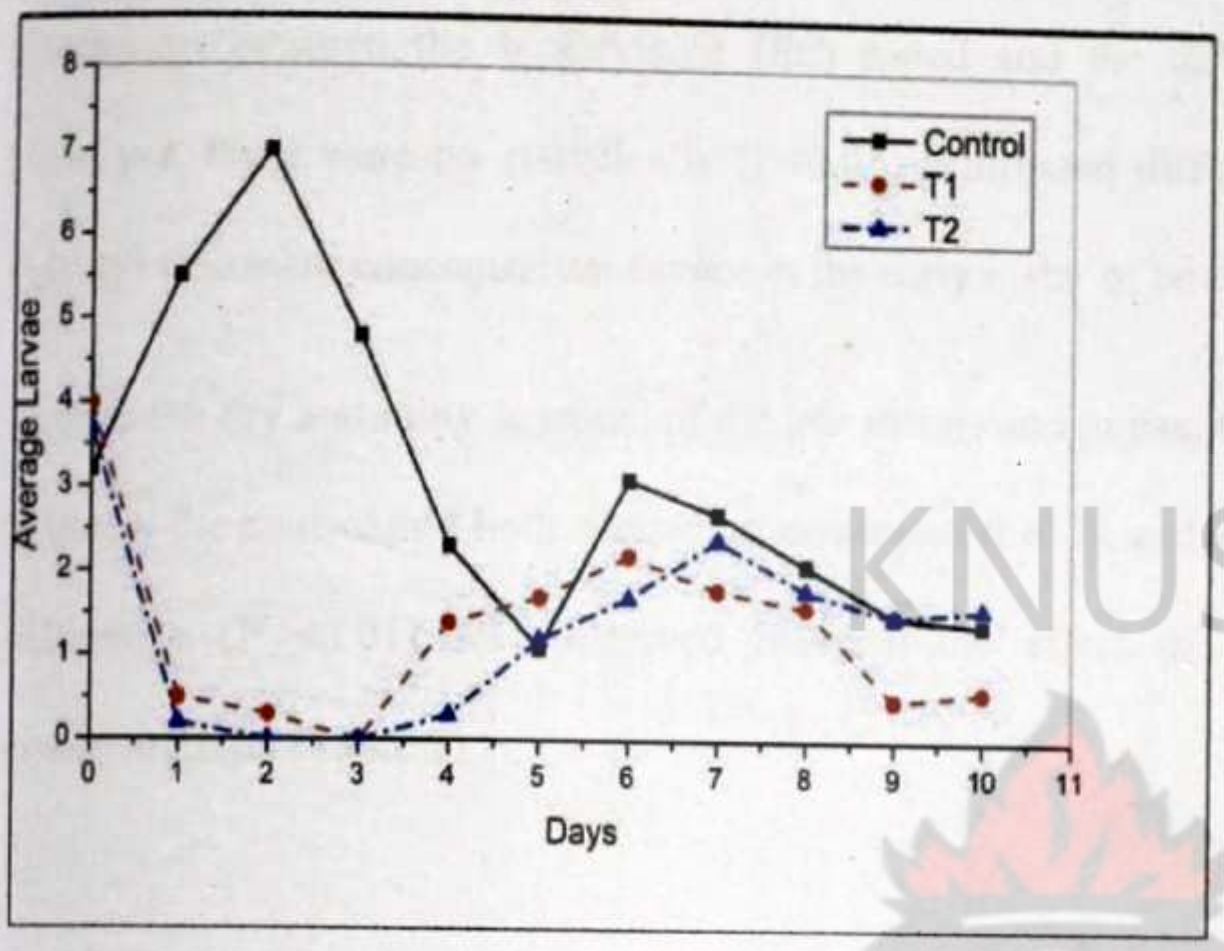


Figure 15: Pop dynamics of late instars (dry season)

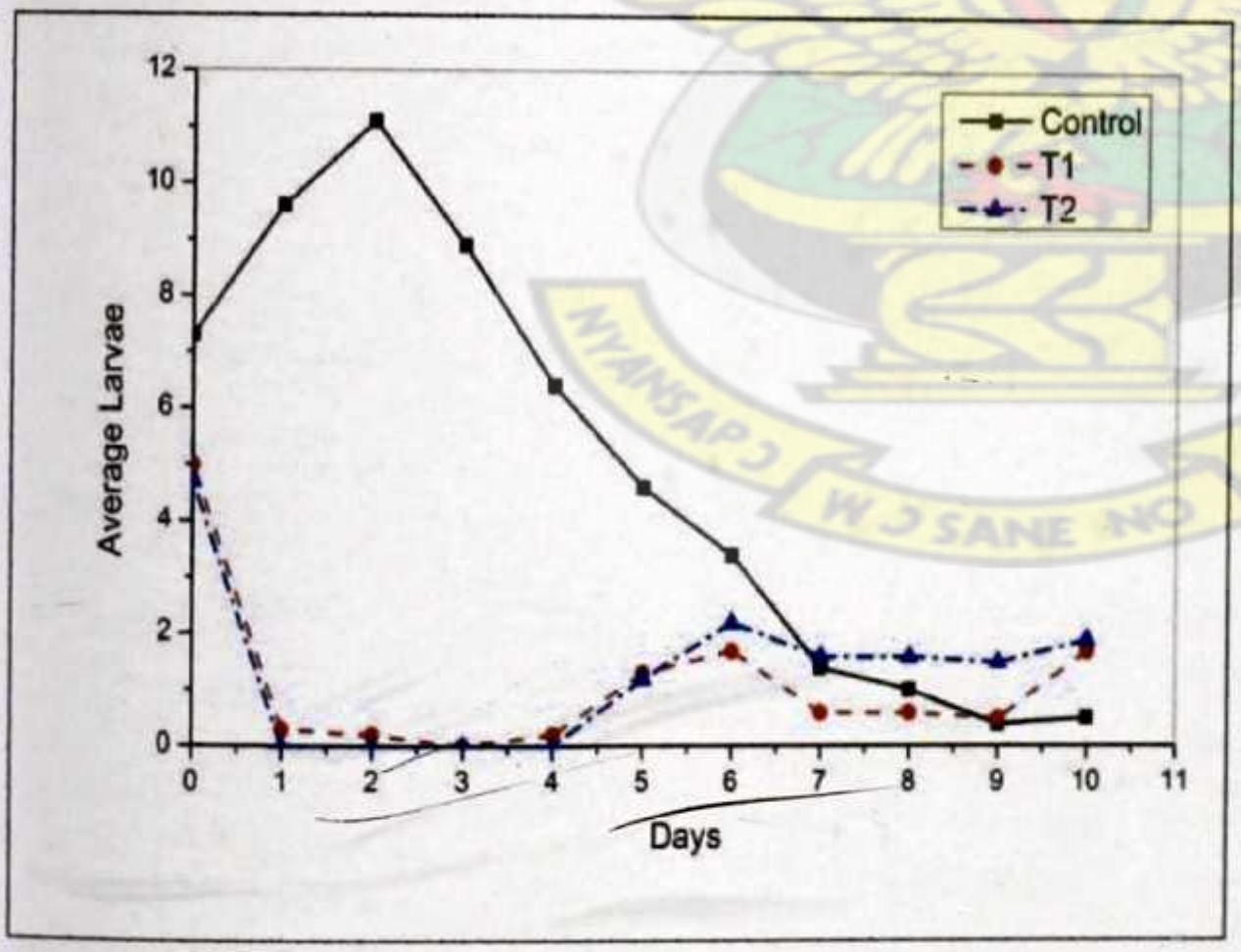


Figure 16: Pop dynamics of late instars (rainy season)

Comparison test of early and late instars and pupae for the various treatments and seasons (α=1%) are presented in Table 3. The results revealed significant differences ($P < 0.01$) in the interaction between the biolarvicide (*Bti*) tested and the different stages of the mosquitoe. However, there were no statistically ($P<0.01$) significant differences between the 0.2mg/l and 0.4mg/l treatment concentration except in the early instar of both dry and rainy season (Table 3).

In both the dry and rainy seasons, of the late instars and pupae, there were significant differences between the control and both concentrations tested (i.e. T1 and T2) but no statistically significant difference ($P < 0.01$) was observed between the effect of 0.2mg/l and 0.4mg/l treatment concentrations (Table 3).

Table 3: Comparison test of early & late instars and Pupae for the various treatments and seasons

Stages	Season	Treatment	Grouped mean	SEM
Early instar	DRY	Control	26.86^{B}	1.163
		T1	30.62^{A}	
		T2	20.41^{C}	
	RAINY	Control	15.364^{D}	
		T1	21.909^{C}	
		T2	13.742^{D}	
Late instar	DRY	Control	24.818^{A}	0.7015
		T1	5.561^{C}	
		T2	6.682^{C}	
	RAINY	Control	15.773^{B}	
		T1	6.652^{C}	
		T2	456.5^{C}	
Pupae	DRY	Control	7.773^{A}	0.3305
		T1	0.409^{C}	
		T2	0.0909^{C}	
	RAINY	Control	5.955^{B}	
		T1	0.409^{C}	
		T2	0.0909^{C}	

Superscripts A, B and C represent statistical difference between the means of the various treatments

4.3.2 DRY SEASON

The initial (Day 0) average number of *Anopheles* larvae per dip before the *Bti* formulation was added (pre-treatment) were 8.2 for the control and 10.2 and 11.6 for the treatment concentrations (T1 and T2 respectively) (Table 4).

Using the *Bti* formulation at 0.2 mg/l concentration, after 24 hours, average number per dip recorded was 0.8 (95% of the larvae died off) whilst at 0.4 mg/l average number of larvae per dip after 24 hour was 0.2 (99% of the larvae died off). The number of larvae remained low till the third day when the average number per dip recorded was 6.5 (0% in larval mortality) which was recorded in the early instar (0.2mg/l concentration).

However, in the control bowls, the average number of larvae per dip rather increased from 8.2 on day 0 to 12.0 on day1, larval density however remained high till the fifth day in the control bowls where it decreased to 3.5 (Table 4).

Considering the late instars only, reduction rates of 51-100% could be observed up to the fourth day after treatment (Table 4). *Bti* was very effective against the late instars reducing the population by 93-97% within 24 hour of post treatment for T1 and T2 concentrations respectively. The reduction even increased gradually to 100% within 72 hours. Generally, larvicidal impact on the late instars remained high up to day 4th post treatment with *Bti*.

Bti was effective against early instars of *An. gambiae* with a reduction of 96%-100% of the larval population within 24 hours. This effect lasted up to day 2 after application (Table 4). The larvicidal impact decreased on the 3rd day, where percentage mortality was recorded at 0% and 53% for T1 and T2 respectively (Table 4).

4.3.3 RAINY SEASON

In the rainy season, the average number of *Anopheles* larvae per dip recorded before the *Bti* formulation was added (pre-treatment) was slightly higher than the numbers recorded in the dry season. The initial (Day 0) average numbers in the control and treatment bowls were 15.2 for the control and 15.2 and 16.3 for the treatment concentrations (T1 and T2 respectively) (Table 5).

Using the *Bti* formulation at 0.2 mg/l concentration, after 24 hours, average number per dip recorded was 0.5 (97% of larvae died) whilst at 0.4 mg/l average number of larvae per dip after 24 hour was 0.0 (100% of larvae died). The number of larvae remained low till the third day when the average number per dip recorded was 15.4 (0% in percentage reduction) of early instar (0.2mg/l concentration). However, in the control bowls, the average number of larvae per dip rather increased from 15.2 on day 0 to 19.0 on day 1. The average number of larvae per dip in the control bowls remained relatively high till the fifth day and decreased to 9.9 (Table 5).

Considering the late instars only, reduction rates of 59-100% could be observed up to the fifth day after treatment (Table 5). *Bti* was very effective against the late instars reducing the population by 95-100% within 24hour of post treatment (Table 5). Larvicidal impact on the late instars in the rainy season remained high up to day 4 post treatment with *Bti* (Table 5).

Bti was effective against early instars of *An. gambiae* with a reduction of 98-100% of the larval population within 24 hours (Table 5). This effect lasted up to day 2 post application (Table 5). The larvicidal impact decreased on the 3rd day, where reduction in larval density was recorded as 0% and 55% for T1 and T2 respectively (Table 5).

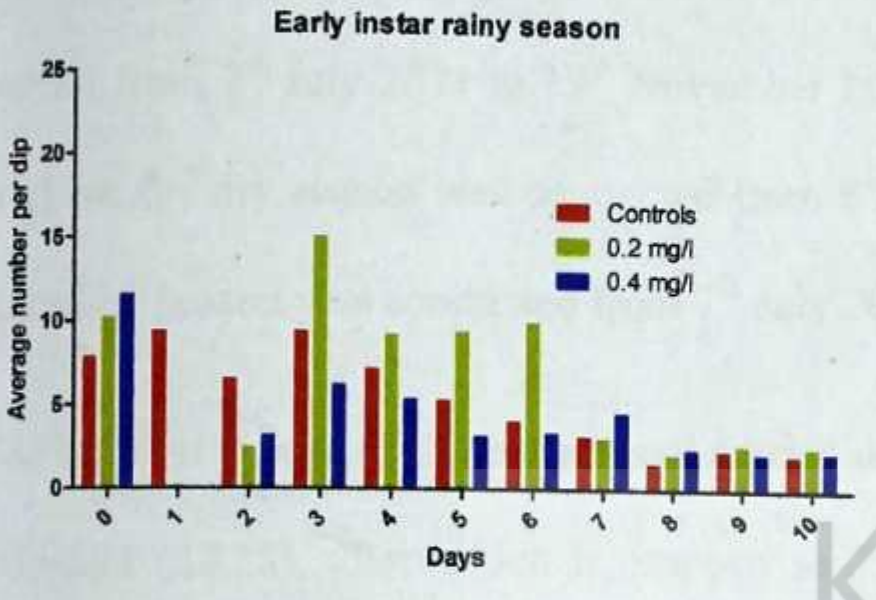
Though the *Bti* formulation resulted in high mortalities on the early and late instars of *An. gambiae* with over 97% reduction within 24 hrs, it showed drastic reduction 48 hrs after application, as dips taken three to four days after treatment indicated quick and continuing re-colonisation of all treated sites by early instars. Both concentration tested (0.2mg/l and 0.4mg/l), were equally effective up to 3 days post-treatment for the total number of larvae and up to 4 days when considering the late instars only (Table 5). There were no statistically significant differences between the 0.2mg/l and 0.4mg/l treatment concentrations (Table 3).

PUPATION LEVELS

Pupation levels were very low in all the treatment containers (Figure 17). Pupation levels determine to a large extent the number of adult mosquitoes that will emerge. All treatments were effective at lowering pupal population and an overall reduction in mosquito emergence was achieved.

Anopheles adults that emerged from pupae collected from the control containers were identified during the study period, using the morphological keys of Gillies and Demeillon (1968) and Gillies and Coetzee (1987), and found to be *Anopheles gambiae* Giles complex.

A



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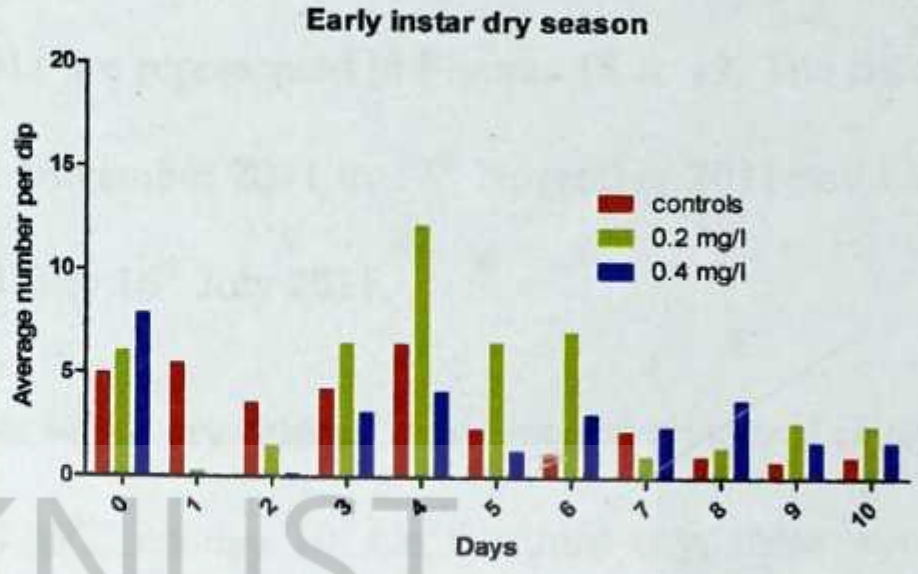

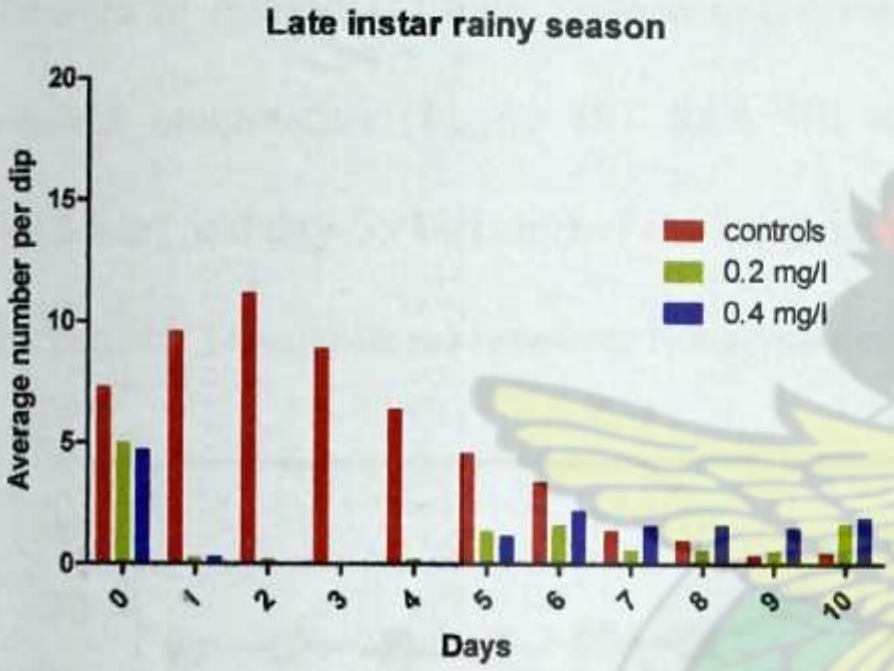

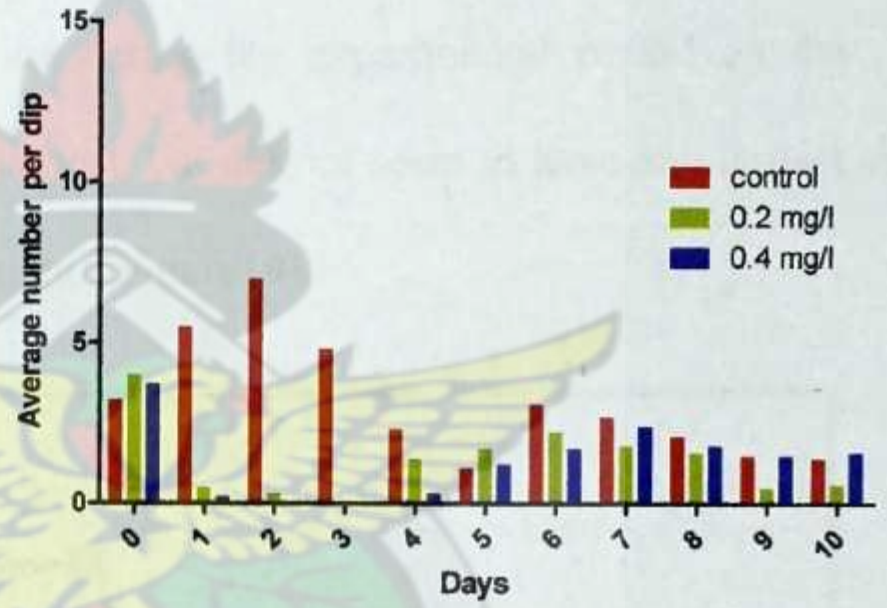

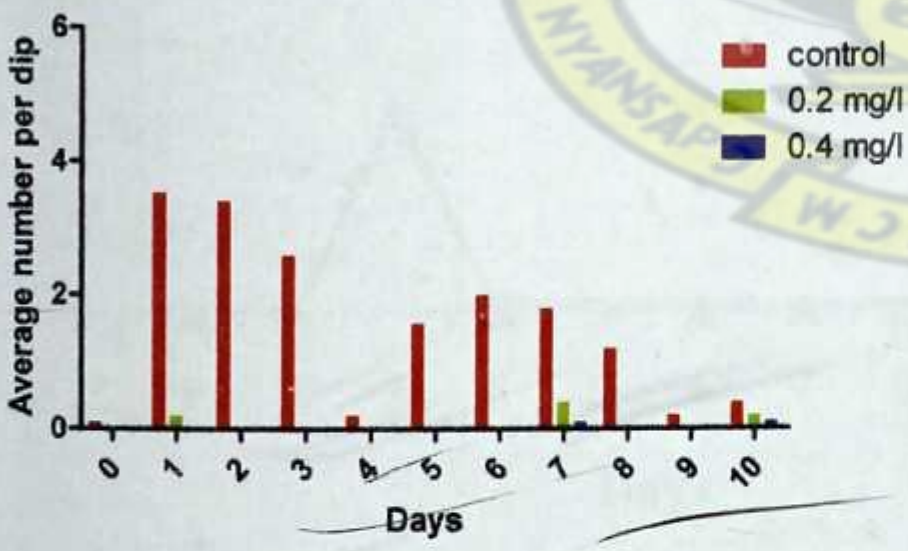

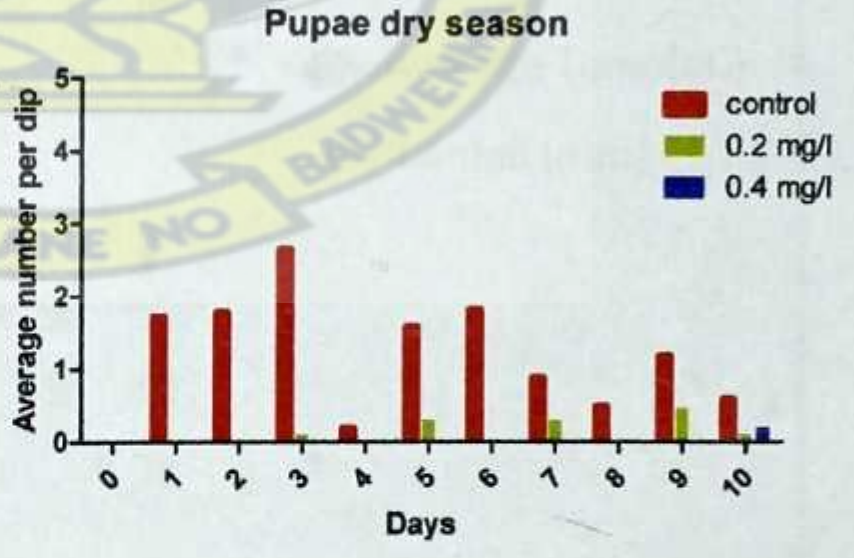

Figure 17: Effect of Bti WDG formulations on densities of late and early instar A. gambiae in simulated field trials with varying conc (s) for dry and rainy season.

CLIMATE

The average minimum and maximum temperature and the daily rainfall patterns during the study period from 7th July 2011 to 17th November 2011 are represented in Figures 18 & 19. The field trial for the dry season was conducted from 8th November 2011 to 17th November 2011 and for the rainy season was conducted from 7th July 2011 to 16th July 2011.

For the first 9 days of the experiment carried out in the dry season, temperatures remained fairly constant (27.25), after which it dropped to 25.85°C on day 10. On the third day, there were showers of rainfall (11.6mm) which lasted for five hours but did not have any impact on the average temperature (Figure 18). Rain fell twice during the experimental period on day 1 (46.5mm) and day 9 (18.1mm).of the rainy season test, but did not seem to have any impact on the results. Temperatures however remained constant. (Figure19).

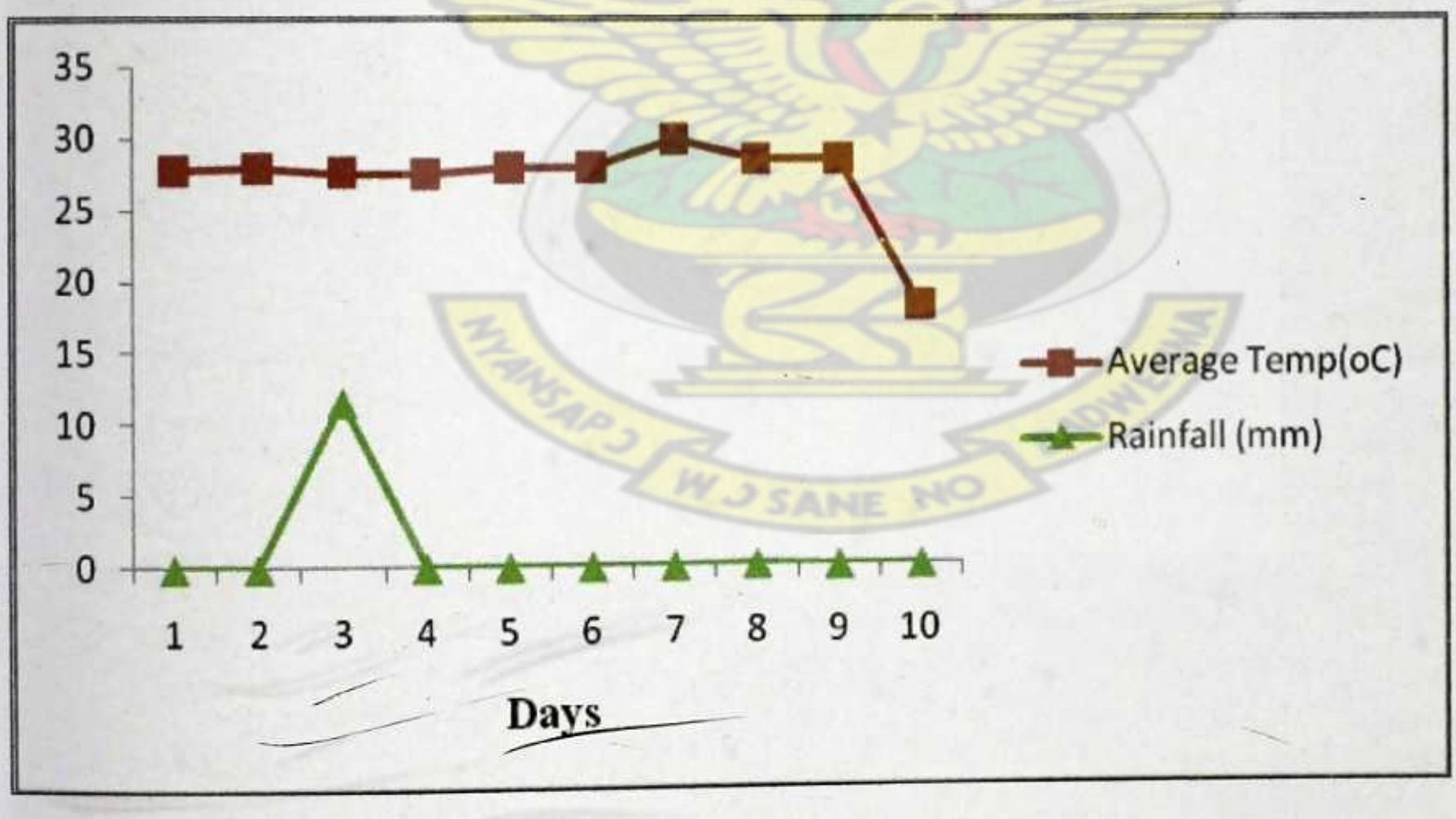

Figure 18: The temperature and rainfall graph (dry season) for the study period

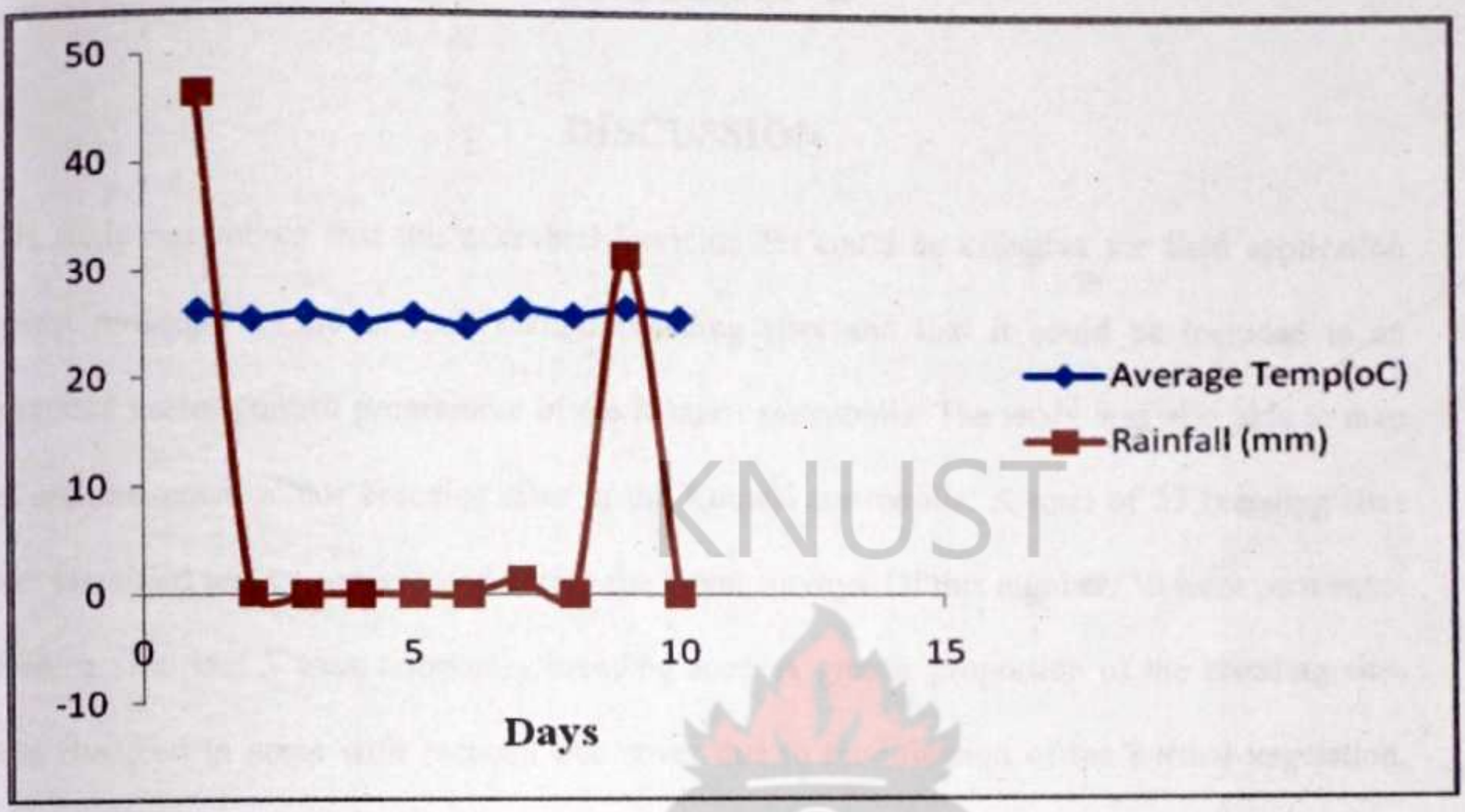

Figure 19: The temperature and rainfall graph (rainy season) during the study period

CHAPTER FIVE

DISCUSSION

This study has shown that the microbial larvicide *Bti* could be effective for field application against mosquito larvae in their natural breeding sites and that it could be included in an integrated vector control programme in the Kumasi metropolis. The study was also able to map out and document major breeding sites in the Kumasi metropolis. A total of 33 breeding sites were identified and geo-referenced during the larval surveys. Of this number, 30 were permanent breeding sites and 3 were temporary breeding sites. A greater proportion of the breeding sites were clustered in areas with reduced tree cover due to modification of the normal vegetation. This corresponds to the findings of Pinault and Hunter, (2012) who also attributed land and human modifications to the creation of man-made breeding grounds for *Anopheles* species.

Asokwa sub-metro showed the highest number of breeding sites (27%). Land use changes; cutting down the vegetation to make way for various construction activities and urban vegetable farming are on the rise within the sub-metropolis and this could explain the high number of breeding sites. Afrane *et al.,* (2004) also reported high *Anopheles gambiae* numbers in open space vegetable farms within the Kumasi metropolis. This finding agree with similar reports of Lindblade *et al.*, (2000) that replacement of natural swamp vegetation with agricultural crops lead to increased temperatures which may be responsible for elevated malaria transmission risk in cultivated areas.

Generally, *Anopheles* larvae were found breeding in either dug out wells, ditches of furrow systems between raised vegetable beds or in human foot prints, sand pools or ponds, edges of streams, drainage channels on sugar cane and temporary pools created after rains. However, ponds were less productive for *Anopheles* larvae. It has also been reported that *Anopheles* females preferentially select small, open habitats for oviposition (Minakawa *et al.*, 1999). Secondly, larval numbers were high in temporary habitats because there is little or absence of larval predation in such temporary habitats (Sunahara *et al.*, 2002) and this explains why most permanent breeding sites that were found with tadpoles and water bugs had very few mosquito larvae or none. Dirty water such as domestic waste water in chocked gutters were characterised by high numbers of *Culex* larvae but no *Anopheles* larvae. This is consistent with the observation made by Calhoun *et al.*, (2007) that *Culex* species principally breed in waste or dirty water.

In the laboratory bioassay, concentrations of 0.026 mg/l and 0.136 mg/l resulted in 50% and 95% mortalities respectively after 24 hours of exposure to *Bti* WDG (VectorBac@, 3000 ITU/mg), similar to 0.039mg/1 (LC_{50}) and 0.132mg/1 (LC_{95}) in Gambia (Majambere *et al.*, 2007) and 0.021mg/1 (LC_{50}) and 0.21mg/1 (LC_{95}) in Kenya (Fillinger *et al.*, 2003). The similarities in vulnerability of the *Anopheles* larvae to the *Bti* in studies conducted in East Africa (Seyoun and Abate, 1997; Fillinger *et al.*, 2003) and West Africa (Majambere *et al.*, 2007) confirms that biolarvicidal activity is intrinsic to the mosquito but not ecologically determined (Charles and Nielsen-LeRoux, 2000). However the concentration, 0.136mg/1 obtained in this study as the LC_{95} depicts that *Bti* formulation is highly effective against *Anopheles* larvae, in the Kumasi metropolis.

Concentrations of 0.2 mg/l and 0.4 mg/l were subsequently tested in open field trials and the results revealed that both concentrations tested were equally effective up to 3 days post-treatment for the total number of larvae and up to 4 days when considering the late instars only.

The controlled field experiments during both the rainy season and the dry season had the residual effect for Bti to be 3 to 4 days and compares to studies carried out in several areas, in Cuba (Lago *et al.*, 1991), Thailand (Mulla *et al.*, 1999), Kenya (Fillinger *et al.*, 2003) and Gambia (Majambere *et al.*, 2007).

The bowls were exposed to the whole array of environmental factors such as water quality, sunlight, which are also typical for other mosquitoe breeding places and the high numbers of immature stages of *An. gambiae* indicate that water bodies on KNUST campus represent dominant sources of *An. gambiae*. The results of the control bowls demonstrate that there is a steady supply of young instars from eggs which are not affected by the larvicide. The observed fluctuations implied by larval populations have been reported in other studies (Mulla *et al.*, 1999).

The number of larvae that were oviposited in the bowls during the controlled field trial in the rainy season were more in number than the number of larvae that oviposited during the dry season and this is because the *Anopheles* breeds mostly during the rainy season than in the dry season (Appawu *et al.*, 1994) and there are reports of relatively high incidence of malaria in Ghana during the rainy season than in the dry season (Afari *et al.*, 1995).

Anopheles adults that emerged from pupae collected from the control containers were identified during the study period, using the morphological keys of Gillies and Demeillon (1968) and Gillies and Coetzee (1987), and found to be *Anopheles gambiae* Giles complex. The reason could be that abiotic conditions were conducive for *Anopheles gambiae* species.

There was no statistically significant difference between the effects of the two different concentrations tested.

It was observed from the study, that the containers with high larval density recorded lower instar mortality compared to containers with low larval density after the *Bti* formulation was administered. This observation confirms findings made by Becker, (1992) and Nayar *et al.*, (1999). Mulla *et al.*, (1990) also showed that denser populations of larvae (50-100 larvae per dip) will require 1.5-2 times more material than the low-density populations (5-20 larvae per dip) to yield equal mortalities. Therefore, it will be necessary in the field to make subjective adjustments of rates of *Bti* applications depending upon prevailing larval densities (Nayar *et al.*, 1999).

Additionally, containers that had high rates of organic pollution and the presence of colloidal particles had lower larval mortality compared to the other containers that had less colloidal particles. This could be due to the fact that in the presence of organic and floating materials, fewer toxin particles are ingested by the larvae at a time than in the absence of these materials. Moreover, the availability of crystals will be decreased by their adsorption onto suspended particles followed by a slow sedimentation. In both cases (high density and pollution), higher rates of application will be necessary to control mosquito larvae (Mulla *et al.*, 1990; Becker, 1992). Ohana *et al.*, (1987) have also shown that contact with mud of a sporal culture of a mutant resulted in an immediate disappearance of the larvicidal activity but had no influence on viability. Thus inactivation of the toxic activity of *B. thuringiensis* var. *israelensis* in the mud was a reversible process and was due to bacteria adsorption on the soil particle thus making the bacteria and its toxins inaccessible to the larvae.

Similarly, Margalit and Bobroglo (2009) reported that the efficacy of *Bti* against 2nd stage larvae of mosquitoes decreased when organic matter was present in the water, or when sterilised silt was added.

During this study, the dominant predator was tadpole and this might be due to the fact that the trials were conducted on a wetland. It was however observed that in experimental containers where the tadpole numbers increased the number of early and late instars decreased or were relatively lower. The predation prey association could not be specifically determined just by counting larvae alive at any point to the number of tadpoles present because the larvae might have died due to other external influence other than predation. In nature *Anopheles gambiae* typically breed in temporal habitat where predators are not present or their presence is relatively low (Tvedten, 1997).

The number of late instar observed after *Bti* treatment during the rainy season was more than the number noted during the dry season. This may be due to the temperature of the water during the rainy season, which was lower than in the dry season.

Nayar *et al.* (1999) showed that lower temperatures (15°C) of water slows the development of larvae, with the result that larvae consume fewer nutrients (and also less endotoxins) and apparently become less susceptible. Higher temperatures (35°C) accelerate development of larvae, with the result that the larvae consume more nutrients and become more susceptible. Although *Bti* was found to be active at low temperatures, its effectiveness may be reduced in cold water due to a cessation or a low rate of feeding of some species of larvae, larval diapause and a decrease in metabolic rate (Boisvert, 2005).

Similar observations were reported on other mosquitoe species by Mulla *et al.* (1990) and Becker, (1992). Thus, higher field application rates of *Bti* may be necessary during the rainy season due to low water temperatures than during the dry season in order to achieve the same level of control.

Finally, increased sunlight has been shown to lower the efficacy of *Bti* (Becker, 1992). Therefore, in Ghana where the intensity of sunlight as well as the water temperature is high especially in the dry season, the combined effect of higher intensity of sunlight and high temperatures can reduce the potency of *Bti* formulations substantially. This suggests that ideally, field applications of *Bti*, should be made during the later part of the day (after 5:00pm) rather than in the morning hours, particularly during the dry season.

5.1 RESIDUAL EFFECT OF *Bti*

In both the rainy and dry season, there was a reemergence of late instar larvae after the 4^{th} and 5^{th} day post application of the *Bti* (Figure 16). This suggests that *Bti* has very low residual effect and the observation made in this study conforms to findings made by Karch *et al.* (1991) and others in the Democratic Republic of Congo, who found that larval population begun to recover 5-7 days after treatments at the latest, irrespective of the *Bti* concentration applied (2000-5000 ITU/l, in 0.1m water depth). This lack of residual effects of *Bti* has been reported previously by Das and Amalrg, (1997) in Western Kenya.

In the study conducted in the Democratic Republic of Congo, Karch *et al.* (1991) proposed a surface application regime of once every week for *Bti* to achieve >95% reduction on mosquito

emergence from breeding sites. Results from the open field also showed that a very low dosage of 0.2mg/l is required to effectively suppress late instars and the resulting pupae as seen in Table 3 and 4.

This value corresponds well with the LC_{95} of the laboratory tests and represents the optimum effective dosage to control *An. gambiae* in Ghana. This low applications dosages offer the possibility of keeping operational cost low even if weekly treatments, caused by the absence of residual activity, have to be considered.

The results points to the fact that, with commercially available microbial, weekly larviciding using *Bti* will be necessary in Ghana (especially during the rainy season) since the cost of *Bti* is relatively low and the development of resistance is unlikely. From this research, very low dosages of 0.2mg/l and 0.4mg/l lead to the optimal suppression of the mosquito larvae and pupae and this is consistent with results from East Africa (Seyoun and Abate, 1997; Ragoonanansingh *et al.*, 1992).

Nevertheless, it would be useful to explore whether greater persistence could be achieved with alternative products. Organophosphates like temephos, appear to be less useful since they rarely show much persistence compared with microbials. Moreover, organophosphates can have a negative impact on non-target organisms and need careful resistance management. Microbial larvicides have several advantages over other mosquito control agents not only do they have high efficacy but also environmental safety and safety to human consumption, for instance when applied in drinking water (WHO, 1999) makes them powerful vector control agents in Africa and other parts of the tropics. The results show that the major malaria vector in Ghana is highly susceptible to *Bti* under laboratory and field conditions.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The study mapped out breeding sites in the Kumasi metropolis which will provide a reference for future control programs by the Ghana Health Service, Kumasi Metropolitan Assembly and other Policy Makers that may seek to reduce the risk and exposure to malaria in the metropolis. The study also presents the possibility of using GIS tools for health research, in Ghana and Kumasi in particular, where GIS application in the health sector has not been exploited extensively.

Land use changes; construction projects, farmlands and agriculture activities close to water bodies or wetlands accounts for the presence and abundance of breeding sites in the various sub-metros.

Anopheline larvae were found breeding in conduits on vegetable farms, puddles collected in human footprints, rain-pools and in swamps. The Anopheline habitat identified were characterised by clear or turbid shallow and sunlit conditions. In breeding sites where habitat tadpoles, dragonflies, and water bugs, etc were present, very few or no mosquito larvae were found.

The minimum effective dosages of *Bti* formulation with 3,000 International Toxic Units (ITU)/mg were 0.026mg/l for LC_{50} and 0.136 mg/l for LC_{95}.

This study confirms the lack of residual activity of *Bti* and therefore recommend that it be applied weekly especially during the rainy season. The cost of weekly application in consideration of reduction in transmission intensity should therefore be carefully assessed.

Results from the open field trials with *Bti* however showed that a very low dosage of 0.2mg/l is required to effectively suppress late instars and resulting pupae, and such low application dosages offer the possibility to keep operational costs low even if weekly treatments due to the lack of residual activity of *Bti* have to be considered

The target mosquito larvae tested (larvae of *An. gambiae* complex) were extremely sensitive to the *Bti* formulation, with the most sensitive stage being the early instars.

6.2 RECOMMENDATION

Larval control using *Bti* should not be considered as a stand-alone intervention, it represents an approach that could be incorporated into many integrated malaria control programmes, tailored to the local ecoepidemiological setting and fine tuned over time.

The variety and occurrence of breeding sites in the Kumasi metropolis is a reflection of the need for further mapping of all breeding sites within the metropolis. The metropolitan assembly has a vital role to play in organising community-wide health educational programs aimed at increasing awareness on the correct attitudes and practices towards the prevention of malaria transmission and on environmental sanitation. This could promote community participation for effective malaria control in the study area.

Increased efforts from Health Policy Makers on malaria control are necessary to build research and operational capacity in Ghana to integrate the use of microbial larvicide *Bacillus thuringiensis* var *israelensis* into new and ongoing integrated vector control programmes.

Further studies are required to determine the contribution of larviciding in reducing the burden of malaria, by conducting a trial of *Bacillus* formulation in communities and also to demonstrate the economic and epidemiological impact of these interventions on malaria morbidity and mortality.

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LIST OF APPENDICES

Appendix I

Table 4: Effect of Bti WDG formulations on densities of late and early instar *A. gambiae* in simulated field trials with varying conc (s) for dry season (T1=0.2mg/l and T2=0.4mg/l).

	AVERAGE NUMBER PER DIP (Dry Season)									PERCENTAGE REDUCTION					
	Total Instars			Late Instars			Early Instars			Total instars		Late instars		Early instars	
Day	Control	T1	T2	Control	T1	T2	Control	T1	T2	T1	T2	T1	T2	T1	T2
0	8.2	10.2	11.6	3.2	4.0	3.7	5.0	6.2	7.9	0	0	0	0	0	0
1	12.0	0.8	0.2	5.5	0.5	0.2	6.5	0.3	0.0	95	99	93	97	96	100
2	10.6	1.8	0.2	7.0	0.3	0.0	3.6	1.5	0.2	86	99	97	100	66	96
3	9.1	6.5	3.2	4.8	0.0	0.0	4.3	6.5	3.2	43	75	100	100	0	53

4	8.8	13.7	4.5	2.3	1.4	0.3	6.5	12.3	4.2	0	64	51	89	0	59
5	3.5	8.2	2.5	1.1	1.7	1.2	2.4	6.5	1.3	0	50	0	6	0	66
6	4.3	9.4	4.9	3.1	2.2	1.7	1.2	7.2	3.2	0	19	43	53	0	0
7	5.0	2.9	4.9	2.7	1.8	2.4	2.3	1.1	2.5	53	31	47	23	61	31
8	3.2	3.1	5.6	2.1	1.6	1.8	1.1	1.5	3.8	22	0	39	26	0	0
9	2.3	3.2	3.3	1.5	0.5	1.5	0.8	2.7	1.8	0	0	73	14	0	0
10	2.5	3.2	3.4	1.4	0.6	1.6	1.1	2.6	1.8	0	4	66	1	0	0

Table 5: Effect of Bti WDG formulations on densities of late and early instar *A. gambiae* in simulated field trials with varying conc (s) for rainy season (T1=0.2mg/l and T2=0.4mg/l)

	AVERAGE NUMBER PER DIP (Rainy Season)									PERCENTAGE REDUCTION					
Day	Total Instars			Late Instars			Early Instars			Total instars		Late instars		Early instars	
	Control	T1	T2	Control	T1	T2	Control	T1	T2	T1	T2	T1	T2	T1	T2
0	15.2	15.2	16.3	7.3	5.0	4.7	7.9	10.2	11.6	0	0	0	0	0	0
1	19.0	0.5	0.0	9.6	0.3	0.0	9.4	0.2	0.0	97	100	95	100	98	100
2	17.6	2.7	3.2	11.1	0.2	0.0	6.5	2.5	3.2	85	83	97	100	70	66
3	18.3	15.4	6.2	8.9	0.0	0.0	9.4	15.4	6.2	16	68	100	100	0	55
4	13.6	9.4	5.4	6.4	0.2	0.0	7.2	9.2	5.4	31	63	95	100	1	49
5	9.9	10.7	4.4	4.6	1.3	1.2	5.3	9.4	3.2	0	59	59	59	0	59

6	7.5	11.7	5.6	3.4	1.7	2.2	4.1	10.0	3.4	0	30	27	0	0	44
7	4.6	3.7	6.2	1.4	0.6	1.6	3.2	3.1	4.6	20	0	37	0	25	2
8	2.6	2.7	4.4	1.0	0.6	1.6	1.6	2.1	2.8	0	0	12	0	0	0
9	2.8	3.2	3.7	0.4	0.5	1.5	2.4	2.7	2.2	0	0	0	0	13	38
10	2.6	4.3	4.2	0.5	1.7	1.9	2.1	2.6	2.3	0	0	0	0	4	25

Table 6: Average number of pupae per dip (rainy & dry season)

	Rainy Season			Dry Season		
Day	Control	0.2mg/l	0.4mg/l	Control	0.2mg/l	0.4mg/l
0	0.1	0	0	0	0	0
1	3.6	0.2	0	1.6	0	0
2	3.4	0	0	1.8	0	0
3	2.6	0	0	2.7	0.1	0
4	0.2	0	0	0.2	0	0
5	1.6	0	0	1.8	0.3	0
6	2	0	0	1.8	0	0
7	1.8	0.4	0.1	0.9	0.3	0
8	1.2	0.1	0	0.5	0	0
9	0.2	0	0	1.2	0.2	0
10	0.4	0.2	0.1	0.6	0.1	0.2

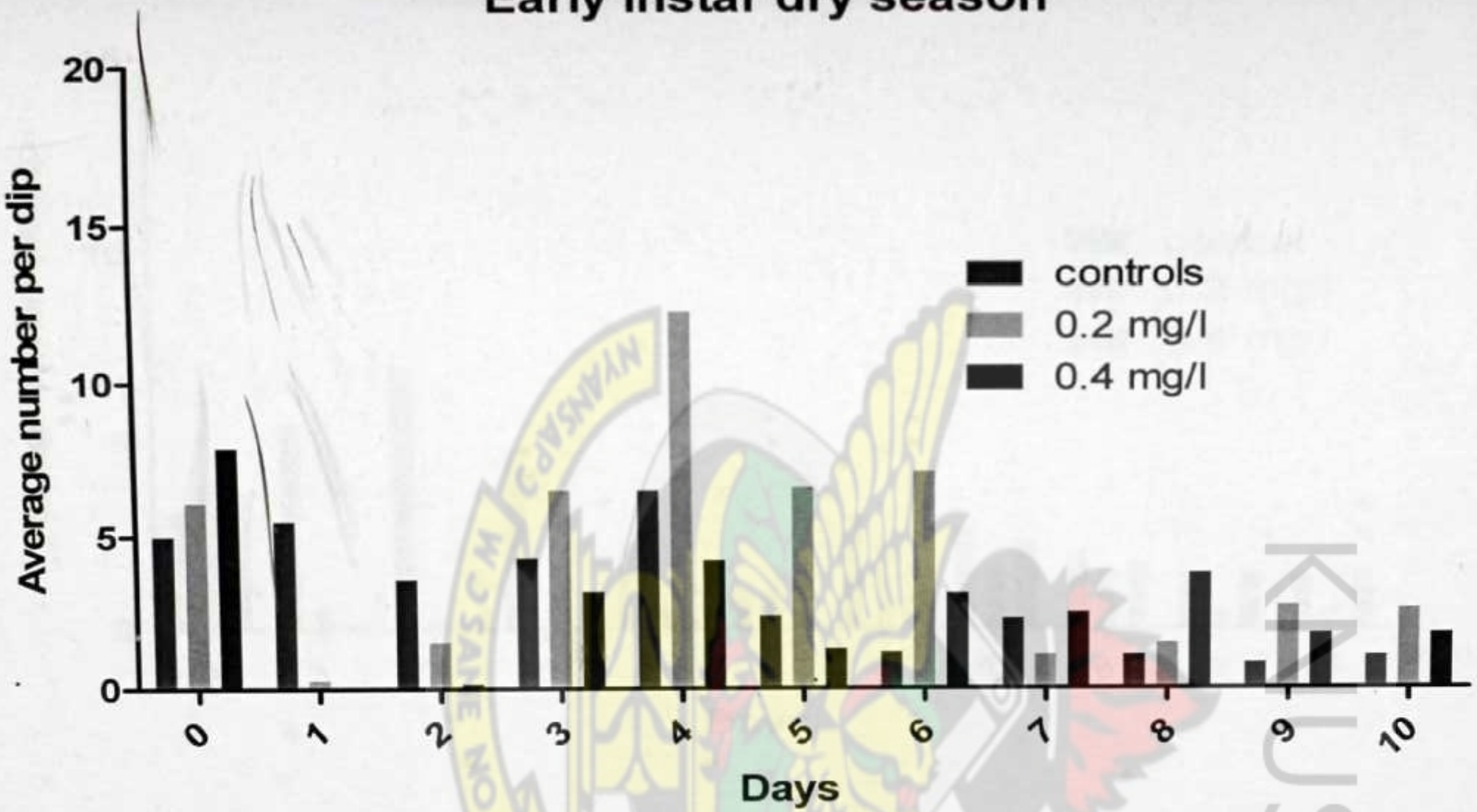

Figure 20: Effect of Bti WDG formulations on densities of early instar *A. gambiae* in simulated field trials with varying conc (s). (T1=0.2kg/ha and T2=0.4kg/ha)

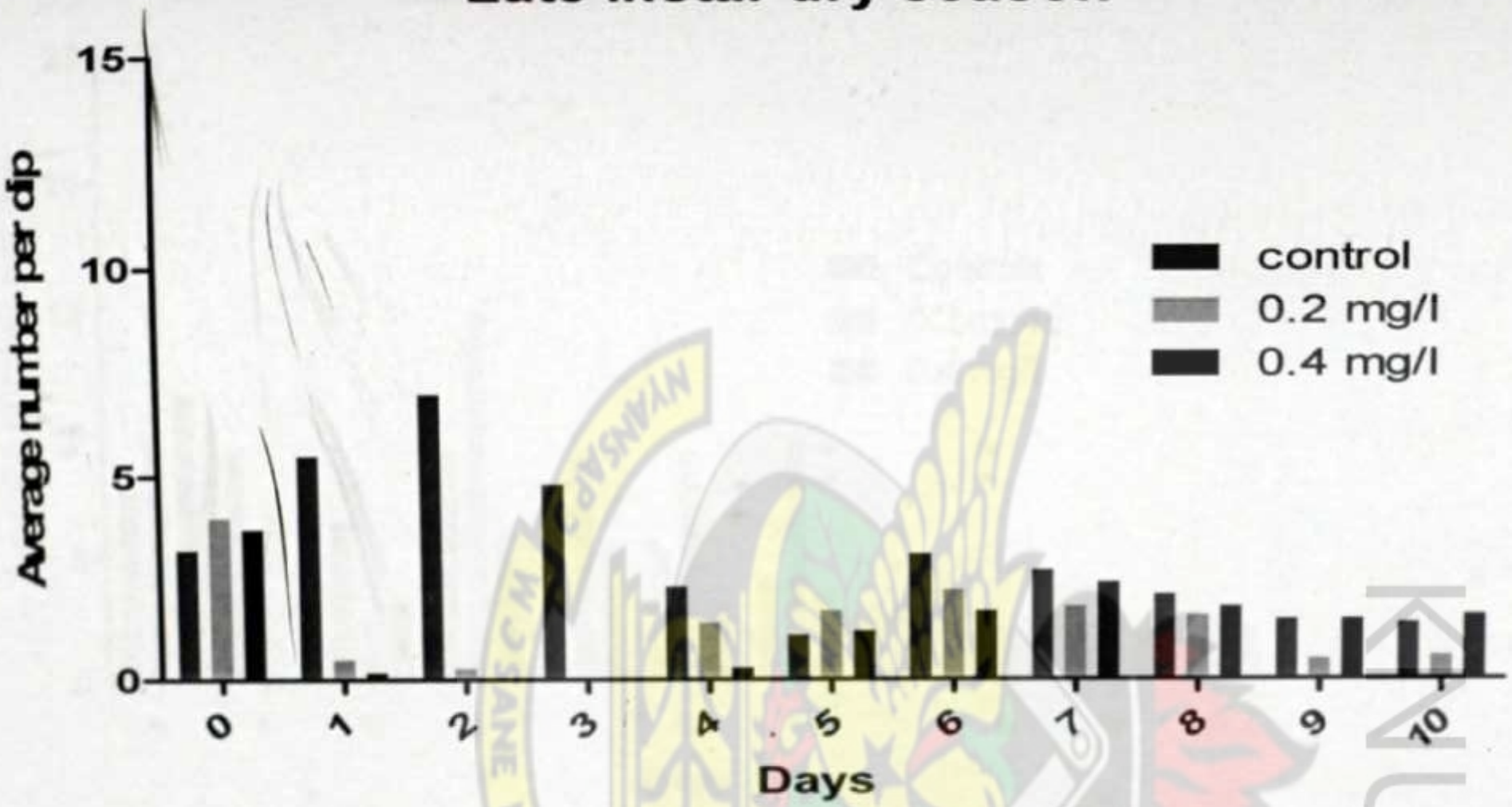

Figure 21: Effect of Bti WDG formulations on densities of late instar *A. gambiae* in simulated field trials with varying conc (s). (T1=0.2kg/ha and T2=0.4kg/ha)

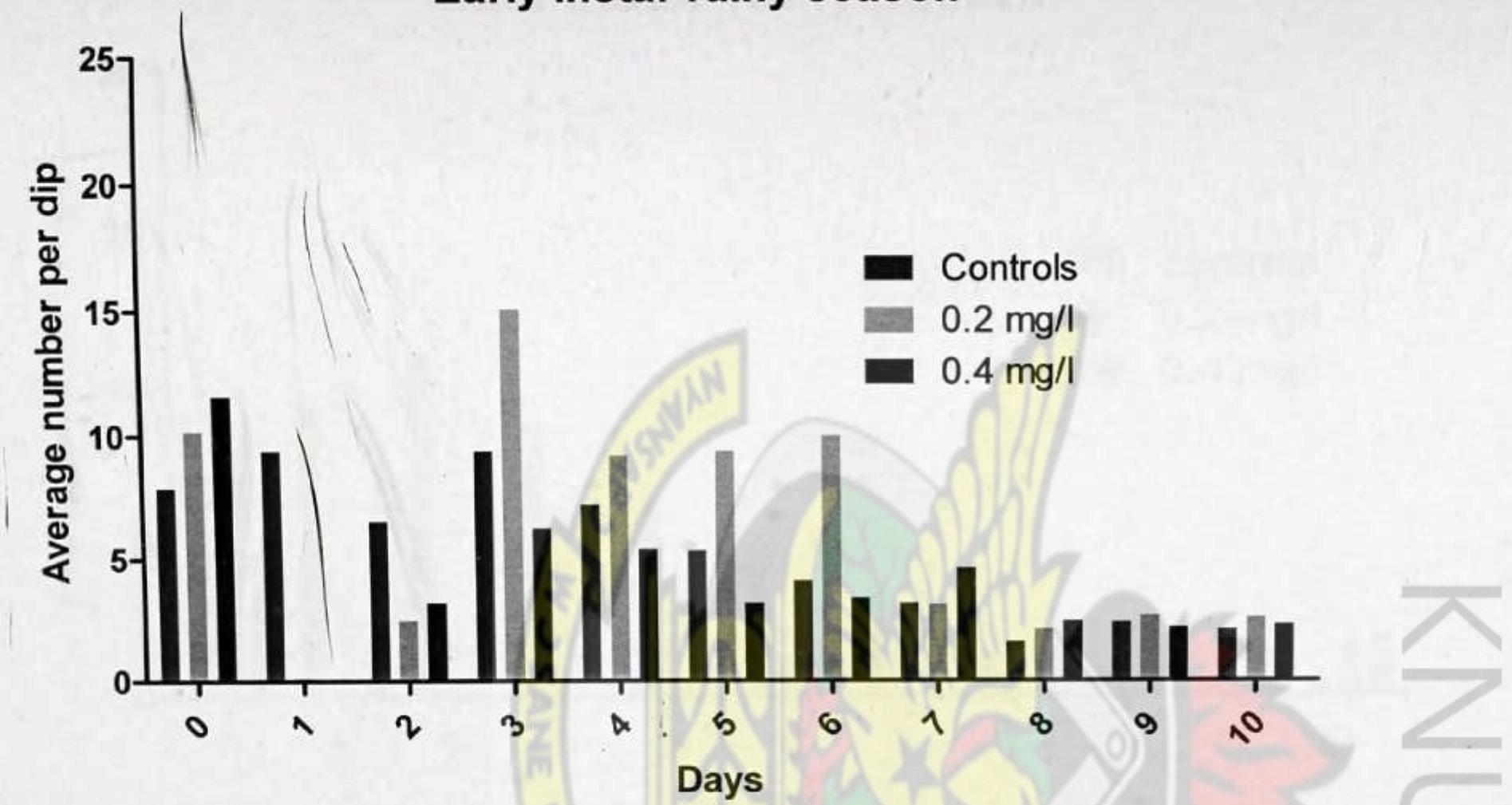

Figure 22: Effect of Bti WDG formulations on densities of early instar *A. gambiae* in simulated field trials with varying conc (s). (T1=0.2kg/ha and T2=0.4kg/ha)

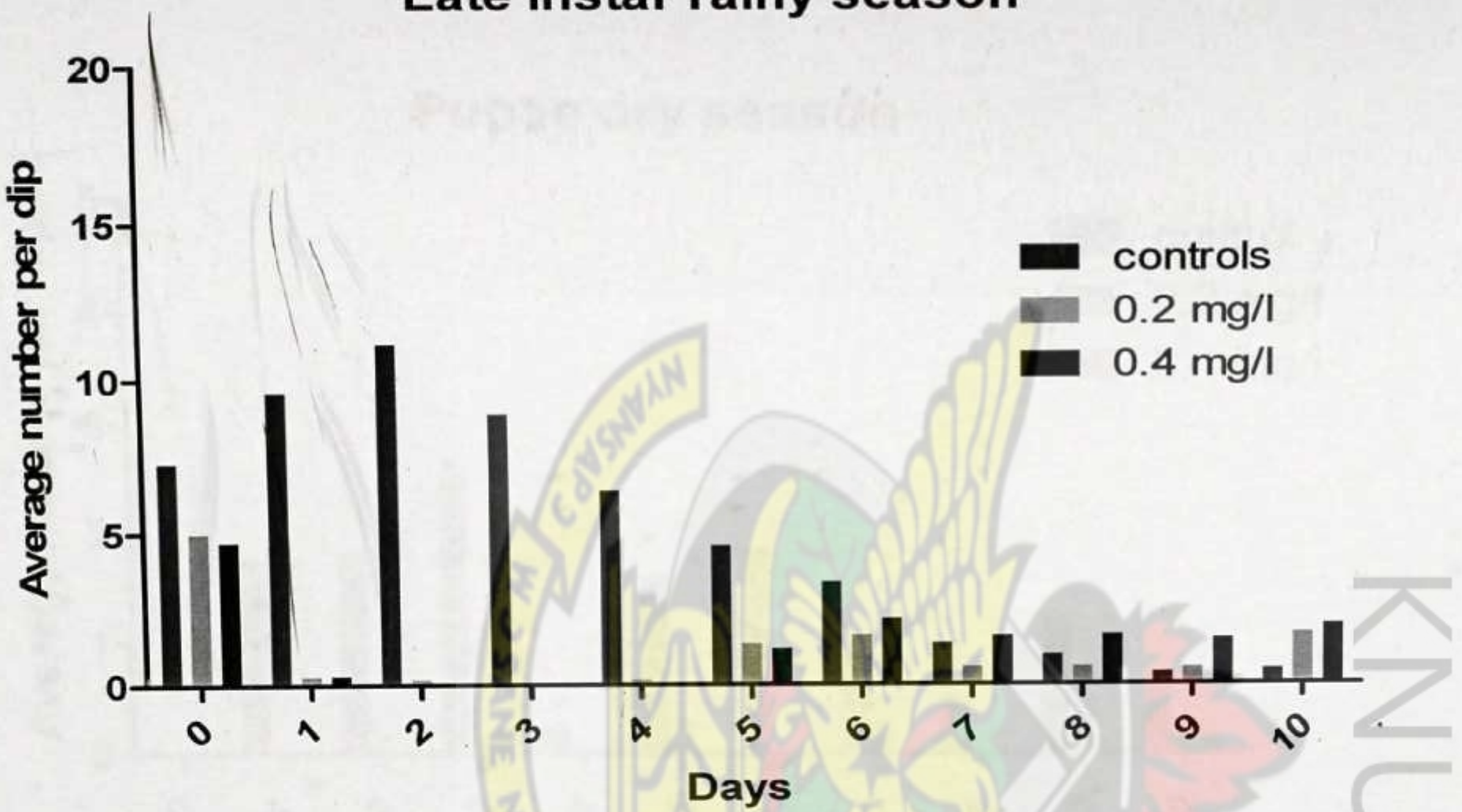

Figure 23: Effect of Bti WDG formulations on densities of late instar *A. gambiae* in simulated field trials with varying conc (s). (T1=0.2kg/ha and T2=0.4kg/ha)

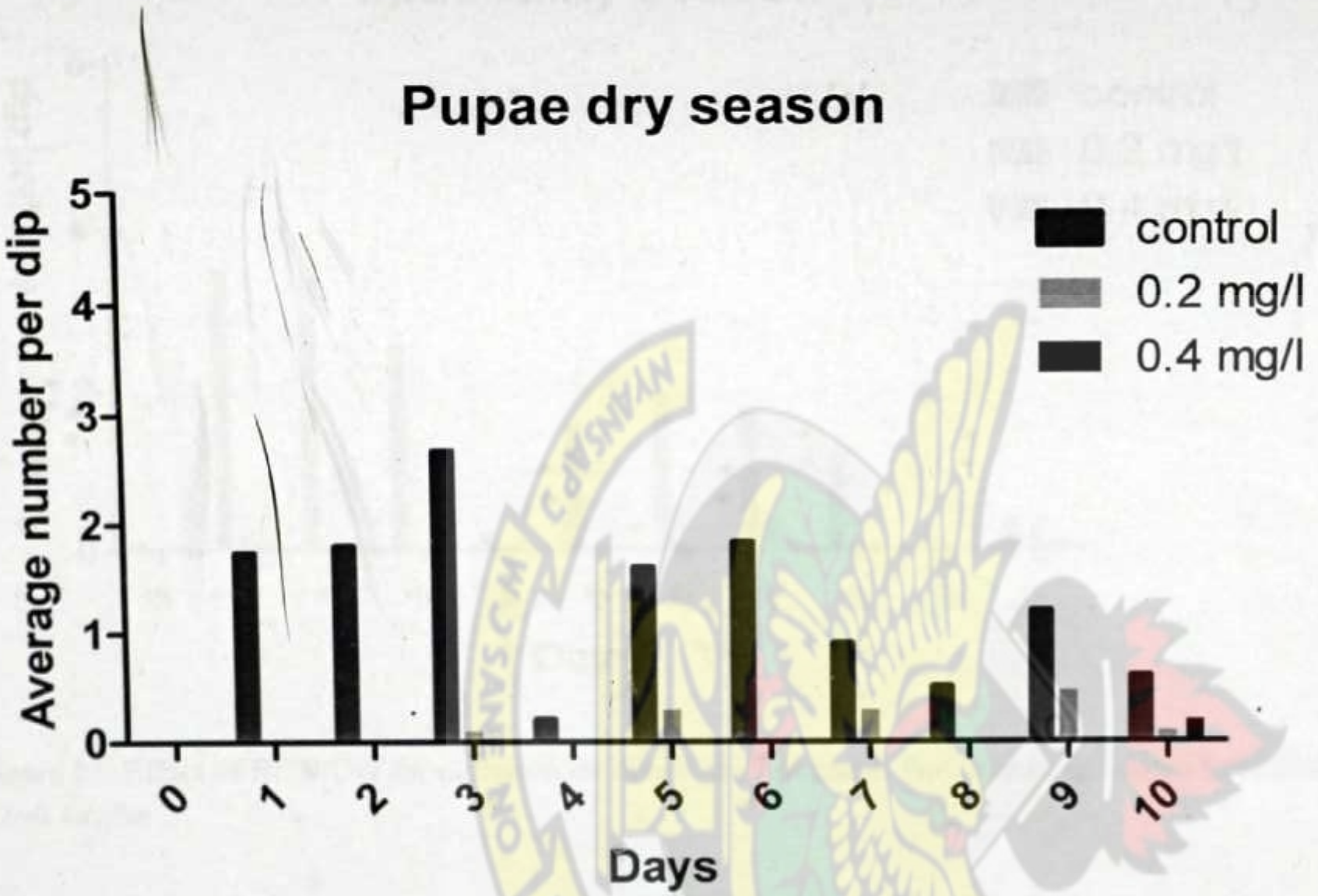

Figure 24: Effect of Bti WDG formulations on densities of pupae *A. gambiae* in simulated field trials with varying conc (s). (T1=0.2kg/ha and T2=0.4kg/ha

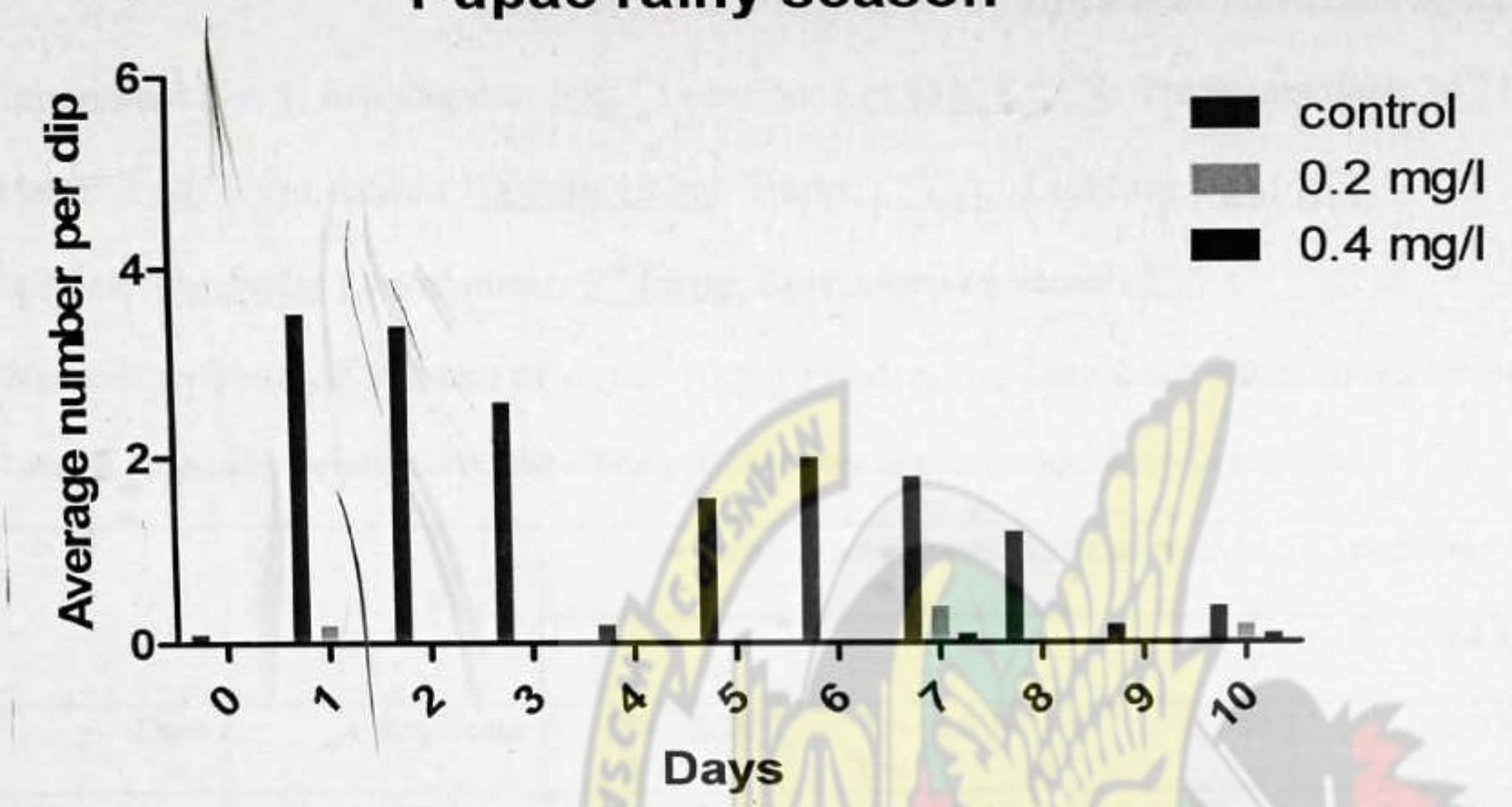

Figure 25: Effect of Bti WDG formulations on densities of pupae *A. gambiae* in simulated field trials with varying conc (s). (T1=0.2kg/ha and T2=0.4kg/ha

Appendix II

Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: 1. Investigator: Rita. Location: Ent.Lab, KCCR. Treatment date: 24th Feb. 2011

Material: *Bti* Formulation: 1000mg/100ml. Temp: 25°C ± 1Lighting: 25L: 25D

Species: *Anopheles* Larval instar: 3rd instar. Larvae/cup or vessel: 25

Water: Tap/Distilled Volume of water: 100ml Food: none. Date stock solution made: 24th Feb

Table 4: : Laboratory evaluation of the efficacy of larvicides against mosquito larvae experiment 1

		No of dead larvae at various conc (mg/L) post exposure (hrs.)						
		24 hrs						
Date	Replicate	0.00	0.001	0.002	0.004	0.008	0.016	0.032
	A	1	10	10	18	20	25	25
	1	0	10	10	17	18	25	25
	2	0	10	11	17	18	25	25
	3	2	10	10	17	18	24	25
	4	1	9	11	18	20	24	25
	Total	**4**	**49**	**52**	**87**	**94**	**123**	**125**

Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: 2. Investigator: Rita. Location: Ent.Lab, KCCR. Treatment date: 9th Mar 2011

Material: *Bti* Formulation: 1000mg/100ml. Temp: 25°C ± 1Lighting: 25L: 25D

Species: *Anopheles* Larval instar: 3rd instar. Larvae/cup or vessel: 25

Water: Tap/Distilled Volume of water: 100ml Food: none. Date stock solution made: 9th Mar

Table 5: Laboratory evaluation of the efficacy of larvicides against mosquito larvae for experiment 2

		No of dead larvae at various conc (mg/L) post exposure (hrs.)						
		24 hrs						
Date	Replicate	0.00	0.001	0.002	0.004	0.008	0.016	0.032
	B	0	10	12	17	19	25	25
	5	1	9	10	16	18	25	25
	6	0	10	10	18	19	25	25
	7	0	10	11	15	18	24	25
	8	0	9	12	17	18	24	25
	Total	**1**	**48**	**55**	**83**	**92**	**123**	**125**

Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: 3. Investigator: Rita. Location: Ent.Lab, KCCR. Treatment date: 23rd Mar 2011

Material: *Bti* Formulation: 1000mg/100ml. Temp: 25°C ± 1Lighting: 25L: 25D

Species: *Anopheles* Larval instar: 3rd instar. Larvae/cup or vessel: 25

Water: Tap/Distilled Volume of water: 100ml Food: none. Date stock solution made: 23rd Mar

Table 6: Laboratory evaluation of the efficacy of larvicides against mosquito larvae for experiment 3

		No of dead larvae at various conc (mg/L) post exposure (hrs.)						
		24 hrs						
Date	Replicate	0.00	0.001	0.002	0.004	0.008	0.016	0.032
	C	0	10	11	18	21	24	25
	9	0	11	10	17	19	25	25
	10	1	10	11	17	17	24	25
	11	1	10	12	18	18	25	25
	12	1	10	12	16	16	25	25
	Total	**3**	**51**	**56**	**86**	**91**	**123**	**125**

LABORATORY EVALUATION OF THE EFFICACY OF LARVICIDES AGAINST MOSQUITO LARVAE (COMBINED)

Table 7: Laboratory Evaluation of the efficacy of Larvicides against Mosquito larvae (Combined)

		No of dead larvae at various conc (mg/L) post exposure (hrs.)						
		24 hrs						
Date	Replicate	0.00	0.001	0.002	0.004	0.008	0.016	0.032
	A	1	10	10	18	20	25	25
	1	0	10	10	17	18	25	25
	2	0	10	11	17	18	25	25
	3	2	10	10	17	18	24	25
	4	1	9	11	18	20	24	25
	Total	**4**	**49**	**52**	**87**	**94**	**123**	**125**
	B	0	10	12	17	19	25	25
	5	1	9	10	16	18	25	25
	6	0	10	10	18	19	25	25
	7	0	10	11	15	18	24	25
	8	0	9	12	17	18	24	25
	Total	**1**	**48**	**55**	**83**	**92**	**123**	**125**
	C	0	10	11	18	21	24	25

	9	0	11	10	17	19	25	25
	10	1	10	11	17	17	24	25
	11	1	10	12	18	18	25	25
	12	1	10	12	16	16	25	25
	Total	**3**	**51**	**56**	**86**	**91**	**123**	**125**
	Average Total	**4**	**49**	**54**	**85**	**92**	**123**	**125**
	Average Mortality	0.16	9.8	10.8	17.0	18.4	24.6	25
	% Mortality	0.64	39.2	43.2	68.0	73.6	98.4	100

LC50: (CL 95%): _____
LC90: (CL 95%): _____
LC99: (CL 95%): _____
Slope: _____ **Heterogeneity:** _____