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EVALUATION OF THE EFFICACY OF WORMATAK® (TEFLUBENZURON AND CYPERMETHRIN) AGAINST *ANOPHELES GAMBIAE* SENSU LATO AND ITS IMPACT ON NON-TARGET ORGANISMS

BY

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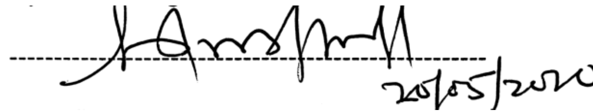
DECLARATION

This is to certify that, this thesis is the result of research undertaken by me, Jeffrey Mawuli Torgby at the Department of Animal Biology and Conservation Science and Vestergaard-NMIMR of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon towards the award of Master of Philosophy degree in Entomology at the African Regional Postgraduate Programme in Insect Science (ARPPIS), University of Ghana, Legon. This thesis has not been submitted either in part or in whole for any degree and all references to other people's work have been duly acknowledged.



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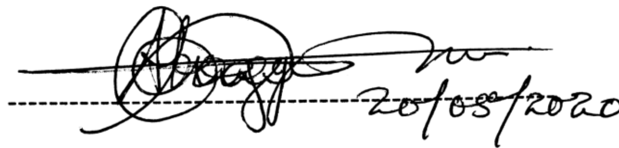

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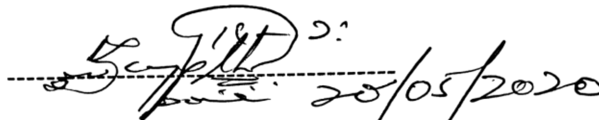

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DEDICATION

I dedicate this work to my parents, Godfrey Torgby and Comfort Abaidoo; all my siblings back home; my beloved, Esther Seyram Amemo; and all my lecturers and friends whose support, patience and encouragement have been very vital during the course of my study.

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ABBREVIATIONS

ACTs – Artemisinin-based Combination Therapies

CSIs – Chitin Synthesis Inhibitors

DDT - Dichlorodiphenyltrichloroethane

EC – Emulsifiable Concentrate

F1 – First Filial

IGRs – Insect Growth Regulators

IRS – Indoor Residual Spraying

ITNs – Insecticide Treated Nets

LC – Lethal Concentration

LD – Lethal Dose

LT – Lethal Time

µM – Micro Molar

ULV – Ultra Low Volume

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ABSTRACT

This study investigated the efficacy of WormAtak® against *Anopheles gambiae* s.l. larvae, pupae and adults (Diptera: Culicidae) collected from Opeibea and Madina in Ghana and its effect on three non-target organisms under laboratory conditions. *Anopheles gambiae* s.l. and the non-target organisms were all exposed to five concentrations of WormAtak® (1.86 µM, 3.71 µM, 5.57 µM, 7.42 µM and 9.28 µM). Mortality was recorded for a period of 72 hours for *Anopheles gambiae* s.l. F1 third instar larvae. Mortality readings for *Anopheles gambiae* s.l. pupae and female adults were taken after 9 hours exposure and 24 hour recovery periods respectively. The results of the experiments indicated that WormAtak® was effective against the developmental stages of *Anopheles gambiae* s.l., with the pupal stages proving more susceptible. WormAtak® concentration of 1.86 µM caused mortalities of 87%, 89% and 95% in *Anopheles gambiae* s.l. F1 third instar larvae from Opeibea, Madina and the susceptible strain respectively after 72 hours. Whilst that of 9.28 µM caused mortalities of 98%, 100% and 100% in *Anopheles gambiae* s.l. F1 third instar larvae from Opeibea, Madina and the susceptible strain respectively after 72 hours. All pupae in all concentrations of WormAtak® from the three populations could not emerge into adults and 100% mortality was obtained in less than 24 hours in all treatments. WormAtak® also had a lethal effect on the female adults of *Anopheles gambiae* s.l. WormAtak® showed detrimental effects on the non-target organisms with 100% mortality occurring in all concentrations of WormAtak® on *Poecilia reticulata*, *Bufo* sp. and *Physa waterloti* after 24 hours. WormAtak® can provide a rapid and effective way for controlling mosquito larvae, pupae and adults but must be carefully introduced in environmentally sensitive areas due to its relatively broad spectrum on non-target organisms.

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CHAPTER ONE

1.0 INTRODUCTION

Mosquitoes are among the most important insect vectors affecting the health of people and animals across the globe. They are arthropods that transmit many diseases, particularly in the subtropical and tropical parts of the world (Rueda, 2007). *Anopheles gambiae* sensu lato was perceived as a species complex only in the 1960s (Yakob, 2011). The *An. gambiae* complex consists of: *Anopheles quadriannulatus, Anopheles merus, Anopheles melas, Anopheles bwambae, Anopheles arabiensis, Anopheles gambiae* sensu stricto*, Anopheles amharicus, and Anopheles coluzzi* (Yakob, 2011). Diseases vectored by mosquitoes have become more prevalent as previously geographically isolated diseases have spread on a global scale due to urbanization, globalization, climate change and changes in agriculture (Lee *et al.*, 2018). Malaria and lymphatic filariasis are the major diseases transmitted by members of the *Anopheles gambiae* complex (Yakob, 2011). However, diseases such as Yellow fever, Chikungunya, Dengue, Japanese encephalitis, West Nile, and Zika virus which are transmitted by mosquitoes are also common (Lee *et al.*, 2018). These infections result in great social discomfort, economic loss, incapacitation, disfigurement and mortality in humans affected (Sachs & Malaney, 2002). In spite of unrelenting efforts made to control mosquito-borne diseases, they are still prevalent. They affect hundreds of millions of individuals every year, leading to serious suffering and hindered socio-economic advancement (Devi & Raju, 2018).

The greatest challenge facing the fight against malaria in Africa is resistance build up by mosquitoes to insecticides (Rajatileka *et al.*, 2011).Vector targeted control measures have led to great successes in the control of malaria (Sachs & Malaney, 2002). The way forward in the control

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of malaria has been to reduce man and vector contact. So far, Insecticide Treated Nets (ITNs), Indoor Residual Spraying (IRS) and larviciding have been the major approaches used to spearhead this cause (Otchere, 2011).

Great successes have been documented in mosquito control with the usage of larvicides, notably the eradication of *An. gambiae* from Brazil and Egypt (Killeen *et al.*, 2002). The usage of larvicides is an old practice for mosquito control and dates back to 1899, when *Anopheline* larval breeding sites were treated with kerosene by Ronald Ross in Sierra Leone (Bockarie *et al.*, 1999). Research on larvicides with focus on microbials such as *Bacillus thuringiensis var. israelensis (Bti)* and *Bacillus sphaericus (Bs)* (Ocran & Akpabey, 2006) as well as botanicals such as neem products (Okumu *et al.*, 2007) have been documented.

Treatment of mosquito habitats with petroleum products was commonly practiced in the early 1960's as it was perceived that some monomolecular chemical films used to reduce evaporation from water storage reservoirs had the ability to control mosquitoes (Lorenzen & Meinke, 1968). Monomolecular chemical films create one-molecule-thick layer on the water surface, reducing the capability of mosquito larvae to attach their siphon to the water surface due to reduced surface tension (Nayar & Ali, 2003). They also have the added advantage of targeting multiple stages in the mosquito life cycle, as they also affect ovipositing and emerging adults (Batra *et al.*, 2006). As a result, these films provide the combined benefits of larval and adult control, which reduces mosquito density and survival (Killeen *et al.*, 2002).

Another way of controlling insect pest is the usage of chemicals that have lethal effect on insect growth and development (Tunaz & Uygun, 2004). These substances are grouped as "insect growth regulators'' (IGRs) or "insect hormone mimics'' due to their effects on certain physiological regulatory processes which are needed for normal development of insects and their progeny (Tunaz

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& Uygun, 2004). Teflubenzuron, an IGR is a Chitin Synthesis Inhibitor (CSI), which interferes with the accumulation of chitin in the cuticle of an arthropod (Tunaz & Uygun, 2004). Chitin is an essential component of the insect exoskeleton, the cuticle, which is an extracellular matrix that covers the insect. Oetken *et al.*, (2004) reported that although IGRs interfere with molting as an endocrine-regulated process in arthropods, the mechanism of CSIs is purely non-endocrine. Because the synthesis normally occurs at or during the time of molting, CSIs cause death during the molt, which is similar to the effects caused by insect growth regulators. Adult emergence inhibition due to the application of CSIs to aquatic insects has been reported in studies on *Culex quinquefasciatus, Aedes aegypti, Anopheles stephensi* and *Culex pipiens* (Batra *et al.*, 2006). Hence, the formulation of teflubenzuron and cypermethrin stands very promising in the fight against resistance in *Anopheles gambiae* sensu lato.

1.1 Justification

Insecticide resistance is not a new phenomenon: it has been an adverse side effect of mosquito control programs since the introduction of insecticides for wide-scale deployment in the 1940s for malaria control (Brattsten *et al.*, 1986). Resistance development to at least one insecticide have been ascertained in over 100 mosquito species including 39 species of Culicine and 56 species of Anopheline mosquitoes all around the world (Camara *et al.*, 2018). In Africa, resistance to Dichlorodiphenyltrichloroethane (DDT) was first detailed in malaria vectors during the 1950s and 1960s (Brattsten *et al.*, 1986). Pyrethroid resistance has been recognized in *An. gambiae* s.l. and *An. arabiensis* (Chandre *et al.*, 1999). Carbamate and organophosphate resistance has likewise been accounted for in malaria vectors (Camara *et al.*, 2018).

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Insecticide resistance is a major concern in public health and also in agriculture due to the overwhelming socioeconomic impact posed by arthropod disease vectors and pest. Insecticide application is the most rapid and effective vector control tool available at the moment (Coleman *et al.*, 2018). Hence, it is imperative to manage insecticide resistance meticulously so as to circumvent the menace of possible field failure. Resistance to insecticides by insect vectors is a difficult issue to reverse in vector control. Successful vector management could be done by a combination of distinctive correlative insecticidal classes to improve efficacy, diminish cost and reduce ecological effects (Glazer *et al.*, 1992). The combined utilization of control specialists with free method of activity not exclusively can be increasingly successful, yet in addition reduces the improvement of insect resistance. Generally, agrochemicals refer to pesticides including, insecticides, fungicides, nematicides and herbicides (Keiser *et al.*, 2005). It may also include hormones, synthetic fertilizers, concentrated animal manure and other chemical growth agents (Keiser *et al.*, 2005).

WormAtak® is an insecticide sold as an Emulsifiable Concentrate (EC) or Ultra Low Volume (ULV) formulations made up of Teflubenzuron (50 g/l) and Cypermethrin (20 g/l). Teflubenzuron is an Insect Growth Regulator (IGR) which restrains the formation of chitin in larvae which have ingested it; making the integument become delicate, and prompting mortality during ecdysis (Ascher & Nemny, 1984). Teflubenzuron has its activity essentially influencing the immature larval phase of mosquito and mortality happens mostly in the pupal stage. It causes morphogenetic variations; for example, expanded pupa, mostly exuviated grown-up with legs or mid-region still connected to the pupal case and pupa-adult intermediates are generally observed (Chui *et al.*, 1993). Cypermethrin is a pyrethroid utilized as an insecticide in agriculture and for household purposes. It is a quick acting neurotoxin in insects. It is effectively degraded in soil and plants, yet

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can be viable for a considerable length of time when attached to indoor dormant surfaces (Maund *et al.*, 2002). Exposure of cypermethrin to daylight, water and oxygen will quicken its disintegration. . Cypermethrin is a broad-spectrum insecticide, and thus has the ability to kill beneficial insects including targeted insects (Maund *et al.*, 2002). Cypermethrin is toxic to fish, honey bees and aquatic insects. Aquatic insects are highly susceptible to cypermethrin, but when applied according to recommended rates around residential site, they pose little risk to aquatic life (Maund *et al.*, 2002).

While insects can become resistant to other insecticides, they are less likely to become resistant to IGRs (Knight, 2010). The mixture of a synthetic pyrethroid and an insect growth regulator can help reduce mammalian toxicity of the synthetic pyrethroid since IGRs have low mammalian toxicity. These attributes would make WormAtak® a promising product not only for the control of agricultural pest but also as a malaria vector control agent. WormAtak® has been introduced in controlling the Fall Armyworm, *Spodoptera frugiperda.* The product is therefore mostly used in agricultural areas. However, due to irrigation, there could be the existence of possible habitats for *An. gambiae* s.l. and other aquatic insects where they breed in collected pools of water. The application of the insecticide then has a residual effect on these larval habitats. Therefore, this study would help bring to light the effect of WormAtak® on aquatic insects and help determine if the product could be employed in controlling *An. gambiae* s.l. based on their susceptibility to WormAtak®.

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1.2 Main objective

To assess the efficacy of WormAtak® (Teflubenzuron and Cypermethrin) on *Anopheles gambiae* s.l. and also the effect on non-target species that co-exist with *An. gambiae* s.l.

1.2.1 Specific objectives

i. To determine the susceptibility of populations of larval, pupal and adult *Anopheles gambiae* s.l. to WormAtak®.

ii. To determine the effect of WormAtak® on three non-target organisms.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Anopheles gambiae* s.l. borne diseases

2.1.1 Malaria

Malaria is an infection caused by protozoan parasites belonging to the genus *Plasmodium*. Of over 150 known species of *Plasmodium* documented, only five species (*Plasmodium falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi*) are responsible for all human infections (WHO, 2016). *Plasmodium falciparum* accounts for majority of the infections, the most severe form of the disease and mortality (Greenwood *et al.*, 2008). *Plasmodium vivax* and *P. ovale* can cause relapses after months of infection and they are associated with substantial morbidity but fewer severe complications which are usually self-limiting in healthy individuals (Greenwood *et al.*, 2008). *Plasmodium vivax* accounts for most cases in Central and Southern America and Asia but is less prevalent in Africa (Greenwood *et al.*, 2008). About 4% of evaluated cases all-inclusive were brought about by *Plasmodium vivax*, yet outside the landmass of Africa this extent was 36% (Baird, 2013). *Plasmodium vivax* is the most predominant parasite in the Americas (64%), 40% in the Eastern Mediterranean locales and 30% in South-East Asia (Autino *et al.*, 2012). Most instances of malaria brought about by *Plasmodium vivax* happen in the WHO South-East Asia Region (58%), trailed by the WHO Eastern Mediterranean Region (21%) and the WHO African Region (10%) (WHO, 2016). *Plasmodium vivax* malaria cases diminished by over 45% between 2010 and 2016. Eighty percent of all malaria cases were represented in fifteen nations with Nigeria having the highest (WHO, 2016). *Plasmodium ovale* is predominantly in Africa (Greenwood *et al.*, 2008). *Plasmodium malariae* has wide global distribution and its infection causes little

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morbidity and almost no mortality, however, renal complications may result if untreated. *Plasmodium knowlesi* is restricted to small geographical areas in Southeast Asia (Daneshvar *et al.*, 2009).

Approximately 30-40 species of *Anopheles* mosquitoes transmit human malaria under natural conditions (Marshall & Taylor, 2009). In Africa, *An. funestus* and *An. gambiae* complexes are the malaria vectors (Marshall & Taylor, 2009). The *Anopheles gambiae* sensu lato species complex is made up of eight sibling species of mosquitoes that are morphologically quite difficult to distinguish. It includes *An. gambiae, An. coluzzi, An. arabiensis, An. melas, An. merus, An. amharicus, An. bwambae* and *An. quadriannulatus* (Coetzee *et al.*, 2013). Although, the sibling species exhibit eco-ethological and genetic heterogeneity in their ability to transmit malaria (Della Torre *et al.*, 2002). Within this complex, *An. gambiae* s.s. Giles is the most important malaria vector because of its widespread distribution, highly endophilic and anthropophilic behaviour, and longer life span (Della Torre *et al.*, 2002).

In 2016, malaria cases in the WHO African Region recorded were 90%, and in the WHO South-East Asia Region, malaria cases were 7% and the WHO Eastern Mediterranean Region had 2% malaria cases (WHO, 2017). A reduction in malaria cases of more than 20% compared with 2015 was estimated to occur in 16 countries which were counted as part of the 91 countries that were considered to have indigenous malaria cases in 2016, while there was an increase of a similar magnitude in 25 countries (WHO, 2017). The WHO regions of the Americas and Africa accounted for nearly 70% of the countries that had increases of more than 20% in 2016 compared with 2015 (WHO, 2017). High-burden countries which were estimated to be twenty-nine which accounted for 85% of malaria cases in 2016 had a reduction of more than 50,000 malaria cases when compared with 2016 (WHO, 2017). Twenty-four had increases estimated at 50,500 (Chad) and

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more than one million (Rwanda and Nigeria) malaria cases, while five had decreases of between 856,000 (Madagascar) and 151,000 (Gambia) (WHO, 2017). In 2016, it was evaluated that 445,000 deaths linked to malaria had happened globally, of which 407,000 deaths (around 91%) were in the WHO African Region (WHO, 2017). Roughly all deaths were estimated at 80% in 2016 which happened in 15 nations, which are part of the WHO African Region, aside from India (WHO, 2017)(Figure 1). The Democratic Republic of the Congo, Nigeria, India and Burkina Faso represented 47% of all deaths attributed to malaria globally (WHO, 2017)(Figure 2).

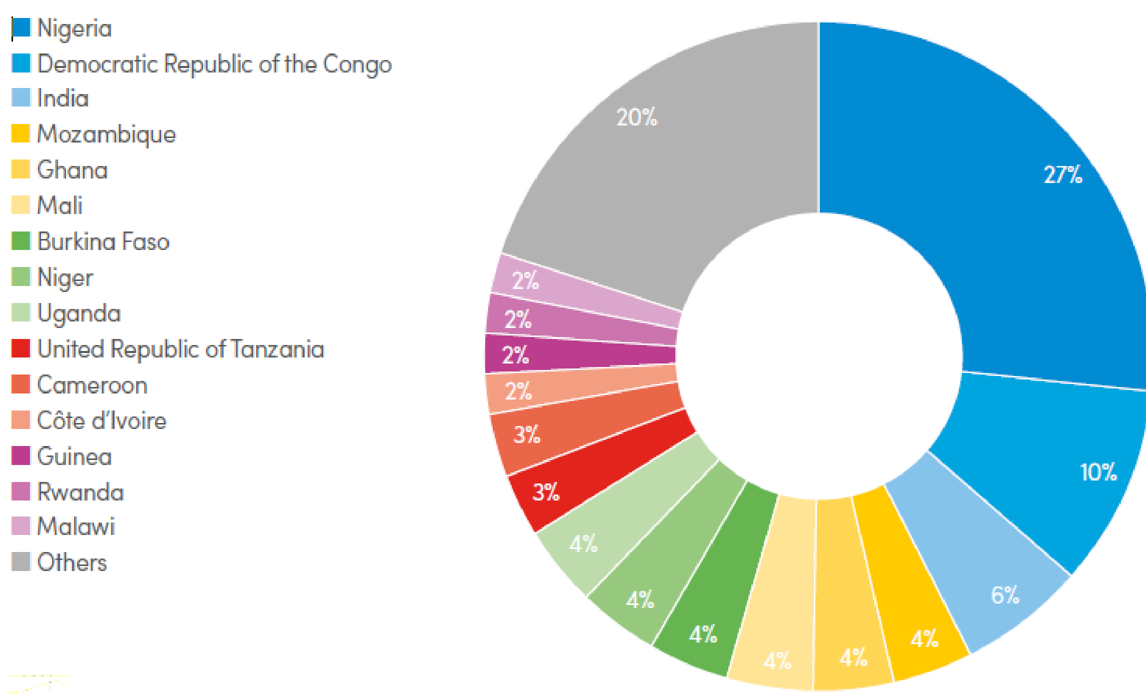

Figure 1: Estimated country share of total malaria cases, 2016. Source: (WHO, 2017).

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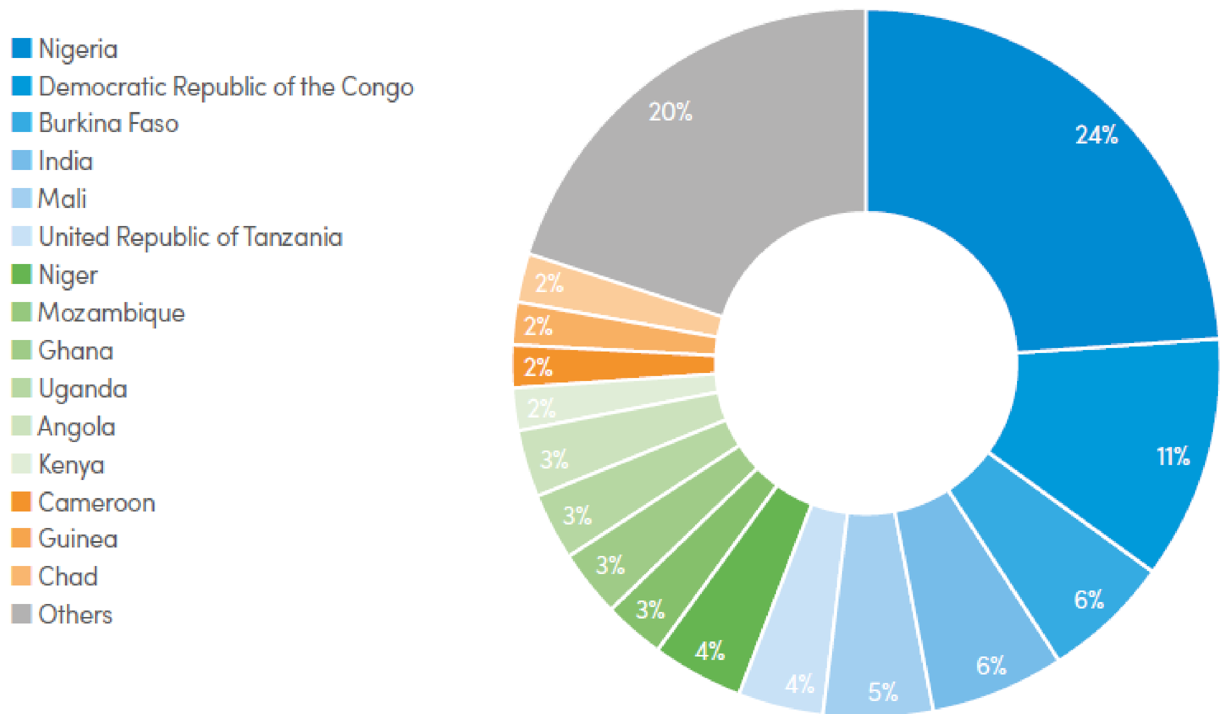

Figure 2: Proportion of estimated deaths attributable to malaria globally in 2016. Source: (WHO, 2017).

Malaria imposes a huge economic burden in tropical Africa through high healthcare costs, reduced economic productivity and low levels of foreign investment (Sachs & Malaney, 2002). Economists believe that malaria is responsible for a growth penalty of up to 1.3% per year in some African countries (Collins & Paskewitz, 1995). Despite the fact that financing for malaria has remained generally stable since 2010, the degree of interest in 2016 is a long way based on what is required to achieve the principal achievement which is a decrease of at any rate 40% in malaria case rate and death rates internationally when contrasted with 2015 levels (WHO, 2017). To achieve this goal, the assessed yearly financing would need to increase to US$ 6.5 billion every year by 2020. The US$ 2.7 billion invested resources into malaria in 2016 speaks to not exactly half (41%) of that sum (WHO, 2017). Legislatures of endemic nations contributed 31% of the funding (US$ 800 million) in 2016 (WHO, 2017).

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Chemotherapy is being used as the front-line defense in containing and treating malaria. However, the development of resistance by *P. falciparum* to drugs has been one of the main causes of poor malaria program performance for decades particularly in Africa (Cheeseman *et al.*, 2012). Artemisinin-based combination therapies (ACTs) have been promising due to their efficacy. However, the recent emergence of *P. falciparum* resistance to ACTs in South-East Asia may threaten to derail malaria control as experts speculate that the resistance may spread to Sub-Saharan Africa as happened in the case of chloroquine (Cheeseman *et al.*, 2012). The development of safe and effective anti-malarial vaccine has been a major health priority in controlling malaria in the tropics. After decades of intensive research, the most promising pre-erythrocytic vaccine (RTS,S/AS01) being developed is currently in the third phase (Phase III) of the ongoing controlled clinical trials in Sub-Saharan Africa expected to end by 2025 (Cheeseman *et al.*, 2012). Vector control is one of the major approaches in malaria control globally (Burkot et al., 2019). This approach helps in reducing *Plasmodium* transmission in malaria-endemic regions in Africa (Cheeseman *et al.*, 2012). Vector control methods work by limiting the contact between humans and vectors, thus reducing the vector density or increasing the adult vector mortality (Burkot et al., 2019).

2.1.2 Lymphatic filariasis

Lymphatic filariasis is a parasitic helminth disease that is a genuine general medical problem in tropical regions (Taylor *et al.*, 2010). The filarial nematodes that cause these illnesses are transmitted by blood-feeding insects and produce incessant and long haul contamination through concealment of host invulnerability (Taylor *et al.*, 2010). It is brought about by contamination with the parasitic nematodes *Brugia malayi, Wuchereria bancrofti*, or *Brugia timori* and is transmitted through the bite of mosquitoes (Specht *et al.*, 2019). Mosquitoes of the genera *Anopheles*, *Aedes*,

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Mansonia, and *Culex* ingest the microfilaria when taking blood meals from individuals and these form into an infective larval stage (Specht *et al.*, 2019). Lymphatic filariasis is a tropical infection that affects around 70 million individuals around the world (Mondiale de la Santé & WHO, 2017). The third reasonable advancement objective calls for end of disregarded tropical ailments, including filariasis, by 2020 (Specht *et al.*, 2019). Sixty three percent of the populace in danger of lymphatic filariasis and half of the general population contaminated worldwide reside in South East Asia (Specht *et al.*, 2019). India harbors 40% of the weight of the disease burden globally (Specht *et al.*, 2019).

Mass medication organization involves yearly conveyance of diethylcarbamazine in blend with albendazole for at least five years in an endemic region (WHO, 2010). These medications are basically microfilaricidal with the objective to accomplish an inclusion of over 65% of the populace (WHO, 2010). It depends on the reason that rehashed mass medication organization will diminish the microfilaria thickness in the network and subsequently end transmission. Up to 2015, the program has given over 6.7 billion medications to more than 850 million individuals in any event once in 66 nations (Specht *et al.*, 2019). Mass organization is evaluated to have restored or anticipated up to 96 million new instances of lymphatic filariasis and turned away more than $100 billion of lifetime monetary misfortune (Ramaiah & Ottesen, 2014). Since 2000, filarial prompted hydrocele have declined by about 49% to 19.4 million, and the cases of filarial initiated lymphoedema by 23% to 16.7 million (Ramaiah & Ottesen, 2014). The number of individuals requiring mass medication tumbled from 1.41 billion in 2011 to 856 million in 2016 (Mondiale de la Santé & WHO, 2017). It is normal that mass organization will never again be required when the predominance of contamination has been diminished to low levels, for example, microfilariae in <1% of the populace or antigenaemia in <2% of the populace (WHO, 2011).

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Long haul care is essential to counteract and treat endless signs of filariasis. Treatment for lymphoedema incorporates great cleanliness (ordinary washing with cleanser and water; skin and nail care), utilization of topical anti-toxins or antifungal specialists, work out, and fitting footwear (Specht *et al.*, 2019). Giving a fundamental bundle of consideration to oversee dreariness appeared to diminish the recurrence of intense assaults of adenolymphangitis that drive the movement of lymphoedema (Specht *et al.*, 2019). Microfilaricidal medications have little advantage in tainted people with lymphoedema and hydrocele (Addiss *et al.*, 2010). Preliminary study including 105 youngsters with filariasis in India demonstrated a conceivable advantage in turning around lymph widening from the get-go over the span of infection and a couple of observational reports have likewise noticed an advantage (Kar *et al.*, 2017).

2.2 Taxonomy and distribution of *Anopheles gambiae* s.l.

Mosquitoes are part of the family Culicidae which are made up of 3,500 species belonging to 41 genera. The family is divided into 3 subfamilies, namely: Anophelinae (Anophelines), Culicinae (Culicines) and Toxorhynchitinae (Marshall & Taylor, 2009). The subfamily Anophelinae contains three genera; *Anopheles, Bironella and Chagasia* but only the genus *Anopheles* is of medical importance (Marshall & Taylor, 2009). There are about 430 species of the genus *Anopheles* (Marshall & Taylor, 2009). Malaria is the most important disease transmitted by *Anopheles* mosquitoes. It is transmitted by different *Anopheles* spp. in various geographical locations. Anopheline vectors are not restricted to malaria-endemic areas, but also in eliminated regions (Sinka, 2013)(Figure 3) thereby making the risk of reintroduction of the disease possible. Some *Anopheles* species are also vectors of filariasis especially that caused by *Wuchereria bancrofti*, but some also transmit *Brugia malayi* and *Brugia timori* while a few species transmit arboviruses that are of minor medical importance (Harbach, 2007).

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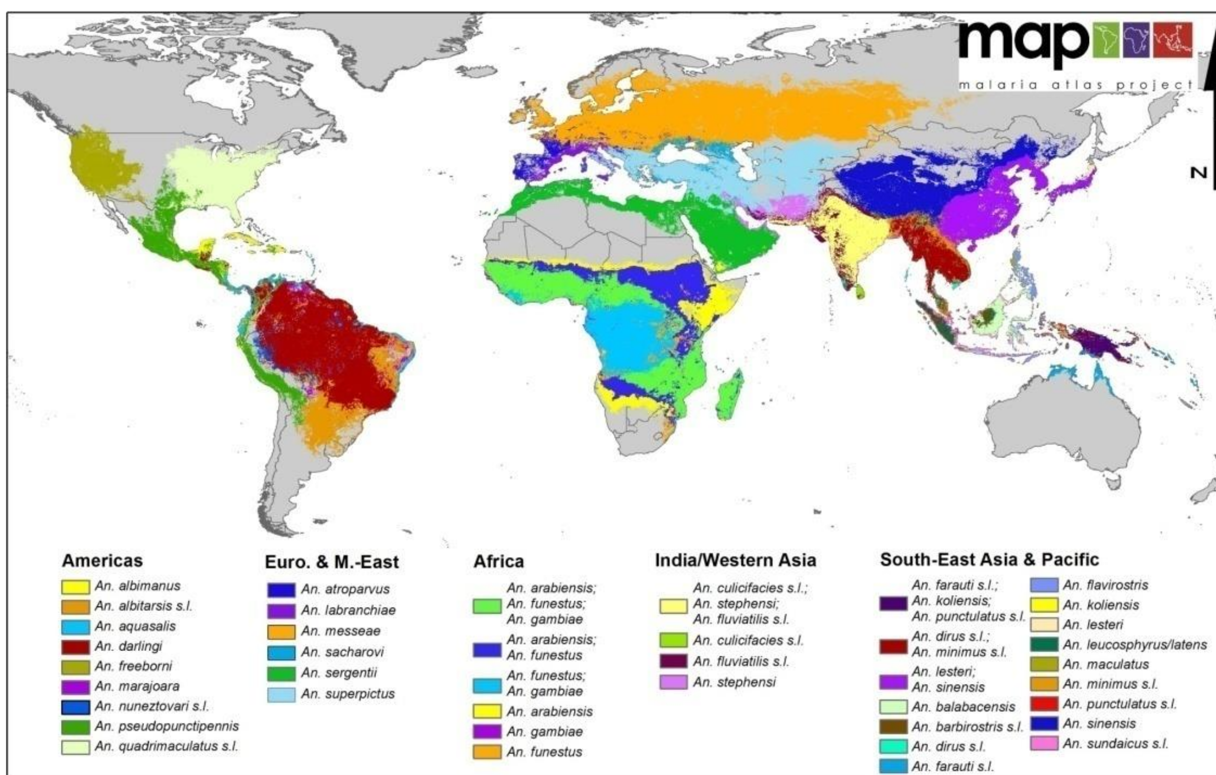

Figure 3: Global distribution of the dominant vector species of malaria. Source: (Sinka, 2013).

Mosquitoes occur throughout the temperate and tropical zones (Harbach, 2007). Other than Antarctica and a few islands, mosquitoes are found in all parts of the world; they are found to inhabit elevations as high as 5,500 meters and down mines at depressions of 1,250 meters below sea level (Sinka, 2013). *Anopheles* mosquitoes also occur both in temperate and tropical regions but do not occur in the majority of the Pacific islands including New Zealand (Harbach, 2007).

2.3 Bionomics of *Anopheles gambiae* s.l.

Anopheles gambiae s.l. undergo complete metamorphosis with four developmental stages: egg, larva, pupa and adult (Figure 4). Female *Anopheles gambiae* s.l. mosquitoes have been discovered to mate once in their life time but are known to produce eggs at intervals (Rudra, 2012). Male and female *Anopheles gambiae* s.l. mosquitoes feed on plant juice which is required for energy and

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flight activities, but only the females are known to blood feed which is required for maturation and development of eggs (Rozendaal, 1997). Development and digestion of blood meal occurs concurrently within 2-3 days in the tropics but takes longer in the temperates because environmental conditions are not favourable for their development (Rozendaal, 1997). Gravid *Anopheles gambiae* s.l. females then search for suitable breeding sites which are normally temporary shallow water puddles ranging from ditches, burrow pits, vegetable farms, human foot prints, animal hoofs etc. *Anopheles gambiae* s.l. female may lay up to 30-300 eggs (Rozendaal, 1997).

Anopheles gambiae s.l. larvae lack siphon, and lie parallel to the water surface to breathe (through a pair of spiracles on the 8th or 10th abdominal segments) and feed as they are surface feeders (Rudra, 2012). Larvae of *Anopheles gambiae* s.l. are frequently found in habitats exposed to sunlight and usually associated with vegetation such as grass and algae (Rozendaal, 1997). *Anopheles gambiae* s.l. larval habitats include pools, puddles, seepages, wells, water-filled car tracks, animal hoof prints, rice fields and marshy areas. However, a few species may breed in tree holes and leaf-axils of epiphytic plants (Grimstad, 2001). Anophelines generally prefer breeding in clean waters unpolluted with organic matter (Grimstad, 2001). *Anopheles gambiae* s.l. larvae are filter feeders and feed on yeast, bacteria, protozoa and numerous other micro-organisms, as well as on decaying plant and animal matter in the water. All *Anopheles gambiae* s.l. pupae are aquatic and comma-like in shape (Rudra, 2012). The pupae do not feed and spend most of their time at the water surface to breathe through their respiratory trumpets. Pupae dive swiftly to the bottom of the habitat when disturbed. In the tropics, it takes 1-3 days for adults to emerge from pupa through the splitting of the pupal skin at one end (Rozendaal, 1997).

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Since adult female mosquitoes need a blood meal to develop their eggs, they have to either feed on vertebrates. Some species feed predominantly on human and are known to be anthropophagic whereas others that feed on animals are zoophagic (Rudra, 2012). These diverse behaviours exhibited by the *Anopheles gambiae* s.l. mosquito are of significant importance in the epidemiology and control of mosquito-borne diseases through planning of appropriate intervention strategies (Rudra, 2012).

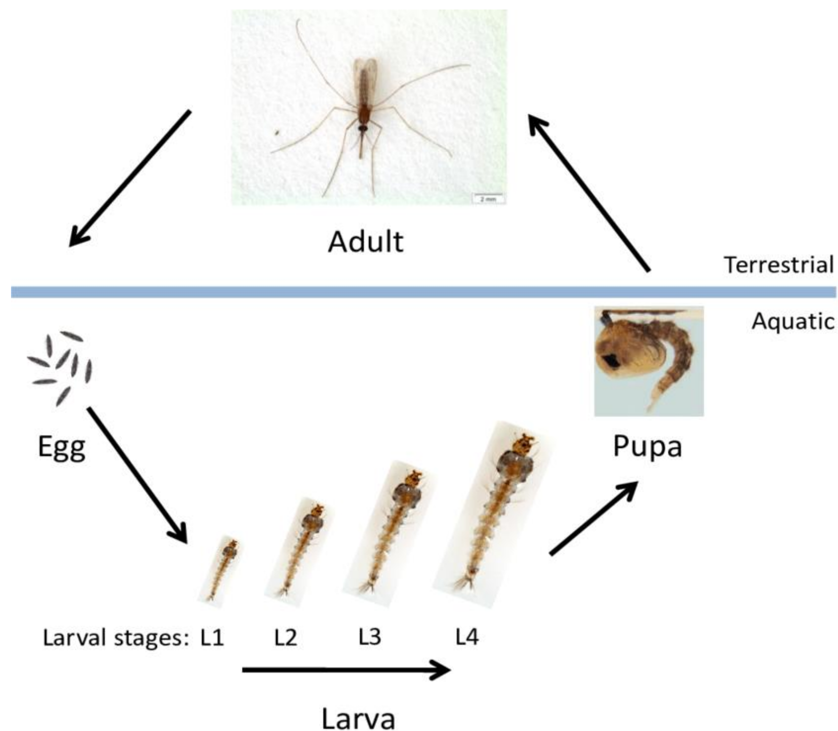

Figure 4: Illustrative diagram of the life cycle of *Anopheles gambiae* s.l. showing egg, larva, pupa and adult stages. Source: https://www.researchgate.net/figure/Stages-of-the-Anopheles-mosquito-fig1_262450744, Accessed 01/03/2019.

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2.4 *Anopheles gambiae* s.l. control methods

Generally, there is no simple universally applicable vector control method due to the wide variety of ecological preferences and behavioral characteristics employed by malaria vectors (Collins & Paskewitz, 1995) and so the vector, the environment and the human behaviour are taken into consideration in determining the suitability of a vector control method in a particular setting (Shafique *et al.*, 2019). Again, as vector control methods are different in terms of their application, cost, and sustainability, the reason for a particular control method will depend on the height of the malaria burden, the possibility of timely and correct application of the required interventions and the feasibility of sustaining the modified epidemiological occurrence (Shafique *et al.*, 2019). Vector control measures include environmental management, larviciding, biological control of larvae or adults and adult control. Vector control methods can be categorized into two based on the life stage of the mosquitoes they target; these are larval control and adult control measures (Shafique *et al.*, 2019).

2.4.1 Environmental Management

Ecological management for vector control includes the arranging, association, completing and observing of exercises for the change as well as control of natural elements or their connection with man with the end goal of anticipating or limiting vector proliferation and diminishing man-vector-pathogen contact (Beier *et al.*, 2008). This methodology, which ought to be done wisely and skillfully, is naturalistic and includes an endeavor to expand and strengthen common components which limit vector reproducing, survival and contact with man (Beier *et al.*, 2008). Natural management for mosquito control covers a wide scope of works and tasks which can be additionally ordered and characterized as: (a) Environmental change: "A type of ecological management comprising in any physical change that is perpetual or durable of land, water and

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vegetation, wiping out or lessening the living spaces of vectors without causing unduly unfriendly consequences for the nature of the human condition" (Utzinger *et al.*, 2001). In spite of the fact that these works are more often than not of a lasting sort, legitimate task and sufficient support are fundamental for their successful working. (b) Environmental control: "A type of ecological management comprising in any arranged repetitive action for creating brief conditions horrible to rearing of vectors in their natural surroundings." Water saltiness changes, stream flushing, guideline of the water level in repositories, dewatering or flooding of bogs or boggy zones, vegetation evacuation, shading and introduction to daylight are instances of natural control exercises (Utzinger *et al.*, 2001). (c) Modification or control of human residence or conduct: "A type of ecological management that diminished man-vector-pathogen contact" (Utzinger *et al.*, 2001). Instances of this sort of methodology incorporate the siting of settlements from vector sources, mosquito sealing of houses, individual assurance and cleanliness measures against vectors, and arrangement of such establishments as mechanical obstructions and offices for water supply, wastewater and excreta transfer, clothing, washing and diversion to avert or dishearten human contact with invaded waters (Beier *et al.*, 2008).

Natural change and control comprise essentially of altering the geographical, hydrological and organic components of mosquito territories in order to render them inadmissible for mosquito rearing (Beier *et al.*, 2008). Ecological management for mosquito control, while it utilizes materials and common procedures existing in nature, cannot be required to be totally free from natural effect (Beier *et al.*, 2008). The biological effect of natural management may, on a fundamental level, be conjectured by breaking down the positive and negative impacts prone to be delivered by every one of the proposed activities of stakeholders (Beier *et al.*, 2008). Notwithstanding, there are no regular criteria for the estimation of environmental impacts that

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would give esteems for a quantitative appraisal of effects. This additionally applies to medical advantages and weaknesses which, albeit unmistakably and for the most part distinguishable, now and then can't be exposed to quantitative investigation (Keiser *et al.*, 2005). The trouble of surveying environmental impacts does not infer that these endeavors ought not to proceed. Evaluations dependent on realities and fair judgment can deliver valuable information to stakeholders. A level of abstract assessment might be required in rating the appropriateness of different options. For the investigation of the connections that might be associated with the examination of effect on a natural framework, the utilization of an organization or lattice might be useful. This can likewise be adjusted to dissect the natural effects of vector control measures being connected by a malaria control program (WHO, 1982).

Much is thought about natural adjustments and its viability in controlling mosquito creation. Real works including the change of the example of land, water and vegetation, did for the most part for different purposes, contributed in numerous regions to the decrease of mosquito abundance (Utzinger *et al.*, 2001). Fortunately, the organizations in charge of improvement of infrastructures, especially those managing water assets, are ending up progressively aware of the need to keep the event and escalation of *Anopheline* borne diseases (WHO, 1982). Projects for the control of diseases caused by mosquitoes can profit much from these improvement plans if vector control experts are permitted to work together in preconstruction overviews, arranging, structure and development. They can propose practical changes with the goal that such plans can add to the decrease and end of mosquito breeding sites (Keiser *et al.*, 2005). Small scale ecological management tasks are once in a while inside the extent of malaria control programs, and can be completed as a major aspect of the general control methodology (Beier *et al.*, 2008). At present just a couple of programs utilize these techniques in their activities. Other accessible strategies

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which produce brisk outcomes have been favored. In any case, the idea of incorporated control is presently more broadly acknowledged and set in motion. Ecological change tasks can be acclimated to suit program prerequisites and assets, and offer a pragmatic commitment to coordinated mosquito control techniques (Beier *et al.*, 2008). As in some other human action, demonstrated strategies are bound to disappointment in the event that they don't fulfill the required guideline of execution due to the absence of keen arranging, clear understanding, faithful application and firm persistence (Beier *et al.*, 2008).

2.4.2 Larviciding

Larval control measures diminish malaria transmission in a roundabout way by decreasing the vector populace thickness close to human residences in this manner affecting the vectorial limit (Walker & Lynch, 2007). Larval control measures as arranged by WHO incorporate the utilization of oils and surface films, microbial larvicides, synthetic organic chemicals, and insect growth regulators (Rozendaal, 1997). In spite of the fact that the viability of larval control measures in affecting malaria transmission is questionable, a few stakeholders believe them to be viable in urban settings where the quantity of mosquito reproducing sites is restricted because of high human populace thickness (Walker & Lynch, 2007). The low versatility and behavioral responsiveness of the larval stages permit compelling inclusion. Again, larval control is moderately straightforward thus can be performed by unskilled personnels, for example, volunteer network of laborers. In endemic areas, notwithstanding, the presence of more mosquito breeding sites and the trouble in finding them make these techniques operationally improper in breaking malaria transmission (Walker & Lynch, 2007). Moreover, *An. gambiae* s.l., for example, have long flight ranges which infers that larval control measures like larviciding must be performed over a wide land zone so as

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to diminish the opportunity of adults flying into control regions from encompassing areas and these can be work and capital demanding (Rozendaal, 1997).

2.4.2.1 Oils and Surface films

Oils kill mosquito immatures by suffocation and harming with poisonous vapor. In most tropical countries, they must be used week after week to guarantee larvae hatched from eggs are killed before they pupate and develop as adults and the prescribed application rates are 9-27 liters for every hectare (Corbet *et al.*, 2000). Utilization of oil is a moderately simple strategy for larval control for little water bodies like ponds, pools, and seepages (Garrett & White, 1977). However it is costly for extensive surfaces. Monomolecular surface films structure an ultra-flimsy layer (around one atom thick) on the water surface, by decreasing surface pressure and disturbing the capacity of the larvae to rest and inhale at the outside of the water, and in this manner choking them out (Corbet *et al.*, 2000). Monomolecular surface films influence all instars of larvae, pupae and ovipositing and developing adults. Early monomolecular surface films were presented during the 1980s and two formulations (Arosurf MSF® and Agnique MMF®) were accessible in the market as mosquito larvicides (Garrett & White, 1977).

A few examinations have been directed to test the viability of these monomolecular films against various types of *Anopheles, Aedes and Culex*. Arosurf® was found to cause 83.3% mortality of the fourth instar larvae and 100% mortality of pupae when tried against *Anopheles albimanus* at the rate of 0.25ml/m^2 while it was discovered that Arosurf® was also viable against *Aedes aegypti* larvae and pupae in small trays at different dosages (Das *et al.*, 1986). Arosurf® was seen to cause critical mortality in larvae, pupae and gravid female adults of *Culex quinquefasciatus*, yet no unfavorable impact was seen on eclosion when egg rafts were dealt with (Levy *et al.*, 1984). The adequacy of Agnique® was assessed at an application rate of 0.25ml/m^2 in lakes in Khartoum,

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Sudan and brought about an 89.4% decrease in instars of *Anopheles arabiensis* and 100% control of mixed mosquito pupae populace following 24 hours of treatment (Bashir *et al.*, 2008). Adequacy of Agnique® was additionally tried against *An. stephensi* in tanks and wells in an Indian urban town, which resulted in over 75% control of instar hatchlings and 100% control of pupae within 24 hours (Batra *et al.*, 2006).

Monomolecular films appeared to be extremely successful when mixed with different larvicides (Levy *et al.*, 1984). Arosurf® when joined with the microbial larvicides *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* at lower application rates give progressively viable control of mixed larval and pupal populations of *Cx. quinquefasciatus* than their individual parts under both laboratory and field conditions (Levy *et al.*, 1984). Levy *et al.*, (1984) also showed Arosurf® to be increasingly viable against mosquito larvae and pupae when mixed with diesel or regular organophosphorus insecticides (temephos). Agnique® likewise turned out to be successful against *Ae. albopictus* when mixed with methropene (an insect growth regulator) (Nelder *et al.*, 2010). Both Arosurf® and Agnique® are enlisted as mosquito larvicides however have not been widely utilized in mosquito control programs. They will in general aggregate around aquatic vegetation and debris and are additionally influenced by wind (Nayar & Ali, 2003).

2.4.2.2 Microbial larvicides

Microbial larvicides are products dependent on the insecticidal crystal proteins created by *Bacillus sphaericus* and *Bacillus thuringiensis* var *israelensis*. As mosquito larvae are filter feeders, they ingest these proteins which tie to explicit receptors on the midgut epithelium upon actuation by compounds in the larval midgut, bringing about spore formation, interference of feeding and homeostasis (Tusting, 2014). Bacterial larvicides are very much suggested for controlling mosquito species that are impervious to synthetic pyrethroids, because of their specific method of

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activity. The poisons delivered by these microscopic organisms are exceedingly lethal to mosquitoes, blackflies and other firmly related Dipteran flies (Lacey & Merritt, 2003). In any case, they are target explicit and accordingly innocuous at prescribed measurements to non-target aquatic invertebrates, insects, fish as well as fowls, animals and humans (Lacey & Merritt, 2003). *Bacillus thuringiensis* var *israelensis (Bti)* is a naturally happening, spore-like bacterium present in soil and oceanic conditions around the globe. It mostly influences certain groups of Diptera in the suborder Nematocera (Lacey & Merritt, 2003). Along these lines, the profoundly explicit delta endotoxin it produces is just harmful to larvae of mosquitoes, blackflies and related flies when they ingest it. For greatest execution, *Bti* should be re-applied after 7-10 days (Tusting, 2014).

Bacillus sphaericus (Bs) is a spore forming bacterium which happens to be in soil and aquatic vegetation in all parts of the world. It is more target explicit than *Bti* as it is dynamic just against individuals from the family Culicidae (Lacey & Merritt, 2003). *Bacillus sphaericus* gives longer leftover impact than *Bti* in conditions with high organic substance (Tusting, 2014). There is an expansion in the utilization of bacterial larvicides for mosquito control, in light of the fact that their objective particularity and capacity to exterminate species that are impervious to synthetic pyrethroids. Microbial larvicides have been used in research and operational mosquito control programs in different biological settings in Africa, for example, Kenya (Fillinger *et al.*, 2003), Gambia (Majambere *et al.*, 2007) and Ghana (Ocran & Akpabey, 2006).

2.4.2.3 Synthetic organic chemicals

Following the revelation of organochlorine insecticides, for example, DDT in the mid-1940s, oiling was to a great extent supplanted by the utilization of these manufactured synthetics to control mosquito larvae (WHO, 1982). In any case, in light of the advancement of opposition, their unfavorable consequences for non-target species, steadiness in the earth and collection in natural

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pecking orders, their utilization as larvicides was ended (Rozendaal, 1997). Organophosphates then again are still prescribed by WHO to be utilized as larvicides because of their less toxicity in the environment contrasted with organochlorines (Tusting, 2014). Some of the organophosphorus insecticides utilized as larvicides include malathion, chlorpyrifos, fenthion and temephos. Notwithstanding, those suggested by WHO include temephos, chlorpyrifos and fenthion (WHO, 2011). Temephos has been used against *Ae. aegypti* larvae in dengue control programs just as in the West African Onchocerciasis Control Program against black flies and the crusade for the destruction of guinea-worm (WHO, 2011). Pyrethroids are harmful to numerous aquatic insects, crustaceans and fish and may likewise select for resistance from adults so are not prescribed as mosquito larvicides (WHO, 2011).

2.4.2.4 Insect Growth Regulators

Insect Growth Regulators (IGRs) are compounds that modify the development of insects by causing metabolic disorders which inevitably result in death of the insects (Rozendaal, 1997). IGRs are used as pesticides, for example, their particularity, influencing generally just target bothers and saving predators and parasites; their low lethality to different types of life including man, domesticated animals, and natural life; and their viability at low concentrations (Schaefer & Wilder, 1973). Insect growth regulators have turned into an essential device for the control of mosquitoes with least ecological effect (Schaefer & Wilder, 1973). Distinctive insect growth regulators have been created to give options to the traditional organochlorine and organophosphate insecticides (Staal, 1975). As per their method of activity, IGRs are comprehensively grouped into two classifications: those that hinder explicitly chitin union and adolescent hormone copies that restrain pupation (Rozendaal, 1997).

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Insect growth regulators are partitioned into two groups: 1. Juvenile hormone mimics, for example, methropene and pyriproxfen, which capture larval and pupal improvement into adults and 2. Chitin synthesis inhibitors, for example, diflubenzuron, teflubenzuron and triflumeron, which repress chitin formation in the juvenile stages (Rozendaal, 1997). Insect growth regulators influence a more extensive scope of invertebrates contrasted with bacterial mosquito larvicides because of their method of action (Tusting, 2014) but have low poisonous quality to warm blooded creatures, feathered creatures and fish. The juvenile hormone mimics are steadier and have longer residua action than the chitin synthesis inhibitors and different larvicides (Yapabandara *et al.*, 2001). A few examinations have demonstrated these products to be viable in research facilities and the field, however broad investigations on the viability of IGRs have not been directed in Africa (Tusting, 2014). As a result of disturbance of chitin synthesis by an IGR, the accompanying morphological variations from the norm are commonly observed: obscuring of the larval skin, expanded pupa (no longer C-molded), halfway exuviated adults with part of the stomach area still in the pupal case, legs of the developed adult still connected to the pupal case, pupa underdeveloped moderately (Schaefer & Wilder, 1973). The measure of teflubenzuron required to control mosquito larvae is altogether small, at the I.tg~L level (Rozendaal, 1997). Due to its method of action (meddling chitin development), teflubenzuron does not show quick knockdown, while morphogenetic variations from the norm of pupal and adult stages take place, and postponed mortality are seen after teflubenzuron application (Rozendaal, 1997).

2.4.3 Biological Control

Biological control includes the presentation of natural operators, for example, predators, parasites and pathogens in larval territories to control or lessen their populace (Chandra *et al.*, 2008). Natural control of mosquitoes was very much drilled in the early piece of the twentieth century before it

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was generally supplanted by the utilization of amazing synthetic concoctions. The overdependence on compound control has caused concerns, for example, insecticide resistance and ecological perils which prompted a restored enthusiasm for the utilization of all the more environmental neighborly strategies, for example, the utilization of natural specialists for mosquito control (Rozendaal, 1997). These organic operators may incorporate insects, viruses, microscopic organisms, protozoa, fungi, plants, nematode worms and fish. The successful utilization of these operators requires a decent comprehension of the science and conduct of the mosquito species to be controlled just as local ecological conditions (Chandra *et al.*, 2008).

With these agents, larvivorous fish are the most generally utilized (Chandra *et al.*, 2008). In other to be effective for use in mosquito control, larvivorous fish species ought to have inclination for mosquito larvae over other sustenance accessible; little size to enable access to shallow water and vegetation; high multiplication rate; resistance to contamination; saltiness, temperature variances and transportation; and ought to ideally be an indigenous species (Chandra *et al.*, 2008). The greater part of the species that have ended up being compelling against mosquito vectors are in the families Poeciliidae and Cyprinodontidae (Chandra *et al.*, 2008). Probably the most successful species that have been presented in different nations are the top minnow or mosquito fish (*Gambusia affinis*) and the guppy (*Poecilia reticulata*) respectively (Chandra *et al.*, 2008). *Gambusia affinis* is progressively adapted to clean waters, while *P. reticulata* is progressively lethal in water contaminated with organic substance (Chandra *et al.*, 2008).

The guppy is more tolerant to higher temperatures than the mosquito fish and might be progressively compelling in rice fields in hot territories, but it can't endure temperatures below 10°C (Chandra *et al.*, 2008). In certain nations like India and Nigeria, some indigenous fish species have appeared as biocontrol specialists for mosquitoes both in research centers and field

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circumstances (Anyaele & Obembe, 2011). Some fish species have demonstrated reasonable outcomes for mosquito larval control and as a protein source for nearby communities. The Mozambique mouthbreeder, *Oreochromis mossambicus* (Tilapia), which is in East Africa, has been effectively utilized in flooded rice fields as a mosquito control operator and as a sustenance source (Tusting, 2014).

2.4.4 Adult Mosquito Control

These measures focus on the adult mosquito which is the most epidemiologically vital mosquito vector population. They sway the vector populace by decreasing the future, viably adjusting the age structure of the vector populace to such an extent that less mosquitoes live sufficiently long to end up infective with the *Plasmodium* parasite (Garrett-Jones *et al.*, 1964). Age is the basic determinant of the capacity of mosquitoes to transmit human pathogens as the pathogens require a time of extrinsic incubation in the mosquitoes before transmission happens (Cook & Sinkins, 2010). In *An. gambiae* s.l., the extrinsic incubation time of *P. falciparum* is around 11 days which includes a critical extent of the normal life expectancy of the vector (Garrett-Jones *et al.*, 1964). Adult mosquitoes experience a high day by day death rate with the end goal that just a little extent of the populace in reality live sufficiently long to transmit malaria thus apparatuses that abbreviate the life expectancy of the mosquito will altogether lessen the limit of the vector populace to transmit malaria (Cook & Sinkins, 2010).

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To clarify the proposition numerically, the vectorial limit condition is utilized (Garrett-Jones *et al.*, 1964):

$$C = \frac{ma^2P^n}{-\ln P}$$

Where

C = vectorial capacity,

m = density of vectors in relation to humans

a = number of blood meals taken on humans per vector per day

p = daily survival probability of vectors (measured in days),

n = extrinsic incubation period in the vector (measured in days).

As indicated by the condition above, vectorial limit increases linearly with vector thickness yet increases exponentially with increment in mosquito life span (Garrett-Jones *et al.*, 1964). Therefore, strategies that target mosquito life span, even insignificant decreases in life expectancy could yield momentous decrease in vectorial limit, and the effect has long haul impact (Cook & Sinkins, 2010). Adult control measures include genetic tools and deployment of insecticides for Indoor Residual Spraying (IRS) and treatment of bed nets (Insecticide Treated Net (ITN)).

2.4.4.1 Genetic Tools for Mosquito Control

Genetic control procedure comprises of a scope of potential strategies that are normally arranged into two namely; populace concealment and populace substitution (Coleman & Alphey, 2004). Genetic methodologies are unmistakable, related with less poisonous buildups in the environment and require less community involvement (Coleman & Alphey, 2004). In any case, ethical and ecological issues relating to discharging of transgenic mosquitoes limit their potential. Populace concealment approaches (Sterile Insect Technique (SIT) and Release of Insects with a Dominant Lethal (RIDL)) includes the mass release of sterile or in part clean male insects into the wild to decrease the objective vector populace (Coleman & Alphey, 2004). This methodology has been

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utilized effectively to control a scope of horticultural pests and disease vectors, for example, tsetse fly, Mediterranean fruit fly, and screwworm fly through SIT (Wang & Jacobs-Lorena, 2013). Field preliminary researches have been conducted using RIDL in *Aedes aegypti* to evaluate its potential in controlling dengue fever (Fu *et al.*, 2010). Release of Insects with a Dominant Lethal (RIDL) is additionally viewed as reasonable for controlling detached populaces of *Anopheles* species, for example, *An. stephensi* and *An. arabiensis* (Coleman & Alphey, 2004). Populace concealment is less inclined to control malaria in Africa because of high inherent regenerative rate and assortative mating of subspecies of *An. gambiae* s.l. (Gwadz, 1994). Populace substitution approach, then again, includes the inundative release of a hereditarily built strain of the objective vector that is unmanageable to the significant pathogen into the wild to supplant the vector populace (Coleman & Alphey, 2004). With regards to malaria control, it infers *Anopheles* species that are unequipped for transmitting human *Plasmodium* parasites (Gwadz, 1994). Despite the fact that, this methodology is viewed as promising in controlling malaria in Africa, the advancement of powerful quality driving components, for example, the utilization of intracellular symbionts, meiotic drive framework, transposable components and under-strength frameworks, that would self-rulingly spread the obstinacy into the *Anopheles* populace is the most testing variable (Wang & Jacobs-Lorena, 2013).

2.4.4.2 Insecticide Application

Insecticide deployment through Insecticide Treated Nets (ITNs) and Indoor Residual Spraying (IRS) and long lasting insecticidal nets (LLINs) have been some of the key components of worldwide malaria control due to its demonstrated efficacy on vector populaces and furthermore they are viewed as the most practical malaria control intervention (Hladish *et al.*, 2018). IRS and ITNs are actualized separately or in mix and the decision relies upon the neighborhood

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epidemiological attributes, worthiness of inhabitants, convenient access to communities (Sine & Doherty, 2010), least inclusion and the speed of the effect required (WHO, 2002). For example, WHO rules have suggested the blend approach in holoendemic circumstances, particularly, in *An. gambiae* commanded settings (WHO, 2002). IRS has been utilized for quite a long time and has contributed fundamentally to the decrease of malaria from a few parts of the world, particularly in areas where malaria is occasionally transmitted and mosquito vectors are indoor-resting (MOP, 2007). In sub-Saharan Africa, 25% to 30% decrease of newborn child death rates through the execution of IRS happened in the Garki Project in Nigeria (Molineaux *et al.*, 1979). Once more, an ongoing expansive scale multi-nation venture in, Swaziland, and South Africa, Mozambique, and Equatorial Guinea, has demonstrated the practicality and effect of Indoor Residual Spraying on malaria in Sub-Saharan African settings (Loewenberg, 2007). In any case, IRS execution is in fact strategically demanding and furthermore requires high acknowledgment to be successful. ITNs have been an extremely successful tool of avoiding man-vector contact in malaria control in Africa. For example, it is accounted for that the presentation of ITNs in Gambia assumed a huge responsibility in decreasing mortality caused by malaria by 42% among youngsters aged 1month to 59 months (Alonso *et al.*, 1991). An audit demonstrated that Insecticide Treated Nets can decrease child mortality by 14% to 29% and malaria cases by 39% to 62% (Pryce *et al.*, 2018). Once more, ITNs have high level of acceptability by householders, as individuals use them for malaria control as well as insurance against other nuisance arthropods, for example, lice and bed bugs, and furthermore high inclusion of ITNs has an overflow impact (WHO, 2002). The effect and supportability of ITNs depend primarily on community interest and level of instruction gotten by householders to change conduct accordingly and effective campaign must be done normally and this could be capital and labor intensive (WHO, 2002). The substantial reduction in malaria

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incidence in Sub-Saharan Africa is attributed to the high coverage of ITN and IRS program (Bhatt *et al.*, 2015) as shown in Figure 5.

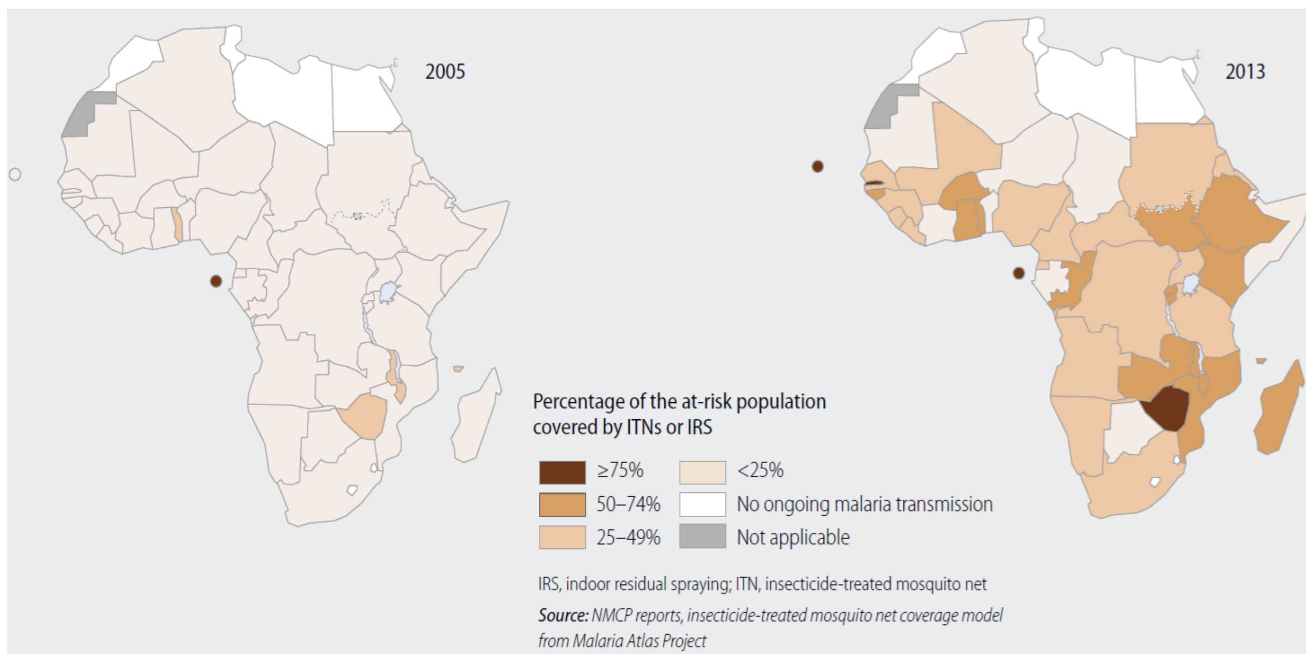

Figure 5: Proportion of the population at risk protected by ITNs or IRS, in sub-Saharan Africa, 2005 and 2013 (WHO, 2014).

2.5 Insecticide resistance

As indicated by the World Health Organization's definition, insecticide resistance is the capacity of an insect to withstand the impacts of an insecticide by getting to be impervious to its dangerous impacts by methods for natural selection and mutations (Ranson *et al.*, 2011). Resistance is a heritable character that depends on hereditary premise and results from the choice of a hereditary change in one or a few qualities happening by movement and/or mutation (Camara *et al.*, 2018). Since the principal instances of DDT resistance with *Ae. tritaeniorhyncus* and *Ae. solicitans* were found in 1947, protection from at least one or more insecticides has been reported in more than 100 mosquito species including more than 50 Anophelines (WHO, 1992). Notwithstanding

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Dichlorodiphenyltrichloroethane, organophosphorus, carbamate and pyrethroid insecticides are altogether been utilized for malaria control, with the pyrethroids overwhelming the market for use in both IRS and ITNs (Yakob *et al.*, 2010). Resistance has developed in the use of every one of these classes of insecticides (Ingham *et al.*, 2018). Pyrethroid resistance in *An. gambiae* s.l. is normal in West Africa including Ghana and different parts of Africa (Ranson *et al.*, 2009). For *An. funestus*, the majority of the recorded pyrethroid resistant cases are in South Africa and Mozambique (Ranson & Lissenden, 2016). Resistance has been recognized in Malawi and suspected in Ghana and Benin (Ranson & Lissenden, 2016). As carbamates are progressively being utilized for IRS, members of *An. gambiae* complex have developed resistance from it in certain countries in Africa including Nigeria, where *An. gambiae* s.s. Giles is now impervious to different insecticides including DDT and deltamethrin (Oduola *et al.*, 2012). Pyrethroid resistance has also been reported in malaria vectors *An. stephensi* and *An. culicifacies* in Asia, just as in Latin America with *An. albimanus* (Brogdon & McAllister, 1998). Organophosphorus resistance has likewise been identified in these malaria vectors just as in *An. arabiensis* in Sudan (Hemingway & Ranson, 2000). Pyrethroid resistance has been noted in *Cx. quinquefasciatus* while resistance to organophosphorus is found in all major Culex vectors (Hemingway & Ranson, 2000). Resistance to pyrethroids has been recorded in *Ae. aegypti* and this mosquito species showed resistance to organophosphorus and carbamate insecticides (Hemingway & Ranson, 2000).

2.5.1 Insecticide resistance mechanisms

Fundamentally four components empower insects to withstand the lethal impacts of insecticides, which are grouped into four classifications, namely; metabolic resistance, target-site resistance, reduced penetration and behavioral avoidance (Ranson & Lissenden, 2016).

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2.5.1.1 Metabolic resistance

Metabolic resistance is the most well-known reason for insecticide resistance in insects and especially mosquitoes (Ranson & Hemingway, 2005). The mechanism depends on the over articulation of enzymes that assist insects to detoxify xenobiotics or insecticides, and three protein families (esterases, P450s and glutathione-S-transferases) are well known (Ranson & Hemingway, 2005). Resistance due to the actions of these proteins have been identified in mosquito populations for all classes of insecticides presently used for vector control, which includes; organochlorines, organophosphates, carbamates, and pyrethroids (Ranson & Hemingway, 2005).

2.5.1.2 Target-site resistance

Target-site resistance (also called target insensitivity) is the second most common type of insecticide resistant mechanism in insects. Insecticides have explicit site of activity in the insect sensory system, which can be changed in resistant strains with the end goal that insecticides would not be able to bind adequately to them (Corbel & N'Guessan, 2013). This insensitivity or reduced sensitivity of the target site is brought about by a point transformation of the gene encoding the protein (Corbel & N'Guessan, 2013). In insects, the transformation in genes encoding for acetylcholinesterase (target site for organophosphates and carbamates) have been found to be ineffectual to the insecticides (Cheung *et al.*, 2018). The G119S Acetylcholinesterase (Ace-1) Target Site mutation in charge of organophosphate and carbamate resistance has been noted in African malaria vector *An. gambiae* just as in *An. albimanus* from Latin America (Cheung *et al.*, 2018). Dichlorodiphenyltrichloroethane and pyrethroid resistance likewise results from change in the sequence of amino acid in the voltage-gated sodium channels of nerve cell membranes in insects prompting decreased affectability of the site to the insecticide (Yellapu *et al.*, 2018).

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Target site resistance or insensitivity is frequently called knockdown resistance (*kdr*) and is the best ascertained type of resistance mechanism, and molecular techniques to recognize it have now been incorporated into insecticide resistance checking measures in malaria control interventions (Ridl *et al.*, 2008). A study done by Boakye *et al.*, (2009) found 81.3% *kdr* allele frequency in bioassay test survivors of *Anopheles gambiae* s.l. which were exposed to deltamethrin and permethrin, which was steady with susceptibility test results. Moreover, reports of high presence of *kdr* gene in *An. gambiae* s.l. populations were gathered from locales that were related with high insecticide use for agricultural purpose (Kabula *et al.*, 2016).

Other resistance mechanism, for example, decreased cuticular penetration and behavioral resistance are distinguished in mosquito populations, yet information on how they meddle with effectiveness of insecticides is as yet not known (Corbel & N'Guessan, 2013).

2.5.2 Insecticide resistance management

Monitoring and surveillance campaigns to identify the resistance status of mosquitoes are fundamental for the structure of intervention procedures to control mosquito-borne diseases (Tusting, 2014). Different resistance management tactics are proposed to defer insecticide resistance in insects. These incorporate rotations, mosaics, blends and combinations of at least two insecticides with various modes of action (Yakob, 2011). Numerous numerical models have been intended to evaluate how these instruments ought to be utilized ideally, despite the fact that they have once in a while been tried in the field because of the down to earth challenges in assessing changes in resistant gene frequencies in several insects (Hemingway *et al.*, 1998). There are currently more prospects to conduct resistant management practices in the field, with the accessibility of various molecular techniques for gene frequency estimation (Corbel & N'Guessan, 2013).

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2.6 Effects of insecticides on non-target organisms

Broad utilization of pesticides in developing countries for agriculture and public health has led to the pollution of the environment and unfriendly effect on plants and animals including people. Wiktelius *et al.*,(1999) explored the impact of various insecticides on some non-target arthropods under laboratory and field conditions in five African nations (Algeria, Nigeria, Tanzania, Uganda and Zambia). It is was recorded that Lindane significantly reduced the numbers of Collembola in over 80% of the field trials for an average of six weeks (Wiktelius *et al.*, 1999). Similarly, spiders were reduced in 53% of the trials for an average of 2.8 week, and ants were reduced in 64% of the trials for an average of 2.5 week (Wiktelius *et al.*, 1999). The lindane treatment significantly reduced organic matter breakdown in over 45% of the trials, whereas endosulfan had no effect (Wiktelius *et al.*, 1999). The latter had little or no effect on non-target arthropods. Endosulfan was found to be harmless to all the non-target arthropod species included in the laboratory screening tests. The order of toxicity was lindane = deltamethrin » chlorpyrifos » endosulfan (Wiktelius *et al.*, 1999). Another study on the impacts of insecticides on stream invertebrates found extremely low portions of the insecticide thiacloprid deadly to the stonefly *Nemoura cinerea* (Beketov & Liess, 2008). Residues of organophosphate insecticides were detected in fish just as in birds (Wiktelius *et al.*, 1999). Dangerous impacts of pyrethroids on non-target organisms have been observed (Smith & Stratton, 1986). The ecological fate and impacts of manufactured pyrethroid insecticides have been abridged. Due to their lipophilicity, pyrethroids have a high rate of gill ingestion, which would be a contributing element in the affectability of fish to aquatic pyrethroid exposures (Smith & Stratton, 1986). Edwards *et al.*,(1987) found that cypermethrin was utilized and disposed of essentially more gradually by fish than by mammals or birds, which may clarify this compound's higher danger in fish than in other organisms. The potential risk of cypermethrin

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to fish is because of its substantial use in numerous aquatic larvicidal programs. The insect juvenile development hormone methoprene utilized generally in the West, is known to mirror the activities of retinoids and incite comparable formative danger in *Xenopus laevis* (Degitz *et al.*, 2003). It is outstanding that pesticides and endocrine disrupter synthetic substances meddle with processes such as egg generation and oocyte development in amphibians (Sower *et al.*, 2000).

Persistent insecticides, for example, organochlorines have side effects such as persistence in the soil, bioaccumulation and bioconcentration in the food chain, and in the tissues of birds, and warm blooded animals, for example, humans (Robin, 2004). International bodies have now put much consideration on pesticides buildups in food, environment and the conceivable damage to people, since quantifiable measures of insecticides are currently being identified in human tissues, blood and breast milk in numerous parts of the world (Ntow *et al.*, 2008).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study sites

Anopheles gambiae s.l. (*Anopheles gambiae* + *Anopheles coluzzi*) and other non-target organisms were sampled from two sites (Opeibea and Madina) in the Greater Accra Region which is found in the coastal savannah of Ghana. Greater Accra, which is in Southern Ghana, is one of the country's most diverse and cosmopolitan region with an estimated population of 1.9 million people (GSS, 2013). This zone is characterized by a bimodal rainfall pattern. March-July is considered as the major rainy season and September-October, as the minor rainy season. Eight hours of sunshine occurs per day (Achonduh *et al.*, 2008). The annual total rainfall is about 700 to 800 mm. The relative humidity and temperatures throughout the year ranges between 65 - 75% and 26 – 30°C respectively (Achonduh *et al.*, 2008).

Opeibea is an urban residential area located within Accra metropolis, the capital of Ghana (Figure 6). The area has an irrigated vegetable growing surface of about five acres, watered by the airport drainage, which serves as source of water for growing of these vegetables (Pwalia *et al.*, 2019). Pits are also dug by the farmers and are used to hold water during the rainy season. During the dry season the pits are filled with water from the drainage with the aid of water pumping machines. Hence, these pits serve as temporal water reservoirs and breeding sites for mosquitoes (Pwalia *et al.*, 2019). The site is also characterized by a massive use of pesticides and herbicides for the control of vegetable pests which have a residual effect on collected pools of water (Pwalia *et al.*, 2019).

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Madina is as well an urban residential area located within Accra metropolis, the capital of Ghana (Figure 6). The area is interspersed with small-scale agricultural activities. Some residents of Madina thus undergo backyard farming of vegetables and other crops such as maize and cassava of which they utilize pesticides. However, the major use of insecticides are in the spraying of rooms against insects especially mosquitoes.

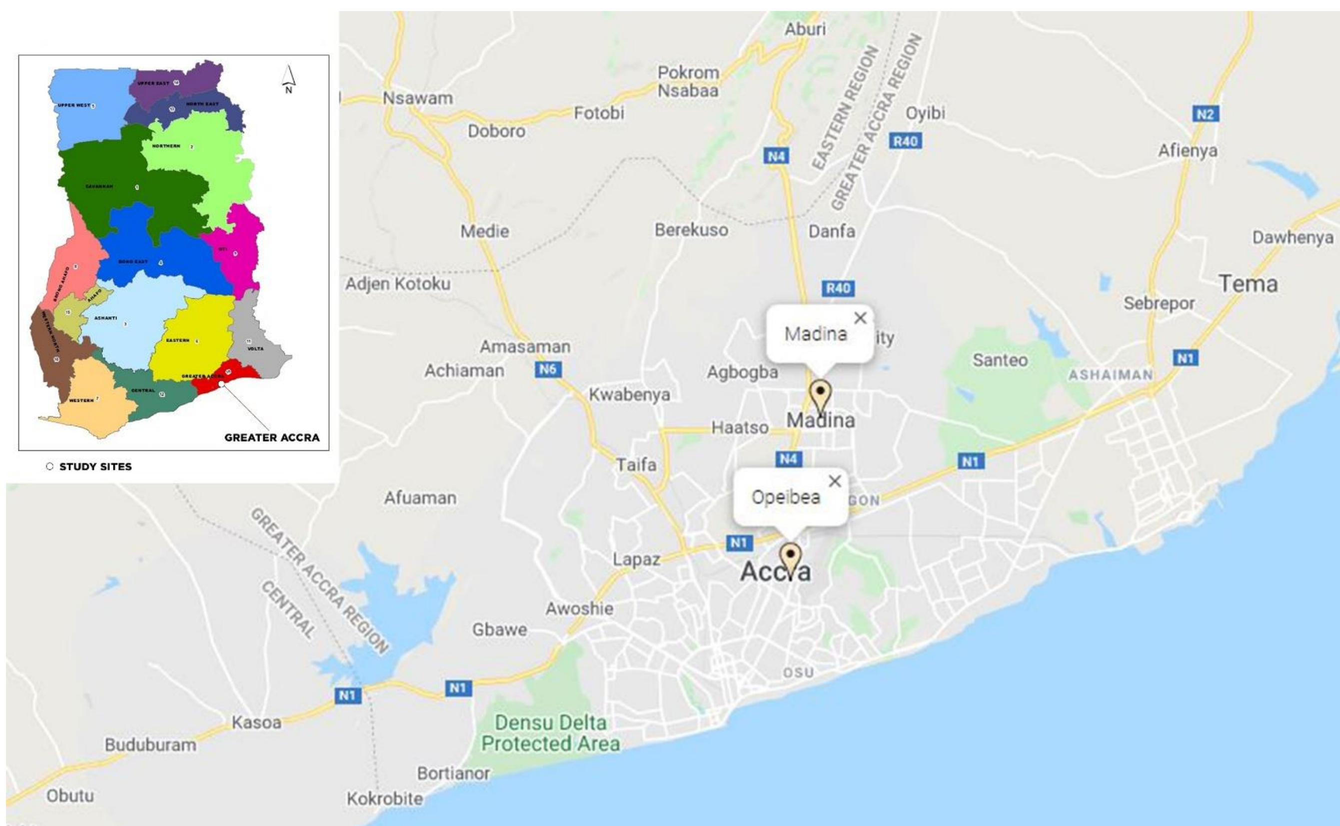

Figure 6: Map showing study sites (Opeibea and Madina) in Greater Accra Region. Source: Google Earth.

3.2 Mosquito collection, maintenance and identification

3.2.1 *Anopheles gambiae* s.l. larval collection

Anopheles gambiae s.l. larvae and non-target organisms were collected from Opeibea and Madina between September – December, 2018. *Anopheles gambiae* s.l. larvae were identified by their lack

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of siphon and parallel position at the water surface. The *Anopheles gambiae* s.l. larvae (both early and late instars) were collected from ponds and shallow wells in vegetable gardens in Opeibea (5°35'55.34"N, 0°10'53.18"W) (Plate 1) and pools, puddles, seepages, wells, water-filled car tracks in Madina (5°40'23.26"N, 0°9'58.99"W) (Plate 2). Susceptible *Anopheles gambiae* s.s. (Kisumu strain) were also obtained from Vestergaard-Noguchi Memorial Institute for Medical Research. Collection was done by the dipping method in the field, using a 500 ml hand-held ladle and were transported to the laboratory in partly-filled, labelled plastic containers. In each site, the samples were collected in at least three locations to improve on the diversity of species population.

Plate 1: Collection points showing *Anopheles gambiae* s.l. breeding sites in Madina.

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Plate 2: Collection points showing *Anopheles gambiae* s.l. breeding sites in Opeibea.

3.2.2 *Anopheles gambiae* s.l. maintenance and identification

Larvae collected from the field and susceptible strains were transported to the insectary at the Department of Animal Biology and Conservation Science (DABCS) at University of Ghana in labelled 4 L transparent plastic containers with perforated lids to allow ventilation. Larvae were transferred into 3 L plastic containers containing 1.5 L of distilled water and raised to pupae and adult stages (Plate 3). This was done separately for the three populations. Each bowl contained about 150 larvae to which 250 mg of Lopis goldfish meals was added every day till pupation. Larvae were raised for 4 to 5 days to pupate. The rearing bowls were secured with fine netting material to keep different mosquitoes from laying eggs in the rearing bowls. The larvae were kept at ambient temperature that went from 25 to 31°C. Pupae were transferred into 220 ml plastic cups with distilled water and put in cages for adults to emerge. Adult mosquitoes were kept at constant ambient temperatures of 28±2°C and RH of 70±10%. They were sustained with 10% (w/v) sugar solution administered with cotton wool which was then placed on top of the mesh on the holding cage (Plate 3).

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Plate 3: Mosquito maintenance; showing rearing of larvae in plastic bowls and maintenance of adults in cages.

Adult mosquitoes that developed out of field collected larvae were morphologically identified. The *Anopheles* adult mosquitoes were identified utilizing the morphological keys of Gillies & Coetzee (1987). *Anopheles gambiae* s.l. adults were distinguished by the nearness of patches or spots on the femur, tibiae and the principal tarsal fragment of the leg with the tibia being barely pale apically. The wings have five unmistakably pale spots on the costal edge of the wing, which are yellow or smooth in shading. They additionally have an indicative white interference on the R1 vein of the third dark territory, which is in some cases persistent with the proximal white zone. Males and females *Anopheles gambiae* s.l. were differentiated by the form of the antennae. In males they are very plumose, while in females they only have a few short hairs.

Adult female mosquitoes that were at least three days old but not more than five days after emergence blood-fed on my hand to lay eggs (Plate 4). Adult female mosquitoes were first starved by removing the cotton sugar ball for at least 12 hours (hrs). The base of the mosquito cage was lined with tissue paper to absorb the blood that drips from the tip of the abdomen of mosquitoes whiles they take blood meal. The light in the insectary was switched off to create darkness which was necessary to foster blood-feeding. Blood feeding was done in the early hours of the morning and evening for 15 - 30 minutes to allow the mosquitoes to take enough blood meal. Cleaning was

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done for any drips of blood on the walls or base of the cage with water moistened cotton buds. The cotton sugar ball was then put back into the mosquito cage.

Plate 4: Blood feeding of 3-5 day old adult female *Anopheles gambiae* s.l.

The sleeve of the mosquito cage was tied loosely to prevent escape of mosquitoes. Two days after blood feeding, an oviposition tray of surface area 0.0058 m^2 was placed in the mosquito cage. The oviposition tray or egging dish was made up of a petri dish lined with filter paper with a ball of wet cotton wool beneath the filter paper. The oviposition tray was removed after 24 hours and the eggs were washed with distilled water into a 350 ml plastic bowl of surface area 0.0090 m^2 to allow eggs to hatch. 40 mg of Lopis goldfish meal was added for 48 hours. After 48 hours, the F1 larvae were distributed into 100 larvae per 250 ml distilled water in other 350 ml plastic bowls each of surface area 0.0090 m^2 to prevent overcrowding. The F1 larvae were then fed with 40 mg of Lopis goldfish meal daily until the third instar which were used for the larval bioassays. The F1 larval populations were also raised to pupae and adults for pupal and adult bioassays.

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3.3 Collection, identification and maintenance of non-target organisms

Various non-target organisms were observed in habitats where the mosquito larvae were collected. Non-target organisms were collected in Madina (5°40’59.80”N, 0°10’28.32”W) because non-target organisms were only observed to be co-habiting with *Anopheles gambiae* s.l. in Madina. These organisms were collected, reared and were used for bioassays. They included tadpoles, snails and fishes which were transported to the laboratory of the Department of Animal Biology and Conservation Science, University of Ghana. The fishes were caught using a small net with a long handle. Tadpoles as well as snails were collected using a hand-held ladle. The fishes were identified as *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae) with the help of morphological keys by Sakurai *et al.*,(1993). *Poecilia reticulata* has many common names such as guppy, mosquito fish, million fish, rainbow fish and gold fish. The snails which are endemic in Ghana were identified as *Physa waterloti*, according to morphological keys by Brown (1994). The sampled tadpoles were identified as *Bufo* sp. according to morphological keys by Du Preez & Carruthers, (2009). The non-target organisms were placed in 350 ml plastic bowls each of surface area 0.0090 m^2 separately containing water from their habitats in the laboratory and maintained for 24 hours before the bioassays (Plate 5). The tadpoles and fishes were fed with 250 mg Lopis goldfish whiles the snails were fed on 40 mg of lettuce.

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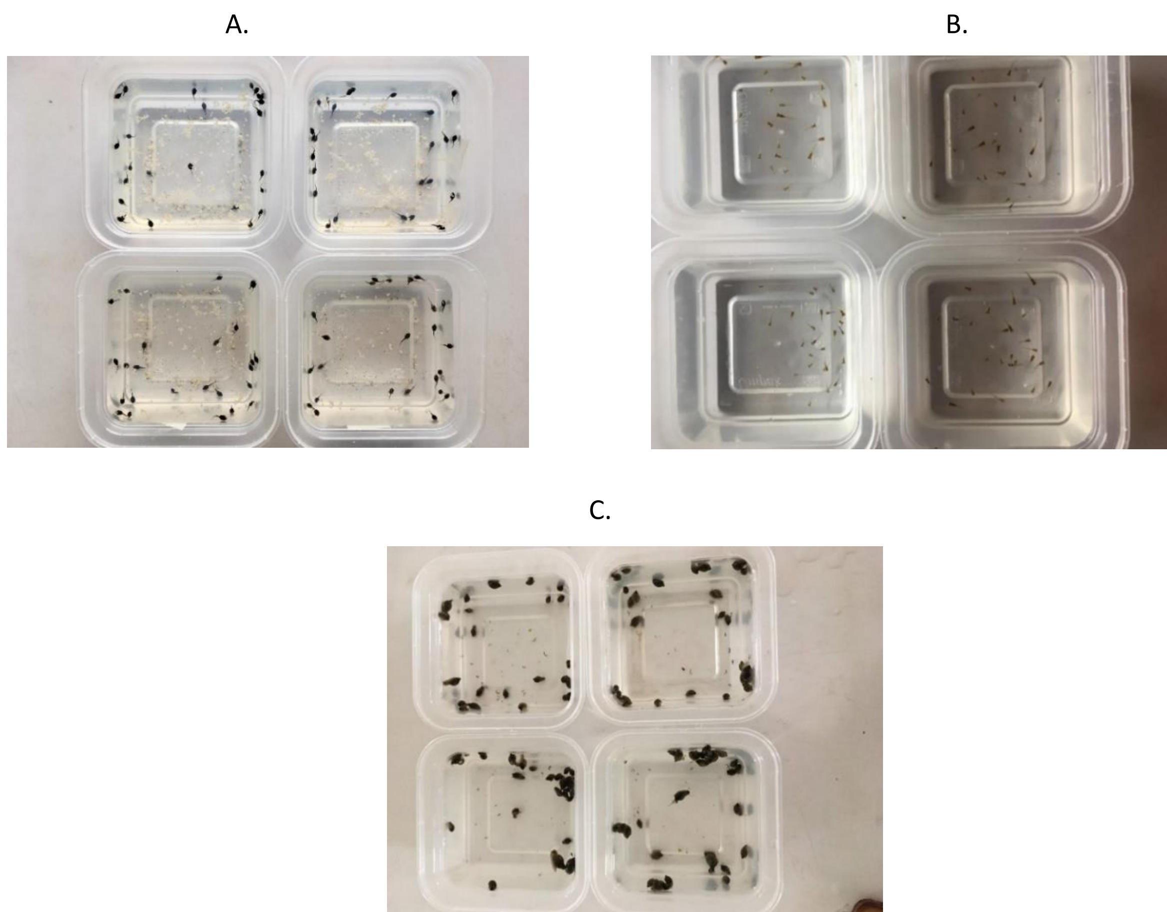

Plate 5: Plastic bowls showing A) *Bufo* sp., B) *Poecilia reticulata* and C) *Physa wtaerloti* .

3.4 Bioassays

3.4.1 Insecticide used for the bioassays

WormAtak® [Emulsifiable Concentrate (EC) and Ultra Low Volume (ULV)] was used in this study. WormAtak® constitutes 50 g/l Teflubenzuron and 20 g/l Cypermethrin (Plate 6). The teflubenzuron and cypermethrin components were manufactured by BASF SE Ludwigshafen Deutschland and Tagros Chemicals India Ltd Chennai India respectively. Teflubenzuron is an Insect Growth Regulator (IGR) which inhibits the synthesis of chitin in larvae which have ingested it; causing the integument to become fragile, and leading to mortality during moulting (Ascher &

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Nemny, 1984). Cypermethrin is a synthetic pyrethroid used as an insecticide in large-scale commercial agricultural applications as well as in consumer products for domestic purposes. It behaves as a fast-acting neurotoxin in insects.

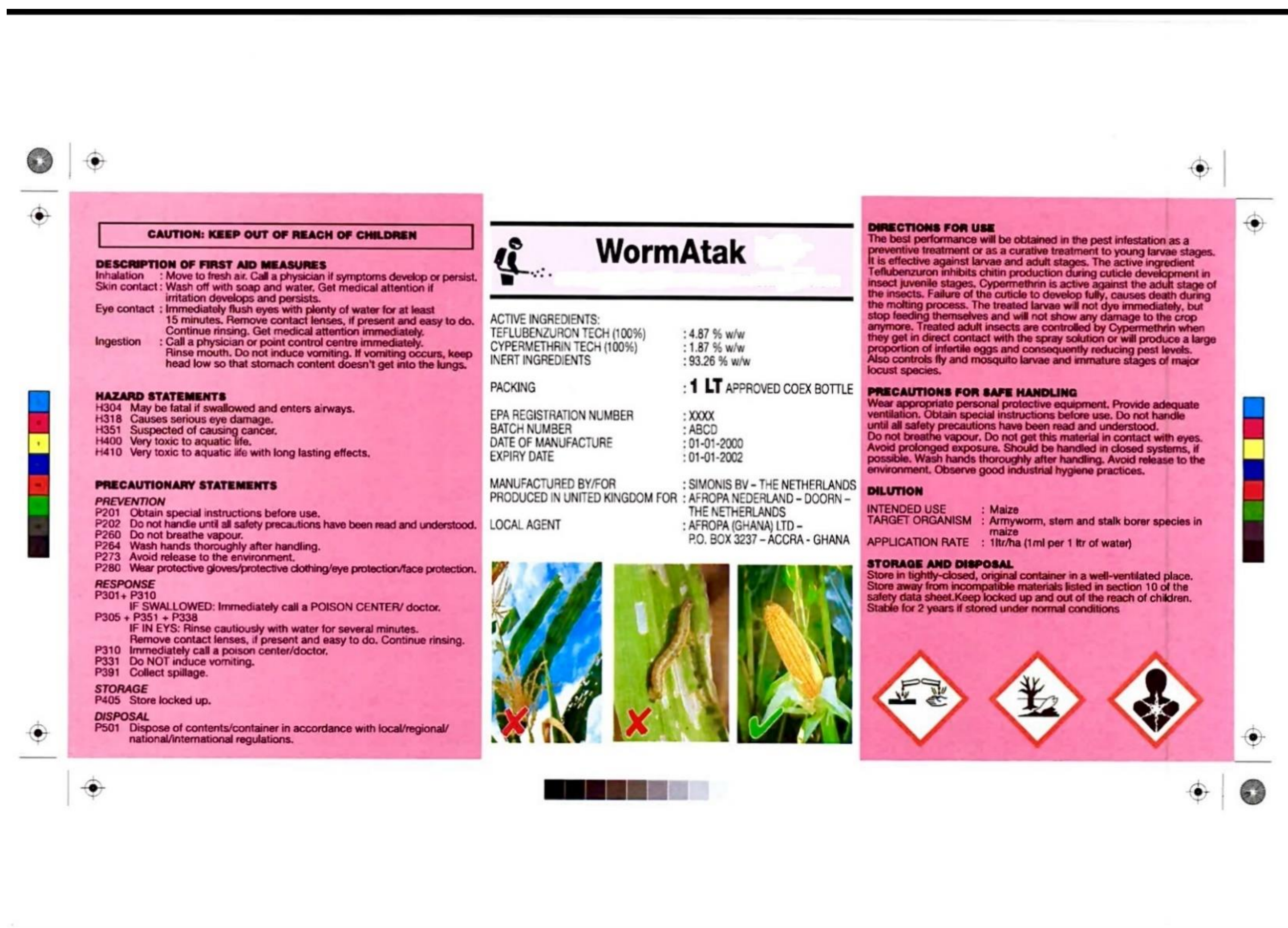

Plate 6: Insecticide label of WormAtak®

3.4.2 Larval bioassays with WormAtak® Emulsifiable Concentrate

Larval bioassays were performed for all F1 third instar larval samples from three populations which were Opeibea, Madina and susceptible strain. For each population, batches of 25 F1 third instar larvae were transferred by means of droppers into four 350 ml plastic bowls each of surface

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area 0.0090 m^2 containing 250 ml of distilled water (Plate 7). Small, unhealthy or damaged larvae were removed and replaced. The depth of the water in the bowl was between 5 and 10 cm; as deeper levels may cause undue mortality. An equal number of controls were also set up simultaneously with 250 ml distilled water. A total of 24 bowls were used in the experiment. The test bowls including the control were held at 28–31 ℃ and at a photoperiod of 12 hrs light followed by 12 hrs darkness (12L:12D). Each test treatment had four replicates.

A 10^{-1} serial dilution of a 0.232 M WormAtak® EC was done by transferring 1ml of WormAtak® EC into a test tube containing 9 ml of dechlorinated water. The solution was shaken thoroughly. Amounts of 20, 40, 60, 80 and 100 µl WormAtak® EC were added to the test treatment bowls containing 250ml distilled water respectively from the prepared stock solution by the aid of a 200 µl micropippette. These gave concentrations of 1.86 µM, 3.71 µM, 5.57 µM, 7.42 µM and 9.28 µM respectively. These concentrations of WormAtak® EC were used based on preliminary experimental results (Appendix 1).

After 24 hour exposure period, larval mortality was recorded to help accertain Lethal Concentration (LC) 50 and 95 values. However, the experimental set-up was left to stand for 72 hours and mortality readings were taken to calculate the Lethal time (LT) 50 and 95 values. During the bioassays, the temperature ranged from 28 – 31°C while relative humidity was 73-79%. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. Dead larvae were those that could not move when they were probed with a needle in the siphon or the cervical region. Moribund larvae were also those incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed.

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Plate 7: Experimental set-up for *Anopheles gambiae* s.l. F1 third instar larvae.

3.4.3 Pupal bioassays with WormAtak® Emulsifiable Concentrate

Pupal bioassays were performed for all samples from three populations which were Opeibea, Madina and susceptible strain. For each population, batches of 10 pupae were transferred by means of droppers into four 350 ml plastic bowls each of surface area 0.0090 m^2 containing 250 ml of distilled water (Plate 8). Small, unhealthy or damaged pupae were removed and replaced. The depth of the water in the bowl was between 5 and 10 cm; as deeper levels may cause undue mortality. An equal number of controls were also set up simultaneously with 250 ml distilled water. A total of 24 bowls were used in the experiment. The test bowls including the control were held at 28–31℃ and at a photoperiod of 12 hrs light followed by 12 hrs darkness (12L:12D). Each test treatment had four replicates.

Concentrations of 1.86 µM, 3.71 µM, 5.57 µM,7.42 µM and 9.28 µM were prepared as described for the larval bioassay and used for the pupal bioassay.

After a 9-hour exposure period, pupal mortality was recorded to help ascertain Lethal Concentration (LC) 50 and 95 values. Mortality readings were taken to calculate the Lethal time

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(LT) 50 and 95 values. During the bioassays, the temperature ranged from 28 - 31°C while relative humidity was 73-79%. Moribund pupae were counted and added to dead pupae for calculating percentage mortality. Dead pupae were those that could not move when they were probed with a needle in the siphon or the cervical region. Moribund pupae were also those incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed.

Plate 8: Experimental set-up for *Anopheles gambiae* s.l. pupae

3.4.4 Adult bioassays with WormAtak® Ultra Low Volume

Bioassay was performed for female adult *Anopheles gambiae* s.l. from the three populations. Twenty-five non-blood fed female adults on average 3-5 days old were aspirated into WHO bioassay tubes and labelled. For each population there were four test treatments and one control setup with four replicates per treatment. Sheets of non-impregnanted papers were rolled into a cylinder shape and then inserted into holding tubes. One sheet was placed in each holding tube, and then fastened into position with a steel spring-wire clip. The holding tubes were attached to

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slides. At least 100 active female adults were aspirated from the cages into the holding tubes for each test treatment. Once the adults were placed into the holding tubes, the sliding unit was closed and then set in an upright position for an hour. This ensured that no adult is damaged before being exposed to WormAtak® ULV.

Concentrations of 1.86 µM, 3.71 µM, 5.57 µM, 7.42 µM and 9.28 µM were prepared as described for the larval bioassay.

Non-impregnanted papers to be used in exposure tubes were dipped into the plastic bowls containing the various concentrations WormAtak® ULV respectively and allowed for a time period of 5 minutes to be well soaked with WormAtak® ULV. The papers were then air dried at room temperature (25°C). Exposure tubes were then prepared and lined with impregnated papers containing WormAtak® ULV.

A control was also prepared using an exposure tube, but with a paper impregnated with Silicon oil alone which was obtained from Vestergaard-NMIMR. The exposure tubes were attached to the vacant side of the slides. The slide unit was opened and adults were gently blown into the exposure tubes. Once all the female adults were in the exposure tubes, the slide unit was closed, and the holding tubes were detached (Plate 9). The adults were kept in the exposure tubes for an hour, after which the holding tubes were attached, and the adults were transferred back into the holding tubes, and provided with 10% glucose (Plate 9). The exposure tubes were detached. The percentage of adults knocked down after the 1 hour exposure period were recorded. The adults were then kept in the holding tubes for 24 hours. This is known as the recovery period. At the end of the recovery period, the number of dead and alive adults were recorded. Mortality was recorded after a 24-hour recovery period with knockdown considered dead. The Lethal Concentration (LC) 50 and 95 of the female adult populations tested were also calculated. The percentage mortality of each test

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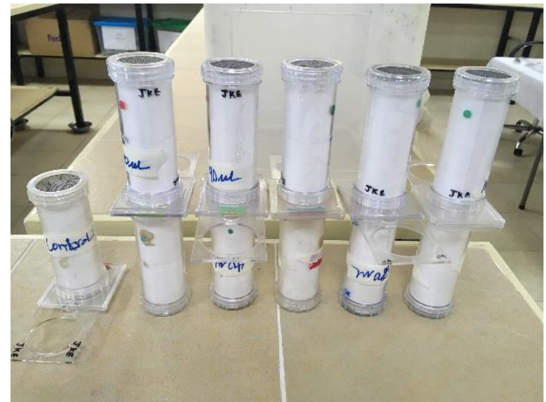
concentration and that of the control was calculated by summing the number of dead adult mosquitoes across each test and its replicates and expressed as a percentage of the total number of exposed adult mosquitoes. Dead adults were counted and stored separately in perforated, labelled 0.5 ml Eppendorf tubes and preserved using silica gel. Female adults that survived were frozen and then also stored separately in perforated, labelled 0.5 ml Eppendorf tubes and preserved using silica gel.



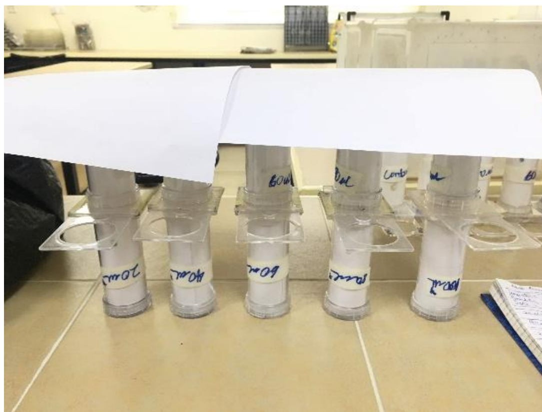


Plate 9: Experimental set-up for *Anopheles gambiae* s.l. adult bioassays.

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3.4.5 Evaluation of the impact of WormAtak® Emulsifiable Concentrate on non-target organisms

Bioassays were performed to assess the impact of WormAtak® EC on 3 non-target organisms observed to co-exist with *Anopheles gambiae* s.l. The three non-target organisms, namely *Poecilia reticulata, Physa waterloti* and *Bufo* sp. were exposed to various concentrations of WormAtak® EC. For each non-target organism, batches of 25 samples were transferred into four 350 ml plastic bowls each of surface area 0.0090 m^2 containing 250 ml of distilled water (Plate 10). An equal number of controls were also set up simultaneously with 250 ml distilled water. A total of 24 bowls were used in the experiment. The test bowls including the control were held at 28 – 31°C and at a photoperiod of 12 hrs light followed by 12 hrs darkness (12L:12D). Each non-target organism was treated separately and each test treatment had four replicates.

Concentrations of 1.86 µM, 3.71 µM, 5.57 µM, 7.42 µM and 9.28 µM were also prepared as described for the pupal bioassay and used for the this bioassay.

After 24 hours of exposure, mortality was recorded to help ascertain Lethal Concentration(LC) 50 and 95 values. However, the experimental set-up was left to stand for 96 hours and mortality readings were taken to calculate the Lethal time (LT) 50 and 95 values. During the bioassays, the temperature ranged from 28 - 31°C while relative humidity was 73 - 79%.

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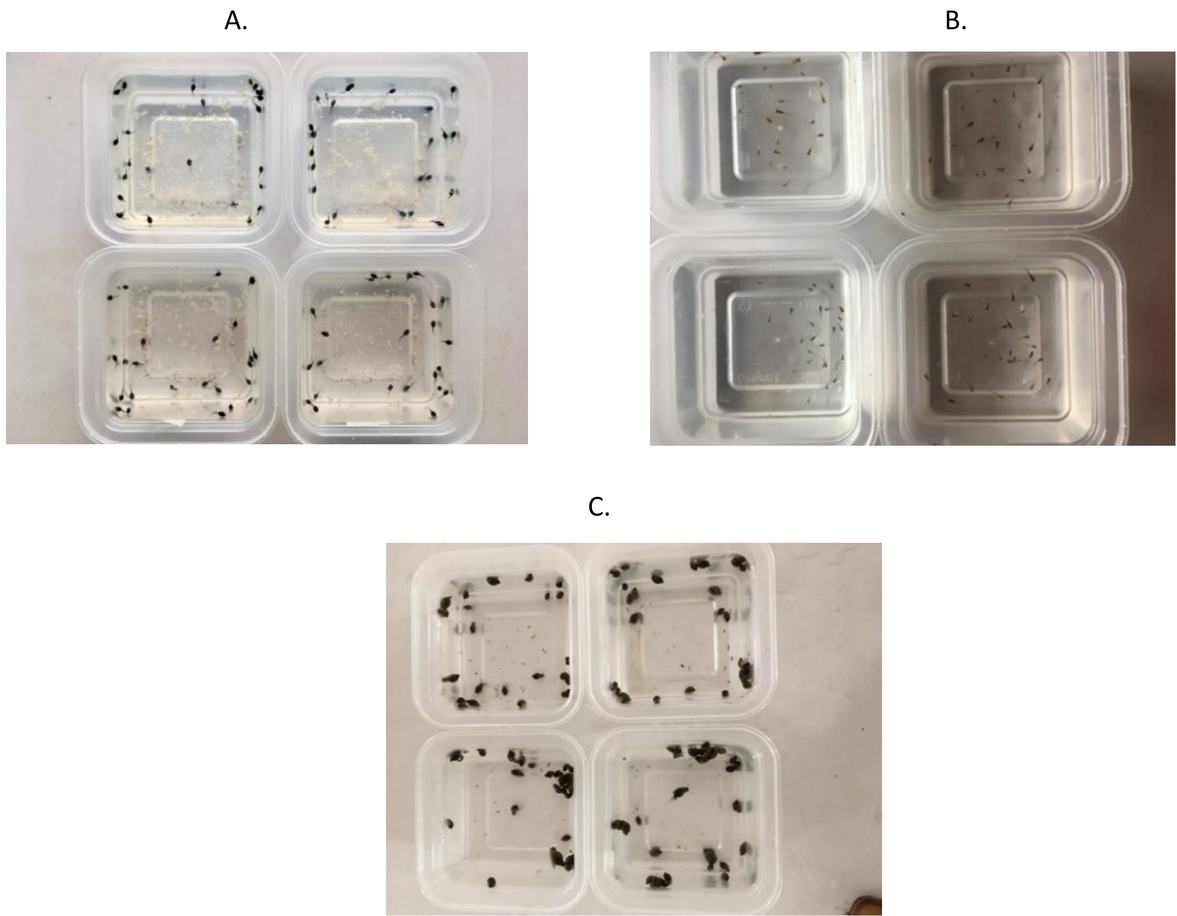

Plate 10: Experimental set-up for A) *Bufo* sp., B) *Poecilia reticulata* and C) *Physa wtaerloti*.

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3.5 Data analyses

Data from all replicates were pooled for analysis. Lethal Concentration (LC) for 50% and 95% of the bioassays were estimated using log probit analysis. The mortality rates obtained in test treatments for the various populations were also compared. Data entry was done using the Microsoft Excel spread-sheet and then imported into the SPSS (version 17) for analysis. All data were assessed for normality following which appropriate parametric or non-parametric tests were conducted. Data were square-root transformed and a one-way analysis of variance (ANOVA) was used to detect significant differences among WormAtak® treatments on mosquito populations and on non-target organisms. The means were separated using the post hoc least significant difference (LSD) test. All levels of statistical significance were determined at $p<0.05$. A probability value (p-value) of less than 0.05 was considered to be statistically significant at 95% confidence interval. The results were illustrated in charts. Resistance factors (RFs) were also determined by dividing LC_{50} of the field populations by the LC_{50} of the susceptible population.

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CHAPTER FOUR

4.0 RESULTS

About 2,500 *An. gambiae* s.l. samples were used for the experiment from each of the three populations (Opeibea, Madina and susceptible strain). A total of 1,800 non-target organisms were treated comprising of 600 samples each of the three non-target organisms (*Poecilia reticulata*, *Bufo* sp. and *Physa waterloti*). During the field sampling, very few *Anopheles* larvae were seen in the habitat where the tadpoles and snails were present. It was observed that where the fishes were in abundance, no larvae existed.

4.1 Identification of mosquitoes

All *Anopheles* larvae and pupae from Opeibea and Madina that were allowed to emerge as adults were morphologically identified as *An. gambiae* s.l.

4.2 Identification of non-target organisms

Fishes, snails and tadpoles collected from mosquito breeding sites in Madina were identified as *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae), *Physa waterloti* and *Bufo* sp. respectively.

4.3 Effect of WormAtak® Emulsifiable Concentrate on first filial third instar larvae of *Anopheles gambiae* s.l.

The F1 third instar larvae of the three populations showed various levels of susceptibility to WormAtak® EC. After 24-hour exposure of *Anopheles gambiae* s.l. from the three populations to WormAtak® EC, a concentration of 4.8 µM killed 50% of the population from Opeibea as compared to 2.3 µM in Madina and 1.4 µM in the susceptible strain (Table 1). Concentrations of 86.8 µM, 39.4 µM and 7.5 µM caused 95% mortalities in Opeibea, Madina and the susceptible strain respectively (Table 1).

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Table 1: Response of three populations of *An. gambiae* s.l. F1 third instar larvae after 24 hours exposure to five aqueous concentrations of WormAtak® EC.

Population	n	Slope ± SE	LC_{50} (µM)	95% CI	LC_{95} (µM)	95% CI	χ^2	RF
Opeibea	600	1.3 ± 0.2	4.8	3.9 - 5.9	86.8	40.1 - 427.6	0.4	3.4
Madina	600	1.3 ± 0.2	2.3	1.5 - 3.0	39.4	22.0 - 127.9	6.8	1.6
Susceptible strain	600	2.3 ± 0.3	1.4	1.0 - 1.8	7.5	6.2 - 9.8	2.6	--

n = total number of larvae tested. LC = Lethal Concentration. RF = Resistance factor.

The F1 third instar larvae of *Anopheles gambiae* s.l. population from Opeibea showed more tolerance to WormAtak® EC as compared to populations from Madina and the susceptible strain (Table 2). Moreover, after 72 hours exposure to WormAtak® EC, all treatments killed more than 50% in each of the three populations under study. The result of the analysis showed significant difference between treatments and control (One-Way ANOVA test: $F=724.737$; $df = 5, 12$; $p<0.0001$). In addition to mortality, WormAtak® EC retarded the development of the surviving larvae in treated bowls. Despite the lack of achievement of 100% mortality after a period of three days, no larvae pupated in the treatments (Table 2). Meanwhile, all the surviving larvae in the control pupated during this period. Survivorship rates in the Madina population and susceptible strain reduced drastically after 24 hours with the susceptible strain being the highest as compared to the Opeibea population which maintained a steady reduction (Figure 7). After the 72 hour period, treatments with 3.71 µM, 5.57 µM, 7.42 µM and 9.28 µM had 0% survivorship in the susceptible strain as compared to Madina population only having 0% survivorship in the 9.28 µM treatment (Figure 7).

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Table 2: Percentage mortalities and lethal time to death (LT_{50} and LT_{95}, in hours) of three populations of *An. gambiae* s.l. F1 third instar larvae after 72 hours exposure to five aqueous concentrations of WormAtak® EC.

Population	Concentration (µM)	% Mortality	% Pupation	Slope ± SE	LT_{50} (Hours)	95% CI	LT_{95} (Hours)	95% CI	χ^2
Opeibea	0	4.0a	96	0.3 ± 0.3	N/A	-	N/A	-	0.1
	1.86	87.0b	0	2.9 ± 0.4	41.0	40.0 -41.6	179.0	178.1-181.2	21.6
	3.71	90.0b	0	2.2 ± 0.4	29.8	28.1 - 31.5	165.2	163.0 -167.9	20.4
	5.57	91.0b	0	2.1 ± 0.4	26.1	25.0 - 27.2	164.9	162.2 - 165.1	19.1
	7.42	95.0b	0	2.0 ± 0.4	20.8	18.9 - 22.2	135.0	134.3 - 136.8	20.5
	9.28	98.0b	0	2.2 ± 0.4	18.0	17.7 - 19.9	100.4	99.2 - 101.7	18.8
Madina	0	5.0a	95	0.9 ± 0.3	N/A	-	N/A	-	0.1
	1.86	89.0b	0	2.4 ± 0.4	23.5	22.9 - 24.9	117.5	116.8 - 118.3	0.7
	3.71	91.0b	0	2.6 ± 0.4	22.1	21.7 - 23.8	96.7	95.1 -98.1	0.1
	5.57	94.0b	0	2.9 ± 0.4	21.5	20.1 - 22.5	80.7	78.9 - 82.1	0.2
	7.42	98.0b	0	2.1 ± 0.5	15.4	13.2 - 16.2	79.7	78.3 - 80.9	0.0
	9.28	100.0b	0	1.9 ± 0.5	10.9	9.9 - 11.5	78.6	77.2 - 79.8	0.2
Susceptible strain	0	5.0a	95	0.2 ± 0.4	N/A	-	N/A	-	0.0
	1.86	95.0b	0	4.3 ± 0.5	25.6	23.7 - 27.1	61.4	60.1 - 63.0	4.8
	3.71	100.0b	0	3.4 ± 0.8	12.0	11.8 - 13.9	36.8	35.2 - 37.9	1.4
	5.57	100.0b	0	2.9 ± 0.8	9.2	8.1 -10.4	34.3	32.9 - 35.8	1.0
	7.42	100.0b	0	0.6 ± 1.0	0.0	0.0 - 0.5	5.2	4.8 - 6.9	0.0
	9.28	100.0b	0	0.5 ± 1.0	0.0	0.0 - 0.3	4.8	3.6 - 5.2	0.0

Means in the same column followed by the same letters for a particular population in a column do not differ significantly ($p<0.05$) from one another.

N/A: not applicable

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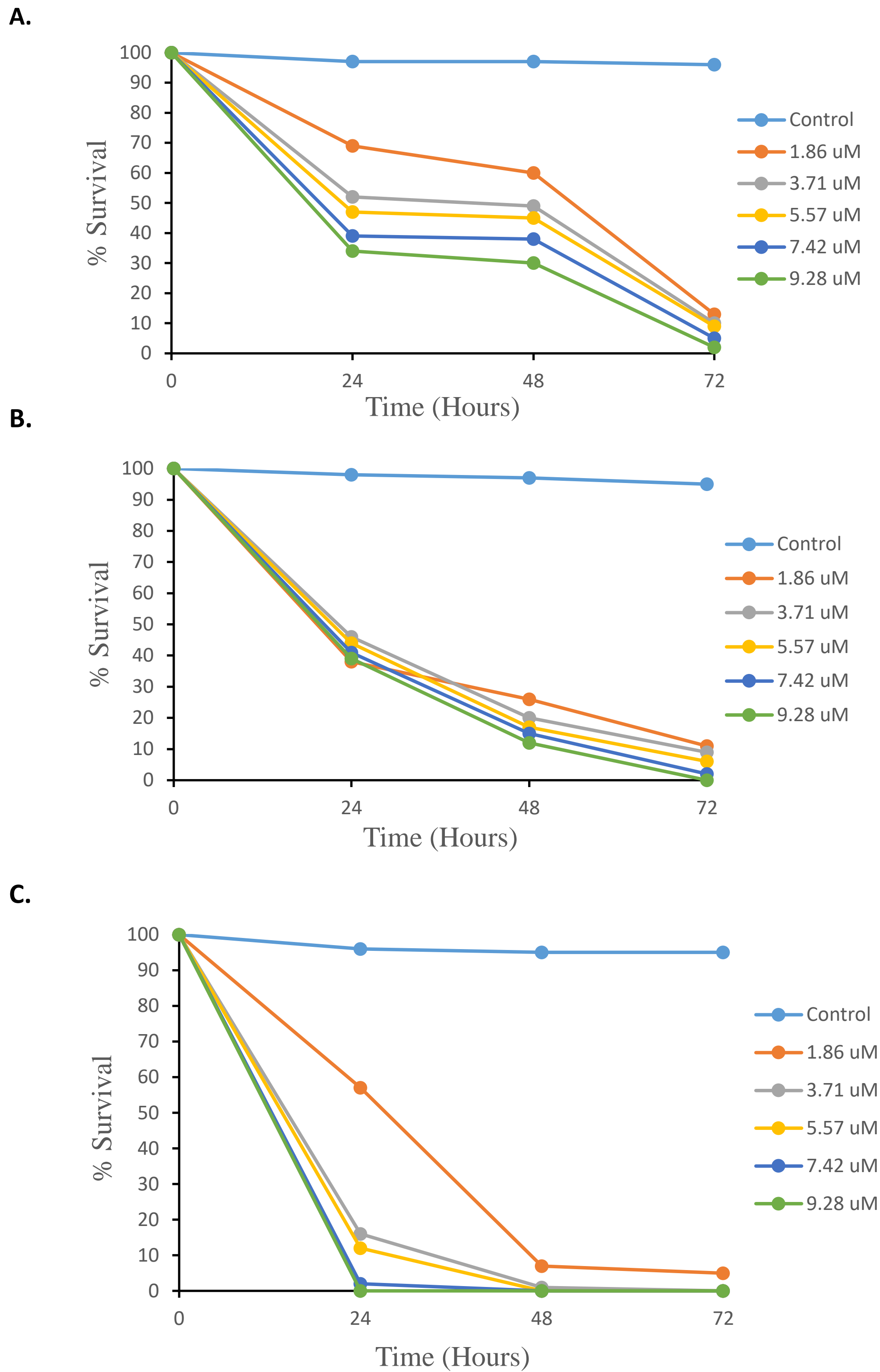

Figure 7: Cumulative survivorship curves of three populations of *Anopheles gambiae* s.l. F1 third instar larvae exposed to five aqueous concentrations of WormAtak® EC for 72 hours.

A) Opeibea B) Madina C) Susceptible strain.

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4.4 Effect of WormAtak® Emulsifiable Concentrate on *Anopheles gambiae* s.l. pupae

Pupae from all the three populations exhibited a continuous rapid movement at the water surface immediately WormAtak® EC was introduced into the treatment bowls. The susceptible strain died faster than populations from Opeibea and Madina. Moreover, after nine (9) hours of exposure to WormAtak® EC, all treatments killed more than 50% in each of the three populations under study. After the nine hours of exposure to WormAtak® EC, the 9.28 µM treatments induced 100% mortality in all three populations. The results showed significant differences between treatments and control (One-Way ANOVA test: $F= 24.799$; $df = 5, 12$; $p<0.0001$). In addition to mortality, WormAtak® EC retarded the development of the surviving pupae in treated bowls. Despite the lack of achievement of 100% mortality after a period of nine (9) hours, no pupae emerged into adults in the treatments (Table 3). Meanwhile, all the surviving pupae in the control emerged.

The survivorship trend was similar for the three populations. The 9.28 µM treatments induced 0% survivorship after nine hours of exposure to WormAtak® EC in the Opeibea population whiles this was attained after 6 hours in the Madina population and the susceptible strain. After the nine (9) hour exposure, the Madina population and the susceptible strain had 0% survivorship in treatments with 5.57 µM, 7.42 µM, and 9.28 µM, whiles in the Opeibea population, survivorship was significantly higher (Figure 8).

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Table 3: Percentage mortalities and lethal time to death (LT_{50} and LT_{95}, in hours) of three populations of *An. gambiae* s.l. pupae after 9 hours exposure to five aqueous concentrations of WormAtak® EC.

Population	Concentration (µM)	%Mortality	% Adult Emergence	Slope ± SE	LT_{50} (Hours)	95% CI	LT_{95} (Hours)	95% CI	χ^2
Opeibea	0	0.0a	100	1.6 ± 0.3	N/A	-	N/A	-	0.0
	1.86	70.0b	0	2.9 ± 0.4	5.5	4.2 - 6.8	20.1	19.0 - 21.2	2.1
	3.71	80.0b	0	3.0 ± 0.4	4.4	3.6 -5.3	15.7	14.9 - 16.5	1.3
	5.57	90.0b	0	2.5 ± 0.4	3.2	2.8 - 4.0	14.7	14.0 - 15.3	2.6
	7.42	90.0b	0	2.1 ± 0.4	2.3	1.9 - 2.9	13.9	12.2 - 14.8	0.1
	9.28	100.0b	0	2.4 ± 0.4	2.0	1.2 -2.6	9.5	8.6 - 10.1	8.6
Madina	0	0.0a	100	2.2 ± 0.3	N/A	-	N/A	-	1.4
	1.86	80.0b	0	3.0 ± 0.4	4.4	3.6 - 5.2	15.7	14.7 - 16.5	1.3
	3.71	90.0b	0	2.5 ± 0.4	3.2	2.7 - 3.9	14.7	13.9 - 15.7	2.6
	5.57	100.0b	0	3.1 ± 0.5	2.1	1.3 - 2.9	7.0	6.2 - 8.3	1.7
	7.42	100.0b	0	2.3 ± 0.5	1.4	0.8 - 2.2	6.9	6.1 - 7.5	3.0
	9.28	100.0b	0	2.6 ± 0.8	1.0	0.5 - 1.7	4.1	3.3 - 5.2	0.8
Susceptible strain	0	0.0a	100	1.2 ± 0.3	N/A	-	N/A	-	0.1
	1.86	90.0b	0	2.5 ± 0.4	3.5	2.8 - 4.2	15.8	15.1 - 17.1	5.3
	3.71	100.0b	0	2.7 ± 0.4	3.0	2.4 -3.7	12.1	11.7- 13.4	0.0
	5.57	100.0b	0	4.7 ± 0.8	2.3	1.7 - 3.1	5.1	4.2- 6.3	3.0
	7.42	100.0b	0	3.8 ± 0.8	1.8	0.7 - 2.1	4.8	3.9 -5.5	1.8
	9.28	100.0b	0	2.6 ± 0.8	1.0	0.5 - 1.5	4.1	3.2 - 5.0	0.8

Means in the same column followed by the same letters for a particular population in a column do not differ significantly ($p<0.05$) from one another. N/A: not applicable.

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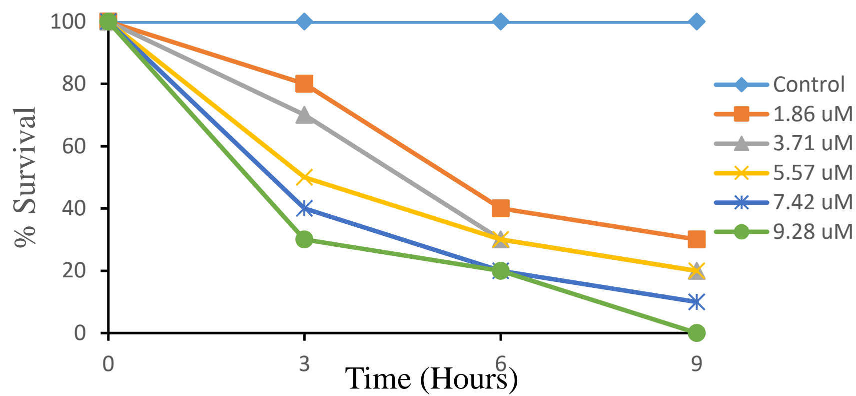

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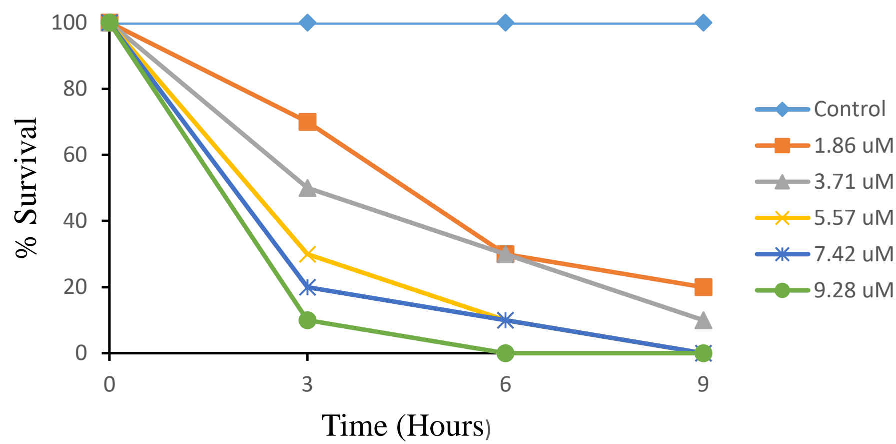

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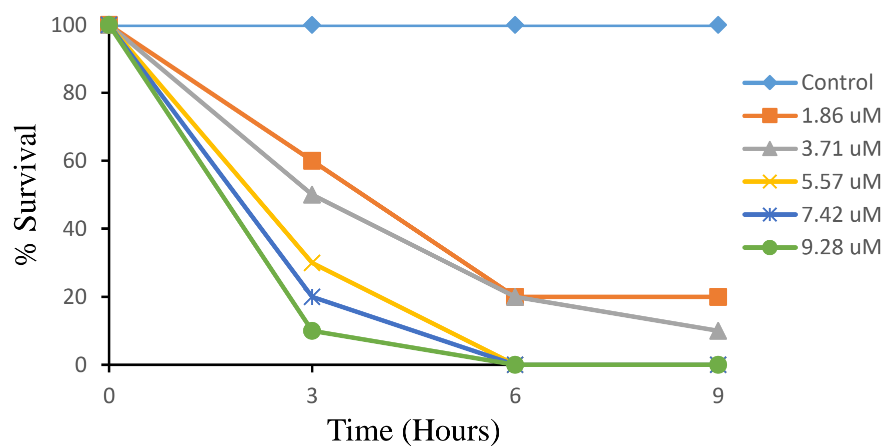

Figure 8: Cumulative survivorship curves of three populations of *Anopheles gambiae* s.l. pupae exposed to five aqueous concentrations of WormAtak® EC for 9 hours.

A) Opeibea B) Madina C) Susceptible strain.

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4.5 Effects of WormAtak® Ultra Low Volume on *Anopheles gambiae* s.l. adults

The *Anopheles gambiae* s.l. female adult populations of Opeibea, Madina and susceptible strain showed various levels of susceptibility to WormAtak® ULV. After 1-hour exposure and 24-hour recovery period of adult female *Anopheles gambiae* s.l. from the three populations to WormAtak® ULV, a concentration of 385.5 µM was estimated to have the potency of killing 50% of the population from Opeibea as compared to 213.6 µM in Madina and 179.9 µM in the susceptible strain (Table 4). Concentrations of 35022.3 µM, 17005.5 µM and 15974.2 µM was also estimated to cause 95% mortalities in populations from Opeibea, Madina and susceptible strain respectively (Table 4). The result of the analysis showed significant difference between treatments and control (One-Way ANOVA test: $F = 14.473$; $df = 5, 36$; $p<0.001$).

Table 4: Response of three populations of *An. gambiae* s.l. female adults after a 1-hour exposure and 24-hour recovery period to WormAtak® ULV.

Population	n	Slope ± SE	LC_{50} (µM)	95% CI	LC_{95} (µM)	95% CI	χ^2	RF
Opeibea	600	0.8 ± 0.4	385.5	383.4 - 387.9	35022.3	35020.1 - 35024.5	0.4	2.0
Madina	600	0.9 ± 0.4	213.6	211.1 - 215.0	17005.5	17003.3 - 17008.1	0.4	1.1
Susceptible strain	600	1.0 ± 0.3	189.5	187.2 - 191.2	15974.2	15971.6 - 15977.1	0.3	-

n = total number of adults tested. LC = Lethal Concentration. RF = Resistance factor.

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4.6 Effect of WormAtak® Emulsifiable Concentrate on non-target organisms

After 72 hours of exposure of the three non-target organisms to WormAtak® EC, the results indicated that, *Bufo* sp. and *Poecilia reticulata* were more susceptible to WormAtak® EC than *Physa waterloti*. *Bufo* sp. and *Poecilia reticulata* from all the treatments exhibited a continuous rapid movement at the water surface immediately WormAtak® EC was introduced into the treatment bowls. After 72 hours of exposure to WormAtak® EC, all treatments killed more than 50% in each of the three non-target organisms under study. The result of the analysis showed significant difference between treatments and control (One-Way ANOVA test: $F= 17.435$; $df = 5, 12$; $p<0.0001$).

Populations of *Poecilia reticulata* and *Bufo* sp. had the same survivorship rates and treatments at all levels of WormAtak® EC induced 0% survivorship in the two populations after 24 hours of exposure. Nevertheless, the control had 100% survivorship. In *Physa waterloti*, survivorship of individuals reduced after 24 hours with the 9.28 µM treatment having only 5 individuals left. After the 72-hour period, concentrations of 3.71 µM, 5.57 µM, 7.42 µM and 9.82 µM had survivorship nearing zero. Survivorship rates were therefore higher in *Physa waterloti* as compared to *Poecilia reticulata* and *Bufo* sp. The survivorship curves of the treatments followed a similar trend (Figure 9).

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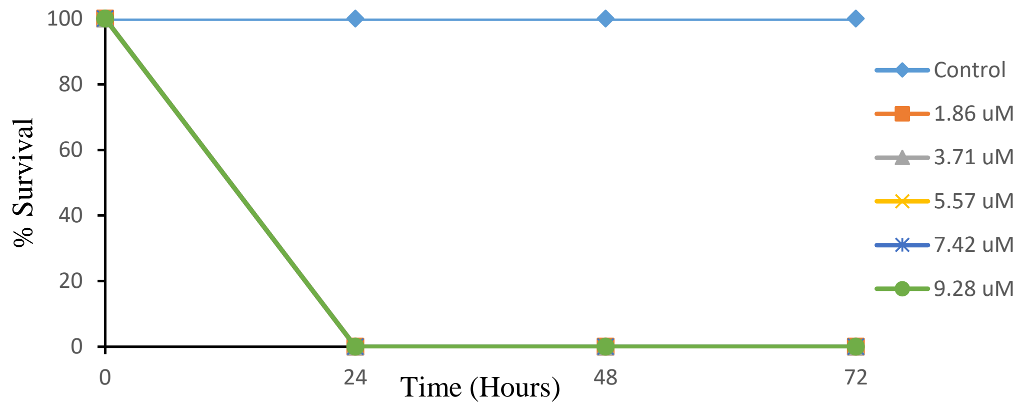
A.



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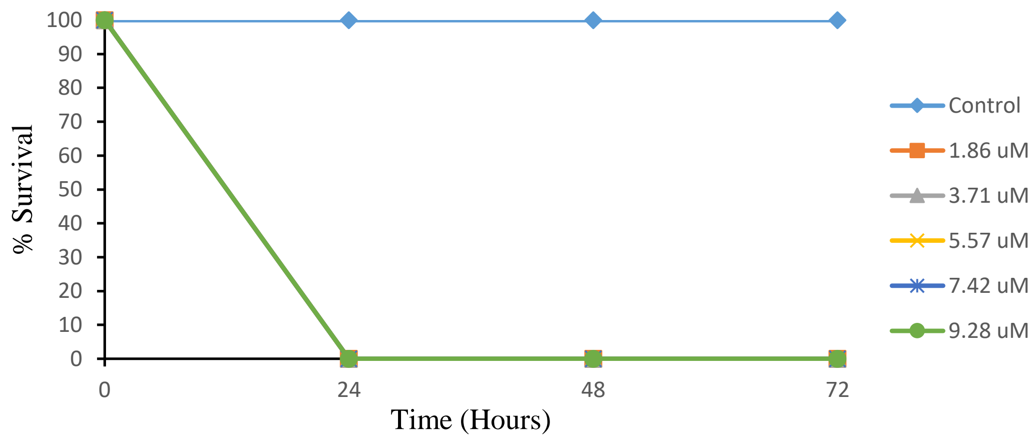

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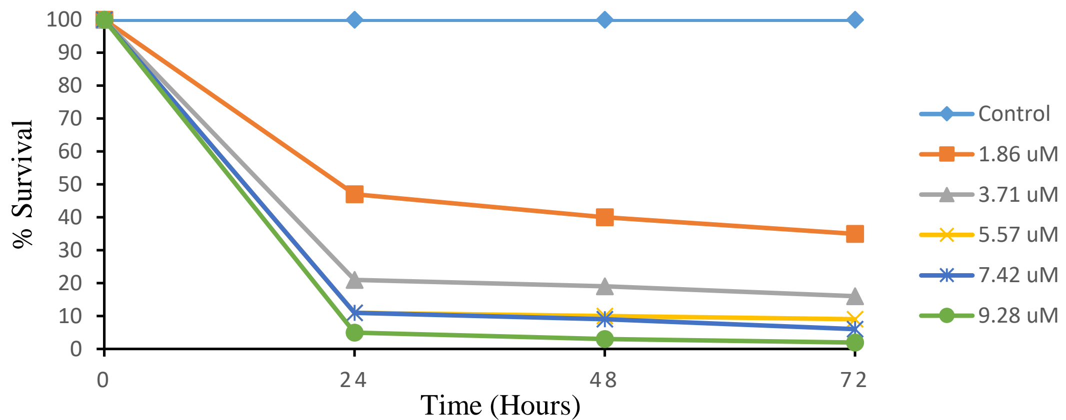

Figure 9: Cumulative survivorship curves of three non-target organisms from Madina exposed to five aqueous concentrations of WormAtak® for 72 hours.

A) *Poecilia reticulata* B) *Bufo* sp. C) *Physa waterloti.*

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CHAPTER FIVE

5.0 DISCUSSION

5.1 Effect of WormAtak® Emulsifiable Concentrate on first filial late instar larvae of *Anopheles gambiae* s.l.

The study revealed that WormAtak® EC had a killing potential on F1 third instar larvae of *An. gambiae* s.l. populations from Opeibea, Madina and the susceptible strain from Vestergaard-NMIMR. All concentrations of WormAtak® EC caused significantly higher mortalities than the controls on *An. gambiae* s.l. populations from Opeibea, Madina and the susceptible strain. The Population of *An. gambiae* s.l. from Opeibea proved to be less susceptible to WormAtak® EC as compared to Madina populations. From this study, all concentrations of WormAtak® EC caused mortality of F1 third instar larvae; however, mortality that resulted from the lowest concentration (1.86 µM) was statistically lower than those caused by the concentrations above it. The higher concentrations, from 5.57 µM to 9.28 µM, produced quite similar efficacy against larvae of all the three populations after 72 hours exposure. These observations could be due to the fact that the 1.86 µM concentration of WormAtak® EC was not high enough to cause very high mortality. However, increasing the concentration above a certain optimal threshold (5.57 µM) does not necessarily result in commensurate mortality effects on immatures stages of the mosquitoes. None of the F1 third instar larvae of *Anopheles gambiae* s.l. populations from Opeibea, Madina and the susceptible strain developed into pupae during the exposure period as was observed in a study done by Chui *et al.*,(1995) where *Ae. aegypti* (Bora-bora) larvae which were exposed to teflubenzuron did not survive during larval moulting to develop into pupae. Teflubenzuron works in controlling mosquito larvae by interfering with the moulting process, killing the larvae when they moult and

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thus, acting more rapidly than the juvenile hormone analogues (Rozendaal, 1997). Since the larvae are filter feeders, the direct ingestion of the chemical in the breeding medium being compromised by WormAtak® EC causes larval integument to become fragile, resulting in death (Ascher & Nemny, 1984). There was fast erratic wrangling by the larvae when WormAtak® EC was introduced at the different concentrations which stopped after the death of the larvae as was also observed in a study carried out by Ikpeama *et al.*,(2017) where *Anopheles* larvae were exposed to cypermethrin.

5.2 Effect of WormAtak® Emulsifiable Concentrate on pupae of *Anopheles gambiae* s.l.

WormAtak® EC treatment was more effective against the pupae than larvae of all three mosquito populations investigated in this study. No pupae emerged into adult and all pupae were killed within 9 hours by all the different concentrations of WormAtak® EC. Mulla *et al.*,(1982) reported that several pyrethroids including cypermethrin were highly active against mosquito pupae. A study by Helson & Surgeoner (1986) stated that cypermethrin was an excellent mosquito pupicide at 20 g ai/ha as it was observed that 90 to 100% mortality of pupae was obtained at this dosage. In the study only 3 pupal exuviae were collected in the post treatment samples from the 3 pools compared to 200 pupal skins in the corresponding untreated control pools. Just 3% of the pupae in the bioassay cages emerged in the treated pools compared to 83% in the untreated pools. WormAtak® treatment caused pupae to become extended and were no longer in a comma-shape as was also observed by Schaefer & Wilder (1973). Insect Growth Regulators (IGRs) have been reported to be lethal to pupae of mosquitoes. A study done by Assar *et al.*,(2019) also showed that teflubenzuron completely inhibited adult emergence of *Culex pipiens*.

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5.3 Effect of WormAtak® Ultra Low Volume on adult *Anopheles gambiae* s.l.

The study revealed that WormAtak® ULV had a killing potential on female adults of *Anopheles gambiae* s.l. populations from Opeibea, Madina and Susceptible strain from Vestergaard-NMIMR. *Anopheles gambiae* s.l. from all three populations experienced significantly higher mortality than the controls at all concentrations of WormAtak® ULV. The *Anopheles gambiae* s.l. population from Opeibea were comparatively less susceptible to WormAtak® ULV than the Madina population. However, the mortality observed in the adults were much lower than in the larval and pupal populations for the same concentrations of WormAtak®. The low mortalities in the female adults of *Anopheles gambiae* s.l. may because they just had contact with WormAtak® and did not directly ingest the chemical. However, the larval and pupal populations had their whole body surface submerged into the WormAtak® solution which was not the same for the female adult population. Hence, the minimal effects of WormAtak® on *Anopheles gambiae* s.l. female adults. A study done by Hoppé *et al.*,(2016) revealed that cypermethrin caused 80–100% mortality between 0.2 and 2 mg AI litre^{-1} and knockdown activity at approximately 2 mg AI litre^{-1} against adult female *Anopheles* mosquitoes. Swale *et al.*,(2018) reported that Novaluron (IGR) can be used in the control of *Anopheles* sp. through a method that is novel, cost efficient, long lasting and requires minimal human intervention due to its long residual effects. WormAtak® however deserve further evaluation as a potential candidate for IRS or ITN applications in malaria vector control. Selection criteria to be considered in such an evaluation will include the ease of formulation and polymer coating or incorporation, activity and durability of the treated polymer or the IRS formulation, potential for cross-resistance, cost efficiency, toxicity and ecotoxicity (Hoppé *et al.*, 2016).

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5.4 Effect of WormAtak® Emulsifiable Concentrate on non-target organisms

The effect of WormAtak® EC on *Poecilia reticulata* indicates that WormAtak® EC has the potential to kill *Poecilia reticulata*. *Poecilia reticulata* access the water encompassing them through their gills. However, *Poecilia reticulata* appears to lack the chemical framework that hydrolyzes pyrethroids and since pyrethroids including cypermethrin have a high rate of gill ingestion (Polat *et al.*, 2002), they are greatly affected which explains the 0% survivorship after 24 hours of exposure. The reason for no survivorship may be attributed to the activity of cypermethrin as reported by Medeiros *et al.*,(2013) who found teflubenzuron to be practically non-toxic to *Poecilia reticulata*. Hence, the uncontrolled use of the insecticide would adversely affect the survival of these biological agents in malaria control.

Similar to its effect on *Poecilia reticulata*, WormAtak® EC had adverse lethal effects on *Bufo* sp. collected which were coexisting in *Anopheles gambiae* s.l. breeding sites. *Bufo* sp. recorded a 0% survivorship after 24 hours of exposure. *Bufo* sp. also access the water encompassing them through their gills. Due to the lipophilic nature of cypermethrin, they are absorbed at a high rate through gills making the chemical more toxic to them. A study by Pancharatna *et al.*,(2010) revealed that, an IGR (Novaluron) disrupts the normal amphibian development when present in minute concentrations in the aquatic medium where amphibians live and reproduce. Hence, it may be ascertained that both cypermethrin and teflubenzuron were responsible for the low survivorship rate. In view of this, inappropriate use of this chemical can reduce amphibian populations.

WormAtak® EC proved to also have a lethal effect on *Physa waterloti*. This confirmed a study done by Schooley & Quistad (1979) where diflubenzuron which is an IGR was persistent in aquatic snails after its introduction into their breeding medium and thus had a lethal effect on them. However, the survivorship rates in *Physa waterloti* were much higher than that of *Poecilia*

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reticulata and *Bufo* sp. This could be due to the possession of shells. It was realized that after application of WormAtak® EC, *Physa waterloti* coils back into the shell and hence reduces body surface contact to the treated medium. Some of the treated individuals of *Physa waterloti* had their shells discolored after the 72 hour exposure period. A study done by Tripathi & Singh (2004) found out that cypermethrin altered the oxidative metabolism in hepatopancreas and ovotestis tissues of snails and thus stress conditions result in less availability of oxygen and in turn less Adenosine triphosphate production in tissues, adversely affecting oxidative metabolism. Hence, the low survivorship rates in *Physa waterloti* may be due to the combined effects of teflubenzuron and cypermethrin in WormAtak® EC.

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CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The findings of this study indicated that WormAtak® EC kills all larval, pupal and adult stages of *Anopheles gambiae* s.l. populations. However, it was more effective against the late instar larvae and pupal populations as compared to the adults. It also revealed that any concentration from 1.86 µM can effectively control both larvae and pupal populations. WormAtak® EC proved to have adverse effects on non-target organisms investigated even at lower concentrations. Hence, this combination can be very useful in terms of achieving general mosquito control in aquatic breeding sites and in indoor residual spraying.

6.2 Recommendations

- Although WormAtak® has proved to be effective against mosquito larvae, pupal and adult populations in this laboratory study, it is necessary to evaluate its field efficacy in Ghana to establish its residual activity in the West African ecological settings.
- Evaluation of the harmful effects of WormAtak® on non-target organisms in this study was limited to just three species. Further research needs to be carried out to study the effects of WormAtak® on a broader range of non-target species.
- The long term effects of WormAtak® should also be evaluated, since the current study only focused on short term effects on mosquitoes and non-target organisms.

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APPENDICES

Appendix 1: Preliminary results on mortality count for *An. gambiae* s.l. F1 third instar larvae after 72 hours exposure to WormAtak® EC through an aqueous solution method.

Serial dilution	Amounts of WormAtak®	Alive	Dead
10^{-1}	5µL	96	4
	10µL	88	12
	20µL	76	24
	40µL	68	32
10^{-2}	5µL	100	0
	10µL	100	0
	20µL	100	0
	40µL	96	4
10^{-3}	5µL	100	0
	10µL	100	0
	20µL	100	0
	40µL	100	0
10^{-4}	5µL	100	0
	10µL	100	0
	20µL	100	0
	40µL	100	0
10^{-5}	5µL	100	0
	10µL	100	0
	20µL	100	0
	40µL	100	0
Control	---	100	0

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Appendix 2: Mortality count for three populations of *An. gambiae* s.l. F1 third instar larvae after 72 hours exposure to WormAtak® EC through an aqueous solution method.

Populations	Conc. (µM)	No. exposed (replicates)	Cumulative percentage mortality at different time intervals (in hours)		
			24 hours	48 hours	72 hours
Opeibea	1.86	100 (4)	31	40	87
	3.71	100 (4)	48	51	90
	5.57	100 (4)	53	55	91
	7.42	100 (4)	61	62	95
	9.28	100 (4)	66	70	98
	Control	100 (4)	3	3	4
Madina	1.86	100 (4)	52	74	89
	3.71	100 (4)	54	80	91
	5.57	100 (4)	56	83	94
	7.42	100 (4)	66	85	98
	9.28	100 (4)	75	88	100
	Control	100 (4)	2	3	5
Susceptible strain	1.86	100 (4)	43	93	95
	3.71	100 (4)	84	99	100
	5.57	100 (4)	88	100	100
	7.42	100 (4)	98	100	100
	9.28	100 (4)	100	100	100
	Control	100 (4)	4	5	5

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Appendix 3: Mortality count for three populations of *An. gambiae* s.l. pupae after 9 hours exposure to WormAtak® EC through an aqueous solution method.

Populations	Conc. (µM)	No. exposed (replicates)	Cumulative percentage mortality at different time intervals (in hours)		
			3 hours	6 hours	9 hours
Opeibea	1.86	100 (4)	20	60	70
	3.71	100 (4)	30	70	80
	5.57	100 (4)	50	70	80
	7.42	100 (4)	60	80	90
	9.28	100 (4)	70	80	100
	Control	100 (4)	0	0	0
Madina	1.86	100 (4)	30	70	80
	3.71	100 (4)	50	70	90
	5.57	100 (4)	70	90	100
	7.42	100 (4)	80	90	100
	9.28	100 (4)	90	100	100
	Control	100 (4)	0	0	0
Susceptible strain	1.86	100 (4)	40	80	80
	3.71	100 (4)	50	80	90
	5.57	100 (4)	70	100	100
	7.42	100 (4)	80	100	100
	9.28	100 (4)	90	100	100
	Control	100 (4)	0	0	0

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Appendix 4: Mortality count for three populations of *An. gambiae* s.l. female adults after a 1-hour exposure and 24-hour recovery period to WormAtak® ULV.

Populations	Conc. (µM)	No. exposed (replicates)	Cumulative percentage mortality
Opeibea	1.86	100 (4)	4
	3.71	100 (4)	5
	5.57	100 (4)	6
	7.42	100 (4)	9
	9.28	100 (4)	10
	Control	100 (4)	1
Madina	1.86	100 (4)	6
	3.71	100 (4)	8
	5.57	100 (4)	10
	7.42	100 (4)	11
	9.28	100 (4)	15
	Control	100 (4)	2
Susceptible strain	1.86	100 (4)	8
	3.71	100 (4)	10
	5.57	100 (4)	12
	7.42	100 (4)	13
	9.28	100 (4)	19
	Control	100 (4)	2

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Appendix 5: Mortality count for three non-target organisms after 72 hours exposure to WormAtak® EC through an aqueous solution method.

Populations	Conc. (μM)	No. exposed (replicates)	Cumulative percentage mortality at different time intervals (in hours)		
			24 hours	48 hours	72 hours
Poecilia reticulata	1.86	100 (4)	100	100	100
	3.71	100 (4)	100	100	100
	5.57	100 (4)	100	100	100
	7.42	100 (4)	100	100	100
	9.28	100 (4)	100	100	100
	Control	100 (4)	0	0	0
Physa waterloti	1.86	100 (4)	53	60	65
	3.71	100 (4)	79	81	84
	5.57	100 (4)	89	90	91
	7.42	100 (4)	89	91	94
	9.28	100 (4)	95	97	98
	Control	100 (4)	0	0	5
Bufo sp.	1.86	100 (4)	100	100	100
	3.71	100 (4)	100	100	100
	5.57	100 (4)	100	100	100
	7.42	100 (4)	100	100	100
	9.28	100 (4)	100	100	100
	Control	100 (4)	0	0	0