CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Mosquitoes belong to the large family of dipteran insects called Culicidae. They comprise the largest group of blood sucking insects which attack not only humans, but also many kinds of vertebrates, including mammals and birds. They cause the greatest harm of all vector borne diseases in Nigeria transmitting malaria, yellow fever, dengue fever and filariasis with malaria being the major public health problem and a cause of poverty in the country (FMH, 2009).

Current efforts at mosquito control in Africa and indeed Nigeria have gradually drifted away from the more traditional larviciding and environmental management to disease management and domestic adulticiding since the discovery of dichlorodiphenyltrichloroethane (DDT). Despite the recorded successes due to the ease of application of these measures, disease management and adulticiding alone have failed to interrupt mosquito vector burden on a nationwide basis. These approaches relied more on the use of chemicals alone resulting in several deleterious effects on man and the ecosystem.

The prolong use of some of these chemicals has led to deleterious effects on non-targets species including plants and animals (Brieger, 2007). DDT was highly persistence in the environment and accumulated in food chain leading to legal issues following reactions from many environmentalist particularly, the *Silent Spring* publication by Rachel Carson in 1962 (Pedigo, 2004). There were incidences of cross resistance with the use of synthetic larvicides

particularly, pyrethroids (Walker and Lynch, 2007) that probably led to mosquito resurgence and emergence of new strains of mosquitoes and related parasites. As a consequence of these problems, there is the continual establishment of new cases of mosquito related diseases and subsequently death particularly amongst children under the age of five and pregnant women (FMH, 2005).

The aforementioned weaknesses brought about the introduction of Integrated Vector Management (IVM) that forms the 21st century disease vector control method proposed by World Health Organization (WHO) in the early 1980s and based on the principles of Integrated Pest Management (IPM) of the agricultural sector. Integrated vector management approach was to resolve not only technical issues pertaining to vector control implementation, but also, to take into consideration health systems constraints as well as, environmental concerns related to the judicious use of insecticides (Chanda *et al*., 2008).

It is almost impossible to attain a total elimination of mosquito vectors from any ecosystem therefore, good mosquito management practices that consider environmental issues must be adopted within the purview of IVM to effectively suppress their population. The integration of improved chemical and non-chemical larval control methods could offer sustainable supplement to other mosquito vector and pathogen control efforts (Killeen *et al*., 2000).

Integrated Vector Management is the targeted use of different vector control methods alone or in combination to achieve the greatest disease control benefits in the most cost effective manner while minimising negative impacts on the ecosystem such as the depletion of biodiversity and adverse side effects on public health (WHO, 2008a). It is based on the premise

that effective control requires the collaboration of various public and private agencies including communities. Integrated Vector Management aims at addressing the deficiencies in vector control and improves efficiency, cost-effectiveness, ecological soundness, sustainability and inter-sectoriality.

The larval stage of mosquito vectors is the most vulnerable stage because they are confined by nature in various aquatic media thus, making control practices more effective and highly sustainable. When mosquito larvae are effectively targeted, they help break the transmission chain in mosquito life cycle thereby resulting in a more sustainable control programme. To achieve an effective and long lasting mosquito control programme, which is one of the major challenge facing mosquito vector control programmes in Africa and indeed Nigeria, there is need to reprioritize larval control practice and adopt the multi-prong approach inherent in IVM System. Efficacious chemical larviciding method can be combined with biological control agent such as the use of fish, *Poecilia reticulata* (guppy).

Guppy, though native to South America, have formed conspicuous members of our freshwater ecosystem. They have been credited for their very high larvivorus profile against mosquito vectors, indeed, the greatest of all the larvivorus fishes (Ekaneyeka, *et al*., 2007). Some of the reasons include their ease of culturing, their habit as surface feeders, their high prolificacy and extreme hardiness.

Many larvicides ranging from organophosphates (Lawal & Samuel, 2010) to newer chemical groups such as bio-larvicides (Stevens *et al*., 2005; Hertelin *et al*., 2010) have been used successfully for mosquito vector control. These chemicals or larvicides when applied

repeatedly to effectively control mosquito larvae often accumulate in water bodies resulting in increased exposure of non-target aquatic organisms including *P. reticulata*. The synergistic/antagonistic effects of the mixture from various larvicides are hardly interpreted and predicted exclusively from the chemical analysis hence, the use of biomarkers on *P. reticulata* to further assess the toxicological impact of these xenobiotics on the non-target biological component of the integrated control approach. Furthermore, fish species are excellent biological monitors of environmental pollutants (Lopez-Barea, 1996; Van der Oost *et. al*., 2003).

Biological markers are used as indicators of a biological state. They are evaluated as the indicators of normal biological processes, pathogenic processes, or response to any particular stress stimuli (Padmini *et al*., 2011). The determination of the status of biomarkers in fish provides evidence of exposure to chemical pollutants. They also indicate a toxic effect in the tissue or body fluids at the level of the whole organisms through biochemical, cellular, physiological or behavioral variations (Taleb *et al*., 2009).

Histological biomarkers include behavioral responses which usually reflect sub-lethal toxicity of chemicals (Little, 1990; Drummond *et al*., 1986); induction or frequencies of some cell types such as the micronucleus cell (MN) which are small fragments of intra-cytoplasmic chromatin that arise from chromosome breaks or whole chromosomes, after the action of clastogenic substances or spindle-poisons, that do not migrate during anaphase (Campana *et al*., 1999; Cavas and Ergene- Gozukara, 2003a); the Binucleated cells (BN) which are cells with two nuclei of relatively equal size bounded as in mature cells or unbounded as in im-

mature cells (Cavas and Ergene- Gozukara, 2003a; Cavas and Ergene-Gozukara, 2005a); Polychromatic erythrocytes (PCE) which are young cells without visible cytoplasmic boundary (Cavas and Ergene-Gozukara, 2005b; Cavas, 2008), and Normo-chromatic erythrocytes (NCE) which are normal cells with distinct cytoplasmic boundary (Cavas and Ergene-Gozukara, 2005b). In furtherance, the ratio of PCE to NCE and the presence of ultrastructural changes in exposed organisms are additional examples of histological biomarkers used to assess the effect of xenobiotics on organisms at cellular and subcellular levels of organization respectively.

The gill and intestinal tissues are the prime sites for uptake and absorption of nutrients and fluid in fish. These tissues provide free access to various chemicals and metals from the external aquatic environment into the internal environment of the fish. The gastrointestinal tract particularly encounters the seemingly contradictory challenges to limit and stop the effects from these chemicals by desquamation of its intestinal epithelium mucus (Sorensen, 1991). In the case of continuous or high exposures, xenobiotics are further transferred from the gut epithelia via blood to other tissues for storage and detoxification in kidney and liver (Marijic and Raspor, 2007) probably to protect the organism. Therefore, the health of these tissues is pivotal in the proper functioning of *P. reticulata* as a biological component in an integrated mosquito larval control.

Usually damage to tissues begins as molecular malfunction within specific organelles (Wayne *et al*., 2009) that may gradually progress into a disease or chronic disorder. The nucleus and mitochondria cells were selected for study because they contain the genetic ma-

terials that define the general activity of an organism as well as, help in the production of energy in the fish. It is well-known that mitochondria defects follow maternal inheritance and these defects may be transmitted to the fish offsprings (Wayne *et al*., 2009). It is therefore pertinent to investigate the impact of some selected larvicides on guppy fish to ascertain their fitness for use as predatory agent against mosquito larvae following the fish pre-exposures to the chemicals.

The evaluation of the effects of chlorpyrifos, actellic and spinosad using suite of biomarkers will allow the examining of specific target organs including the guts and gills that are responsible for vital functions, such as respiration, excretion, accumulation and biotransformation of xenobiotic in fish (Gernhofer *et al*., 2001). It will also provide data on the toxicity profile of these natural and synthetic larvicides under acute and sub-lethal toxicity studies.

Guppies play an important role of regulating mosquito vector population in an ecosystem Anogwih and Makanjuola, (2010) and must be protected from elimination therefore an in vivo evaluation of the cytological and histological effects of the selected larvicides on the intestinal and gill cells of the fish species will form the basis for an establishment of a dosage solution that is compatible to apply in an aquatic ecosystem particularly during an integrated mosquito larvae control practice that involves the use of a biological control agent.

Most toxicity studies on *P. reticulata* exposed to larvicides have focused mainly on acute lethal bioassays (Hertlein *et al*., 2010; Bond *et al*., 2004; Lawal and Samuel, 2010). It is therefore assumed that employing a suite of biomarkers under sub-lethal toxicity study will

provide more detailed evaluation of the impacts of the selected larvicides at behavioural, cellular and ultracellular levels of organization.

The main goal of this research is therefore, to effectively interrupt mosquito larvae population through the use of selected larvicides and a fish component, *P. reticulata*, combined in a compatible manner.

1.2 STATEMENT OF PROBLEM

The gradual drift away from using the more traditional larviciding and environmental management to disease management and domestic adulticiding has contributed largely to mosquito control failures evidenced by the continual establishment of new cases of mosquito related diseases and death (FMH, 2009).

Earlier mosquito vector controls were adulticidal and based on single approach involving principally the use of chemical larvicides (Bond *et al*., 2004; Breiger, 2007). These larvicides are often applied at concentrations that are detrimental to non-target aquatic organisms including *P. reticulata* that can serve as an additional control agent for mosquito larvae. The identification of a compatible larvicide(s) and the concentration of larvicide that will effectively kill the target species (mosquito larvae) with minimal damage on the non-target species (*P. reticulata*) will reduce the deleterious effects on the non-target aquatic organism.

The extent at which predator diets are explained by passive components of predation (predator-prey density relationship) as opposed to active choice by the predator (attack probability) is a major gap in the field of foraging ecology which may inhibit the successful use of

P. reticulata as biological control agent for field mosquito larvae control. The determination of an optimal feeding density between *P. reticulata* and mosquito larvae species and the understanding of the fish-larvae interaction under laboratory trials will help circumvent this problem in the field of foraging ecology.

Therefore, the main goal of this research is to effectively interrupt mosquito larvae population through the use of a double barrel approach of selected larvicides and a fish agent: *Poecilia reticulata* combined in a compatible manner.

1.3. PURPOSE/OBJECTIVE OF RESEARCH

1.3.1. Purpose of Research

To control mosquito larvae through an integrated approach involving the use of selected larvicides and *P. reticulata* in a compatible manner.

1.3.2. Research Objectives

1. To establish an optimal feeding density for *P. reticulata* on mosquito larvae;
2. To determine *P. reticulata* prey preference;
3. To investigate the acute toxicity of some selected larvicides-:Chlorpyrifos, Actellic and Spinosad on mosquito larvae and *P. reticulata* respectively;
4. To assess the sub-lethal effects of the larvicides on *P. reticulata* using suite of biomarkers (Micronucleus and Nuclear Abnormal cells, Behavioral responses and Ultrastructural responses);
5. To investigate the impact of integrating *P. reticulata* with larvicides on mosquito larvae population.

1.4. RESEARCH QUESTIONS

1. What is the optimal feeding density of *P. reticulata* on mosquito larvae?
2. Does *P. reticulata* prefer alternative preys to mosquito larvae?
3. What concentration of the selected larvicides will bring about a 50% mortality of the exposed fish and mosquito larvae respectively?
4. What are the sub-lethal effects of the selected larvicides on *P. reticulata*?
5. What is the impact of integrating *P. reticulata* and selected larvicides on mosquito larvae population?

1.5. SIGNIFICANCE OF RESEARCH

Disease management and adulticiding alone have failed to suppress mosquito vector population below levels that are of public health importance hence, the need to reprioritize larviciding in a more environmentally friendly manner. By inference, to control mosquito at their larval stage when they are confined by nature in various aquatic media is more effective. Larval control by comparison may be more tasking and have less dramatic effect at any given level of coverage than adulticiding but the most outstanding success ever attained in the history of mosquito vector control was through an integrated approach that relied overwhelmingly on larval control (Killeen *et al.*, 2002a).

The use of single approach in mosquito vector control has also failed evidenced by the present challenges mosquito vectors still present in public health. Therefore, there is the need to adopt a multi-prong approach inherent in IVM tenets essentially, by combining *P. reticulata* and selected larvicides in a compatible manner against mosquito larvae species.

A detailed evaluation of the selected larvicides on the *Poecilia reticulata* and mosquito larvae species will assist in the identification of a compatible larvicide(s) and concentration that will effectively kill the mosquito larvae (target species) with minimal damage on *P. reticulata* (non-target species).

1.6. OPERATIONAL DEFINITION OF TERMS

Larvicides: These are chemicals of synthetic or natural origin that are used to kill insect larvae.

Bio-larvicide: Natural larvicides derived from biological sources e.g. spinosad.

Synthetic larvicides: These are larvicides formulated from the combination of various compounds e.g. actellic and chlorpyrifos.

Prey: A smaller organism fed upon by larger organism e.g. mosquito larvae being fed on by fish.

Predator: A larger organism that feed on smaller ones e.g. fish species that feed on mosquito larvae.

Double-barrel approach: This is the simultaneous use of two control strategies to suppress mosquito vector population such as the use of larvicides and *Poecilia reticulata.*

Histological biomarkers: Indicators of the impact of xenobiotics on different levels of biological organization including cell, tissue, organ and system.

Target organism: An organism that a control strategy is directed towards

Non-target organism: An organism that is accidentally affected by a control strategy.

Xenobiotics: Any chemical substance that is foreign to an organism.

CHAPTER TWO

LITERATURE REVIEW

2.1. HISTORY OF MOSQUITO CONTROL

The implication of mosquitoes in disease transmission by Patrick Manson and other scientists in the 19th century was a great landmark in a worldwide development of the science of vector ecology and disease control. Following intensive research in 1897, Ronald Ross pointed out the correlation between *Plasmodium* transmission and mosquito bites. Meanwhile Manson in 1896, proved the role of mosquitoes in the transmission of human filariasis while Reed in the early 1900's, made it clear that mosquitoes also transmitted yellow fever (Harrison, 1978). It was realised then, that suppressing the mosquito vectors could attain successful control of these diseases.

Systematic efforts for mosquito control actually started soon after the discovery by Ronald Ross that mosquitoes transmit malaria, although several natural substances, mainly of plant and fungal origin, with insecticidal properties were used before that time, indicating the extent of nuisance caused by these insects (Walker and Lynch, 2007). In many parts of Africa, farmers used extracts from the neem plant *Azadirachta indica* either in crude or refined forms to control insect vectors but their short shelf life necessitated frequent site retreatment in order to cover a sufficiently large time period which resulted in deleterious effects on non-target aquatic organisms (Scott and Kaushik, 2000).

Crude kerosene and distilled petroleum oils were applied over 100 years ago on mosquito breeding sites to asphyxiate the larvae (Gratz & Pal, 1988; Brieger, 2007). Crude petroleum

oils were discovered to be heavy and therefore were replaced with lighter products such as monolayer surface films which may show good efficacy against *Anophelines* under certain conditions (Karanja *et al.*, 1994).

Until the 1940s, the larvicidal poison Paris green (Copper aceto-arsenite) was used extensively as a fine powder that floated on the water surface where it was eaten by *Anopheles* larvae (Rozendaal, 1997). The systematic use of Paris green over approximately 54,000 km^2 of apparently ideal habitat in northeast Brazil during the 1930s contributed to elimination of *An. gambiae s.l.* from the region where it had been accidentally introduced (Killeen *et al.*, 2002b). Although inexpensive and highly effective, use of Paris green is no longer recommended, considering the risks posed by its high toxicity to non-target organisms (Coosemans & Carnevale, 1995).

In the 1940's and 1950's, DDT was introduced as the first synthetic insecticides. The discovery of DDT and its derivatives marked the beginning of new era in the control of insect vectors. In Zambia, between 1930 and 1936, malaria incidence within 4 copper mining communities declined from 457-514 cases to 135-251 cases per 1000 people per year using environmental mosquito control approach alone but when indoor residual spraying with DDT was introduced in 1946, as a supplementary measure, there was another sharp decline in malaria incidence to just 21-30 cases per 1000 people (Utzinger *et al*, 2001). In addition to this health benefit, 6.5million US dollar in direct and indirect medical costs of lost worker production were estimated to have been averted (Utzinger *et al*, 2001). Dichlorodiphenyltri-

chloroethane was not used in Nigeria until the late 70s when it was applied mainly for adulticidal purposes (Don-Pedro, 2007).

As a consequence of the resistance mosquitoes developed to those organochlorine chemicals and most essentially, the accumulation of DDT in food chain, further research was then directed towards the discovery of new insecticides with different mode of action (Ware, 1983). In Nigeria, DDT was later replaced with alternative synthetic insecticides like propoxur, pyrethroids and malathion used for insecticides treated nets (FMH, 2005; FMH, 2009) as well as, temephos used for larviciding since 1970s, but was discontinued in the country due to lack of funding (FMH, 2009), and subsequent effects on non- target aquatic organism (FCCMC, 1998; USEPA, 2002).

Global mosquito control relied on the use of synthetic chemical insecticides for so many years because of their ease of application and effectiveness. The extensive use and lack of adequate knowledge on the side effects of these compounds however, had a tremendous destructive impact on the environment and wild life that led to the extinction of varieties of fish, birds, arthropod predators, insect pollinators which resulted in strong oppositions from environmentalist like Rachael Carson (Pedigo, 2004). When all these side effects became obvious and with the increase in the awareness of environmental safety, alternative control measures were investigated and a revolution to biological control started in mid 60s (Becker *et al*., 2003) and recently, the Integrated Vector Management (IVM) approach which was proposed by World Health Organization (WHO) in the early 1980s and based on the principles of Integrated Pest Management (IPM) in the agricultural sector (USAID, 2006).

2.2. CHEMICAL LARVICIDING AS A MOSQUITO CONTROL APPROACH

The growing interest in larval control of Africa mosquito vectors has stimulated debates about the importance of targeting larval control through identification and treatment of those larval habitats producing most adults (Gu and Novak, 2005; Gu and Novak, 2006; Killeen *et al*., 2006).

Chemical larvicides including Pirimiphos methyl (Actellic) and Chlorpyrifos (Figure 1 & Figure 2) are very effective broad spectrum organophosphorus compounds used in the control of mosquitoes. They generally act as nerve poison by inhibiting the function of acetylcholinesterase enzyme (AChE) leading to the accumulation of acetylcholine which is a neurotransmitter (WHO, 1986). They have low environmental persistence which is typical of all organophosphates therefore they are often applied repeatedly for effective control of mosquito larvae species. This may lead to their being accumulated in water bodies resulting in increased exposure of non-target species including guppy which shares the same habitat as mosquito larvae species.

A range of synthetic chemicals have been used successfully as mosquito vector larvicides (Gratz and Pal, 1988; FCCMC, 1998; Kanda *et al*., 1995; Yapabandara *et al*., 2001) but these compounds have become unpopular due to the resistance effects by mosquito species to synthetic chemicals and the negative side effects to non-target aquatic fish species. A study by Rodríguez *et al*., (2007) to evaluate the resistance of 8 Latin American strains of *Aedes aegypti* to 6 organophosphates (temephos, malathion, fenthion, pirimiphos-methyl, fenitrothion, and chlorpyrifos) and 4 pyrethroids (deltamethrin, lambdacyhalothrin, betacypermethrin, and cyfluthrin) under laboratory conditions showed that despite the susceptibil-

ity of majority of the strains to the organophosphorus compounds, temephos resistance was high however, resistance to pirimiphos-methyl ranged from moderate to high in majority of the strains. Otitoloju and Don-Pedro, (1997) reported that Dieldrin and Cypermethrin were lethal to three species of mosquitoes and non-target tadpoles with *Anopheles gambiae* as the most tolerant larvae species.

The deleterious impacts of synthetic chemicals to non-target fish species including guppies has been demonstrated in different studies. Rice *et al*., (1997), reported that the exposure of 30-day-old Japanese medaka, *Oryzias latipes* to different insecticides including chlorpyrifos resulted in fish mortality, behavioral and morphological changes. In another but similar study, Mahmut *et al*., (2005) reported the acute toxicity of chlorpyrifos methyl on guppy and then concluded that this compound was less toxic to guppy than the other fish species. In a more recent work, Lawal and Samuel (2010) demonstrated that pirimiphos methyl (Actellic), a newer organophosphorus compound, caused fish mortality and behavioural changes at acute toxicity bioassay.

There appears to be a growing interest in the use of alternative insecticides that are more environmentally safe owing to the deleterious effects of synthetic larvicides particularly on non- target aquatic species. Spinosad (Figure 3) is derived from the fermentation of a soil bacterium *Saccharopolyspora spinosa* making it a biological larvicide thus; it is less hazardous to the environment than the synthetic chemicals. It represents a new generation of biorational insecticide developed initially for the control of agricultural pests with reduced spectrum of toxicity compared to the synthetic insecticides (Williams *et al*., 2003). The low toxicity of spinosad to non-target organisms particularly mammals have earned it the name:

'*Reduced Risk Material*' by the US Environmental Protection Agency (Thompson *et al*., 2000), and this has contributed to its continual use by Integrated Pest Management (IPM) practitioners since the 1980s (Williams *et al*., 2003). The action of spinosad on insect is a unique one, acting on the post synaptic nicotinic acetylcholine and GABA receptors (Salgado, 1998; Watson, 2001).

Temarek (2003) reported that spinosad was not affected by the existing resistance mechanism to conventional insecticides. These properties of spinosad, especially its low mammalian toxicity and larvicidal potentials, have contributed to the strong and growing recommendations for spinosad as a replacement for synthetic organophosphates in domestic and urban mosquito larval control (Baghat *et al.*, 2007; WHO, 2005).

Bioassays have shown that although this naturally derived compound is lethal to mosquito species, it is highly safe to *P. reticulata*. Bond *et al*., (2004) reported that *Anopheles spp* were as highly susceptible to spinosad as *Aedes aegypti* following laboratory and field trials of spinosad suspension concentrate on these organisms. In another study carried out at an urban cemetery in Southern Mexico during dry and wet seasons, Perez *et al*., (2007), investigated the efficacy, persistence and oviposition response of spinosad suspension concentrate on *Ae. aegypti* mosquitoes compared to temephos and the bacterial larvicide *Bacillus thuringiensis var. israeliensis* (Bti, VectoBac® AS).Spinosad was found to be as effective as temephos in preventing the development of *Aedes spp.* (mostly *Ae. aegypti*) while VectoBac performed poorly. These compounds however did not repel gravid *Ae. Aegypti* at the concentrations tested and no ovicidal properties were reported (Perez *et al*., 2007). In Egypt,

Baghat *et al*., (2007) demonstrated the toxicity of two formulations of spinosad on some mosquito species and discovered that although the liquid formulation performed better than the dust formulation at the initial stage, the dust form presented a delayed larval mortality which was absorbed up to pupation indicating better initial activity than the liquid form. The acute toxicity studies by Hertlein *et al*., (2010) demonstrated the efficacy of spinosad formulations on various mosquito species and the inability of the compound to cause mortality in the mosquito fish *Gambusia affinis* at concentrations up to 50 ppm AI when compared to fenitrothion that caused 100% mortality at rates of 5 ppm AI.

The need for accurate assessment of the environmental impact of all insecticides, both natural and synthetic, on non-target organisms such as *P. reticulata* is of international concern. Now that spinosad is presently under review for large scale mosquito control usage (WHO, 2005), and replacement for organophosphorus compounds in larvicidal practices (Baghat *et al*, 2007; WHO, 2005), it becomes very necessary to carry out detailed toxicological studies on these compounds.

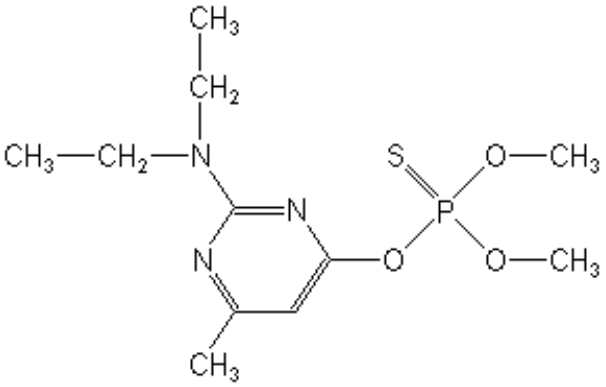


Figure 1: Pirimiphos methyl ($C_{11}H_{20}N_3O_3PS$)

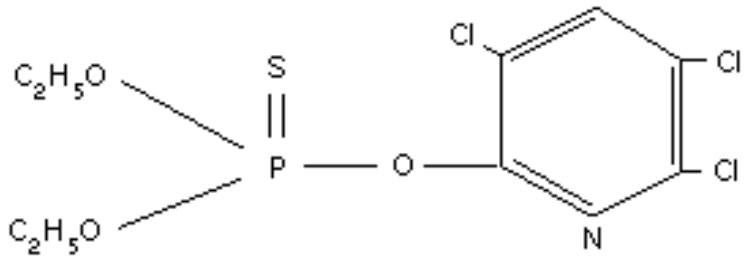


Figure 2: Chlorpyrifos ($C_9H_{11}Cl_3NO_3PS$)

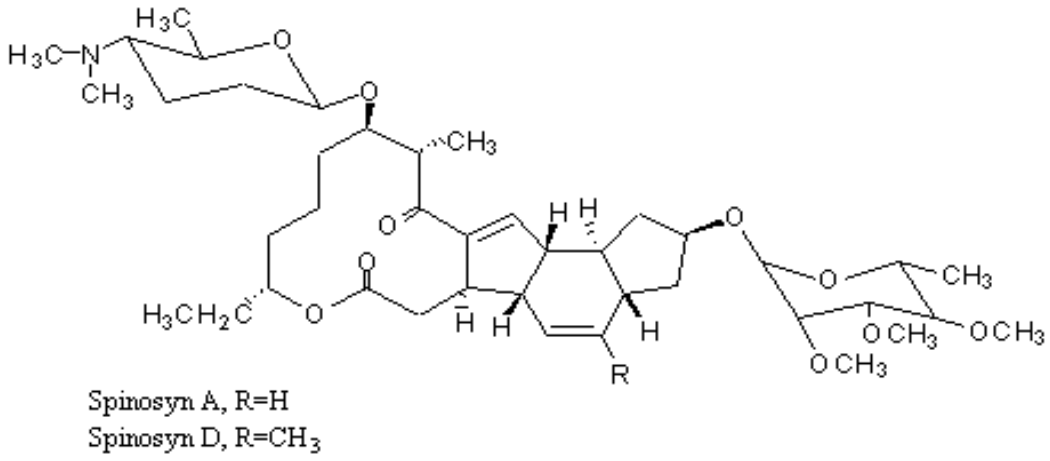


Figure 3: Spinosad

2.3. THE USE OF FISH AGENTS AS A MOSQUITO CONTROL APPROACH

The use of fish particularly in the family *Cyprinodontidae* is the oldest and well-known predatory strategy used in mosquito control dating back to the 19th century (Livadas and Sphangos, 1940). In 1902, some species of fishes were used in mosquito control in India for the first time (Livadas and Sphangos, 1941). *Gambusia affinis*, a native of Texas, and *Poecilia reticulata*, a native of South America, have been used in vector control programs around the world for at least 100 years (Meisch, 1985) but this has not been made popular in Nigeria (FMH, 2009) despite the abundance of the fish species in various open drainages.

To be successful as bio-control agent, certain characteristics are required of the fish species. The fish selected must be a surface feeder, as mosquito larvae must obtain air from the water surface. In addition, the fish must be hardy enough to survive transport to the breeding area, variations of water quality and turbidity, as well as, temperature variations.

Several potential negative environmental impacts are associated with introducing larvivorus fish. The assessment of the adverse effects of introduced fish on indigenous species was observed in Greece where fisherlings taken from Italy and Marseille respectively were introduced to water bodies in Northern Greece and Marathon lakes in Attica to enrich the water bodies. In Marathon area, there was an endemic fish species, *Pseudophoxinellus marathonicus*, which is competitively inferior to *Gambusia spp.* and it became extinct from the water pools because the lather either ate the eggs, hatchlings and fisherlings of *P. marathonicus* or performed better than the local species since it readily acclimatized to the environment (Pecl, 1995). Rupp (1996) has equally reported that the introduction of *Gambusia sp* in

certain habitats actually led to the elimination of native fish species. For this reason, introducing fish into any aquatic natural environments is not recommended. The introduction of larvivorus fish should be limited to man-made environments including underground and overhead tanks, abandoned septic tanks, open and blocked drains, storm water drains, road culverts, irrigation canals, abandoned wells, and commercial fish ponds (Walker and Lynch, 2007), that characterize many local environment in Africa and indeed, Nigeria. A major factor determining the efficacy of larvivorus fish is the suitability of the fish species to the water bodies where vector species breed (Walker and Lynch, 2007).

Larvivorus fish show promise in controlling mosquito borne diseases particularly, malaria which cause the most severe health problem of all mosquito borne diseases. In China, Wu *et al*., (1991) found that stocking rice paddies with edible fish such as carp improved rice yield, supported significant fish production, and greatly reduced the number of malaria cases. Larvivorus fish have been used in both Africa and India to control vectors that breed in human-made water holding structures such as wells, cistern, and barrels. In an urban area in Ethiopia, Fletcher *et al*., (1992) found that the indigenous fish, *Asphanius dispar dispar* (Day) (*Cyprinodontiformes: Cyprinodontidae*, Arabian pupfish), effectively suppressed *An. Culicifacies adenensis* breeding in wells and containers.

In Northern Somalia, a locally developed initiative to control water tank-breeding malaria vectors using the indigenous fish *Oreochromis spilurus spirulus*: Gunther (Perciformes: Cichlidae, River tilapia) found average mosquito larval densities reduced by 50% after 1 month (Mohammed, 2003). On Grande Comoro Island, where the vector *An. gambiae s.s.*

breeds only in human-made reservoirs, the introduced fish, *P. reticulata* (Cyprinodontiformes: Poeciliidae, Guppy), provided year-long suppression of larval and adult mosquito populations and significantly reduced malaria incidence (Sabatinelli *et al.*, 1991). In the majority of the breeding sites on the island, the fish reproduced successfully, thus reducing the need to restock.

A number of studies have also shown that both introduced fish species (*Gambusia affinis* and *Poecilia reticulata*), and indigenous species are effective at suppressing *An. stephensi* populations breeding in containers in India (Menon & Rajagopalan, 1978; Gupta *et al.*, 1992; Rajnikant *et al.*, 1993; Chapman, 2000). In Bangladesh and Cambodia respectively, one *P. reticulata* ate an average of 41.0 and 106 mosquito larvae per day (Elias *et al*., 1995; Dua *et al*., 2007).

Factors such as drain pollution, predator size, predator sex, predator density and the presence of alternative preys have been demonstrated to be capable of affecting guppy consumption pattern (Dua *et al*., 2007; Manna *et al*., 2008; Seng *et al*., 2008; Saliu *et al*., 2011). Anogwih and Makanjuola, (2010) reported that *Chironomus sp*. was the most selected prey type compared to mosquito larvae and earthworm following a laboratory study on the feeding preference of *P. reticulata*.

Despite these factors that could affect the success of *P. reticulata* as mosquito regulating agent, they remain the only proven and sustainable non-chemical agent that can control mosquitoes even beyond laboratory condition (Walker and Lynch, 2007). It is therefore pertinent to device means to incline the prey preference pattern of *P. reticulata* more towards

the target mosquito larvae species through a right estimation of guppy-mosquito density that can be extrapolated to field studies. This in no small measure will further improve the effectiveness of the bio-control fish agent particularly, for integrated larval control practices.

It is true that the use of fish as bio-control agent reduces reliance on chemicals which has greatly contributed to the effect of mosquito vector resistance that has long posed a major challenge to mosquito control programmes, but this method alone may not effectively bring down mosquito larvae burden in highly mosquito endemic zones such as Nigeria. The combination of this effective bioenvironmental method with the use of relatively safe chemical larvicides under the Integrated Vector Management (IVM) tenets is therefore necessary.

2.4. INTEGRATED VECTOR MANAGEMENT AS A MOSQUITO CONTROL APPROACH

The introduction of Integrated Vector Management (IVM) in 1980 by World Health Organization (WHO) formed a new dawn in the science of vector control. The following principles or tenets guide IVM implementation: -

1. It is an evidence-based decision-making tool;
2. It involves the use of a range of interventions often in combination (where more than one control method is employed e.g. the use of chemical and biological control methods) or synergistically (where more than one approach inherent in a control method is employed e.g. the use of Insecticide treated bednets and Indoor residual spray which are different approaches under chemical control method);

3. It involves collaborative efforts between health sector and other sectors e.g. environmental sector;
4. There must be Advocacy and social mobilization;
5. It must be backed up by legislation for adequate enforcement;
6. It involves capacity building both at local and international levels.

2.4.1. Components of IVM

The following basic vector control techniques that are inherent in IVM are adapted for integrated mosquito vector control (Figure 4).

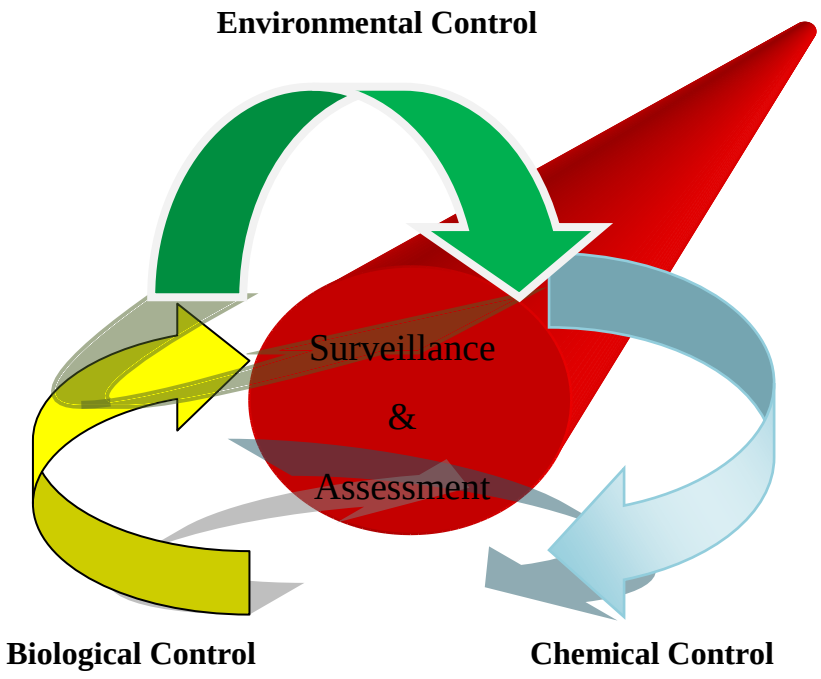


Figure 4: Components of IVM

2.4.1.1. Surveillance and Assessment

Surveillance is an essential activity for planning, operation and evaluation of an efficient mosquito vector management while Assessment is the careful monitoring/evaluation of disease incidence which is important to improve vector control strategies. To achieve an effective mosquito control and disease diagnosis/treatment, assessment must be carried out continously throughout the control programme.

The surveillance activity that is carried out before the intervention study is known as Pre-surveillance. This type of surveillance helps to identify the species of mosquitoes present in a control area, provide general information on locations, densities and disease potentials. Knowledge gained from pre-surveillance activity is used to determine mosquito bionomics and ecology (feeding preference, life cycles, predict larval habitats, adult resting places and flight range), disease incidence and even make preliminary recommendations for control programmes.

Post- surveillance is an activity that is continuously carried out after an intervention study. This type of activity can be likened to a basic inspection programme on mosquito population. In addition to eliciting information similar to those from pre-surveillance, it also serves as an on-going indicator for a successful intervention while adding database of knowledge concerning mosquito fluctuation (abundance and diversity) in the control area. Simple surveillance tools such as traps, filed notebooks, plastic dipper, pipette, aspirators, flashlight, cages, collection jars and sweep nets, to complex ones like mapping equipment e.g. geographic information systems (GIS) abound.

2.4.1.2. Environmental Control

The concept of modifying vector habitat to discourage larval development and/or human vector contact is generally referred to as environmental management or source reduction (Singer *et al*., 2005). The specific techniques of environmental management are grouped into environmental modification, environmental manipulation and modification of human habitations/behaviour (WHO, 1982).

Environmental modification involves a capital intensive investment that is permanent such as building of dams, water and irrigation infrastructure design. In China, India and other Asian countries for instance, irrigation schemes permitting intermittent irrigation of fields as well as, alternation between cycles of irrigated and non-irrigated crops have proven successful in controlling anopheles mosquitoes (Pal, 1982; Lacey *et al*., 1990). In Italy, modification efforts involved substantial investment of money and time to achieve major engineering feats including the 16 km diversion of the Ombrone River into a canal and draining the almost 100,000-ha Pontine marshes using an elaborate series of ditches and canals (Fantini, 1998). In Africa, several recent environmental modification projects have involved the renovation of abandoned drainage systems. In Kitwe, Zambia, a project was begun in the 1950s using lined drains, filling and leveling, and planting of eucalyptus with other water-intensive vegetation to convert a large peri-urban wetland into a public park (Baer *et al*., 1999). New environmental modification projects modeled after this earlier success were begun in 1998 and initial entomological data suggested that this effort reduced adult mosquito densities (Baer *et al*., 1999). A small study in urban sites in Uganda reported statistically significant declines in malaria parasitemia rates associated with reduction of breeding sites through

environmental modification (Lindsay *et al*., 2004). In urban Dar es Salaam, Tanzania, an integrated malaria control programme operated from 1988 to 1996 found restoration and maintenance of drains to be one of the most effective measures for reducing both vector populations and malaria parasite rates among school children in the urban areas (Castro *et al*., 2004). The efficacy of environmental modification to reduce or eliminate malaria vector breeding habitats depends both on the initial design and construction of the project, as well as, on regular maintenance. Poorly maintained drainage projects may actually increase larval breeding habitat (Takken *et al.*, 1990; Castro *et al.*, 2004). It may only be feasible where the water body or wetland considered is clearly the main larval breeding site for the malaria vector species since modification projects tend to require significant initial investments in construction. Furthermore, as noted by Konradsen *et al*. (2004), environmental modification requires a highly species- and site-specific approach, and techniques applied successfully in one region may be ineffective in another. The successes of environmental modifications in the earlier part of the 20th Century were generally obtained only when implemented in concert with other strategies (Kitron & Spielman, 1989; Fantini, 1998; Utzinger *et al*., 2001). For instance, in Zambia, techniques including bank modification and vegetation clearance along the Luanshya River, draining of swamps, later combined with indoor DDT spraying, enabled the development of the Zambian Copper belt, and effectively controlled malaria for several decades (Utzinger *et al*., 2001). Although interest in environmental modification waned with the rise of DDT, the practice is now being re-examined as countries look for more sustainable, less pesticide-intensive approaches to malaria vector control (Walker and Lynch, 2007).

Environmental manipulation involves a recurrent activity requiring proper planning and operation. Earlier researches in Igbo-ora, Western Nigeria, revealed that through health education and community mobilization, malaria was reduced by about 10 – 12%. This has been included in the IVM plan which consisted of filling and/or draining standing or slow flowing waters that may serve as malaria breeding sites (FMH, 2009). At the moment, a document "*Guidelines in Environmental Management for Malaria Control"* was prepared and submitted to the sub-committee on ITNs/Environmental Management of National Malaria Control Committee (NMCC). A workshop was organized in south – south zone in 2004 where 20 participants received training (FMH, 2009).

The feasibility of flushing streams to control *Anopheles culicifacies* Giles was examined in five river systems in rural Sri Lanka in the late 1930s (Konradsen *et al*., 2004) and again in one stream in the mid-90s (Konradsen *et al*., 1998). Stream flushing successfully reduced larval density in cases where local stream and bank conditions facilitated efficient movement of water (Konradsen *et al*., 2004). Flushing has also been used successfully in Mexico to remove Theobald larvae, *Anopheles pseudopunctipennis* associated with rice fields (Rafatjah, 1988). Intermittent irrigation has been suggested to remedy increased malaria vector abundance associated with irrigated agriculture, specifically rice (Van der Hoek *et al*., 2001). Irrigated rice cultivation in particular has been clearly linked to increased malaria transmission in areas of Africa and other parts of the world (Surtees, 1970). Intermittent irrigation involves periodic draining of the fields timed at a frequency to prevent mosquito larvae from completing their developmental cycle. The wet-dry cycles may vary in length from 2 – 3 days to 2 weeks, depending on rice variety and planting system (Keiser *et al*., 2002).

This method has proven successful in rice growing regions in India, China, and other parts of Asia (Lacey & Lacey, 1990). In China, vector control has also been accomplished by simply letting fields dry up naturally (Pal, 1982).

The combined use of the natural insecticide neem (*Azadirachta indica*) and intermittent irrigation in Indian rice fields achieved substantial reductions in culicine larvae as well as, smaller but still significant reductions in anophelines (Rao *et al*., 1995). Environmental manipulation through management of vegetation has also been implemented to reduce vector breeding populations, either by eliminating aquatic habitat or making it less suitable to vector larvae. For example, planting water-intensive tree species such as *Eucalyptus robusta* can reduce standing water in marshy areas (WHO, 1982; Sharma *et al.*, 1986; Sharma & Sharma, 1989; Baer *et al.*, 1999). Vegetation may also be managed to affect light conditions. Such approaches generally require extensive knowledge of the ecology of the local vector population. Malaria experts have long observed that some vector species prefer shaded breeding sites e.g. *An. umbrosus* in Malaysia, whereas others such as *An. maculatus*, *An. gambiae s.l.*, *Anopheles minimus* Theobald, and *An. sundaicus* prefer sunny conditions (Rafatjah, 1988). In a controlled field study, Bond *et al.* (2004) found that manual algal removal from breeding pools along a river in southern Mexico significantly reduced both larval and adult densities of *An. pseudopunctipennis* for up to 6 weeks. Rajagopalan *et al.* (1991) observed similar results in a community-based programme to remove algae from *An. subpictus* coastal breeding sites in south-east India. Although more widely used to control mosquitoes of the genus *Culex*, a vector of lymphatic filariasis, expanded polystyrene beads

(EPBS) have also been applied to control anopheline larvae in water tanks and abandoned wells, particularly in India (Sharma *et al.*, 1985; Dua *et al.*, 1989; Sharma & Sharma, 1989; Dua *et al.*, 1997). Expanded polystyrene beads form a floating layer on the water surface, blocking mosquito oviposition and causing high larval mortality. The advantages of the EPBS method are its simplicity, safety, low cost and persistence. However, the beads which tend to blow off shallow water bodies exposed to wind may be collected by children, hence it may be most suitable for enclosed structures such as cisterns, wells or tanks (Singh *et al.*, 1989). Expanded polystyrene beads were applied specifically for control of *Culex* larvae in the integrated urban malaria control programme in Tanzania (Castro *et al.*, 2004). Although these mosquitoes cannot transmit malaria, their management was important for community acceptance of the malaria control programme.

The efficacy of all environmental manipulations depends on several key factors, including how well the intervention is matched to the specific ecological requirements of local vector population. Effective implementation depends on accurate information on the distribution of breeding sites and establishing ongoing entomological monitoring capacity to modify interventions bearing in mind that environmental conditions including humidity, rainfall and soil composition also affect interventions on the malaria vector and disease transmission (Walker and Lynch, 2007).

Modification of human habitations/behaviour are strategies aimed at preventing mosquito-man contact through better use of window screen, mosquito nets, repellents, protective clothing and promotion of health conscious behaviour.

2.4.1.3. Biological Control

This involves the introduction or manipulation of living organism including predators, parasites and parasitoids to target and kill mosquitoes (Walker and Lynch, 2007; Samanidou-Voyadjoglou, 2007). The use of predators to control mosquito species dates back to the 19th century when the first attempt was made to introduce dragonflies as predator in mosquito breeding places (Lamborn, 1890). Thereafter, it was discovered that several other living organisms, aquatic and terrestrial, could consume mosquitoes as food. Very few of these organisms however, are effective and have been considered as possible biological control agents. The oldest and better-known organism that has been successfully used in mosquito control is fish species particularly the guppy fish (*Poecilia reticulata*).

At present, the main parasites that have been successfully employed against mosquito species are the bacterial pathogens *Bacillus thuringiensis var. israeliensis* (Bti), *Bacillus sphaericus* (Das and Amalraj, 1997) and of recent, Spinosad (Hertlein *et al*., 2010) which represents a new generation of bio-rational products against mosquito vectors developed initially for the control of agricultural pests (William *et al*., 2003). These naturally derived products are called biological larvicides. Some of them are widely sold as patented brands (Scott and Kaushik, 2000; Cetin *et al*., 2005). Karch *et* al., (1991) reported that Bti granules gave good initial control of *An. Gambiae s.l.* larvae in irrigation pond in peri-urban Kinshasha, Zaire (Democratic Republic of Congo, D.R.C) but the residual activity was too short (less than 5days) to provide reliable control. Trials of Bti granules for *An. arabiensis* control in three sites in Eritrea were more promising, showing good control of larvae for 2-3 weeks in all sites except streams and pools with high algal content (Shilulu *et al*., 2003). In urban

Maroua, Cameroon, Barbazan *et al*., (1998) found that a large-scale *Bacillus sphaericus* spray programmes targeting *C. quinquefasciatus* delayed the onset of the transmission period by 2 months, apparently through impact on anophelines. In recent times, Hertlein *et al*., (2010), carried out a comprehensive study on the use of spinosad for the control of mosquito species and reported that the bio-larvicide was highly active to all the species of mosquitoes investigated. They therefore suggested that the bio-rational compound will be very effective in integrated management of mosquito larvae considering its safety to the mosquito fish, *Gambusia affinis* during the acute toxicity study.

The advantages of bio-larvicides in comparison to synthetic chemicals can include their effectiveness at relatively low doses, safety to humans and non-target wildlife (including natural predators of mosquito larvae), low cost of production in some cases and lower risk of resistance development by disease vectors (Yap, 1985; Samanidou-Voyadjoglou, 2007). However, some bio-larvicides tend to be more specific in terms of which mosquito species they can control and in which habitat (Das and Amalraj, 1997). Also their short persistence often requires repeated applications which increase costs and logistical complications (Walker and Lynch, 2007).

2.4.1.4. Chemical control

Chemicals used in the control of insects are generally called insecticides. Insecticides may be applied as larvicides or adulticides to control larva and adult insect species respectively. Majority of the insecticides that are presently used against mosquito vector come under organic insecticides. Organic insecticides can originate from natural products (botanicals) or from synthetic ones.

Botanicals are derived from plant products or extracts such as tannins which are derived from live seeds (Yang *et al*., 2003), roots (Park *et al*., 2005) or decayed plant materials (David *et al*., 2000). Almost in all tropical countries for many years the farmers used botanical extracts, either crude or refined of the neem plant *Azardirachta indica* (Meliaceae) to control mosquitoes with only a few environmental problems (Ishman *et al*., 1991; Sundaram *et al*., 1997). The short life time of the active ingredient of botanicals especially azardirachtin which ranges from 24 h to 6.85 days of the commercial formulation Margosan- O© necessitates the repetition of the applications in order to cover a sufficiently large time period (Samanidou-Voyadjogloul *et al*., 2007). In bioassays transacted by Scott and Kaushik (2000) in specifically designed microcosms, it was found that neem oil (the formulation Margosan-O) did not affect the filter feeding planktonic organisms *Daphnia sp*. and *Culex sp*. In the same experiment, the benthic ecosystems of the artificial microcosms were destroyed since the multiple applications of the neem oil as a result of the short life of the active ingredients (azardirachtin) killed the fundamental invertebrates species *Chironomus riparius* (Diptera: Chironomidae).

Synthetic organic insecticides are of four basic types which include the chlorinated hydrocarbons e.g. DDT; the organophosphorus compounds e.g. pirimiphos methyl; the carbamates e.g. carbaryl; and the pyrethroids e.g. deltamethrin (Omoloye, 2009). Integrated vector management makes judicious use of synthetic insecticides only when other measures are ineffective or not cost effective especially in places with intense disease transmission such as Nigeria. Mosquito control programmes such as indoor residual spray (IRS), space spraying and insecticide treated nets (ITNs) apply the use of synthetic insecticides. These IVM tactics are protective measures that combine the judicious use of chemicals with physical barrier approaches particularly with ITNs.

2.5. PROGRAMMES WITH IVM APPROACH

Historically, programmes with IVM elements have brought about significant reductions in vector populations and disease transmission across a range of transmission settings (Bang *et al*., 1975; Hinman, 1941; Wiseman, 1939). There is evidence that IVM can complement other existing mosquito control strategies such as the use of insecticide treated bednets (ITN) and access to effective treatment by avoiding reliance on any single intervention to reduce the burden of mosquito related diseases (Killeen *et al*., 2000; McKenzie *et al*., 2002; Ross, 1911). There was a demonstration on the feasibility and acceptability of integrated approach in health delivery by community members after a multi-country study carried out between 2005 and 2007 in Cameroon, Nigeria and Uganda respectively involving the integration of various disease interventions including ITNs (WHO, 2008b).

In many respects, the successful implementation of IVM and integrated malaria control in Zambia serves as a prominent success story for all of Africa. The first documented experience in the use of IVM approach was in the copper mining communities of Zambia between 1929 and 1949 when malaria was successfully suppressed for 20 years (WHO, 2008a). Prior to this period, unsuccessful control efforts had resulted in migrant workers abandoning some of the copper mining sites and rumours flourished along the labour routes of the malaria danger associated with copper mining, this having a great toll on the nation's economy. Environmental management strategies like modification of river boundaries, increasing velocity of the river flow to swamp drainages; larviciding with oil were incorporated into existing vector control measures including improvement on housing, water, sanitation, medical treatments/facilities, and bednets. Evaluation was carried out using monthly malaria incidence rates and vector density surveys. Information obtained from this was used for improve the performance of IVM. These measures were implemented in parallel by careful cooperation among health, water managements and planning authorities. Within the period under review, malaria incidence reduced significantly in the 4 communities undergoing control and Zambia was transformed from an insignificant player in copper mining to the third most important copper ore producer worldwide and in 1938, copper represented 55% of taxable natural income (Utzinger *et al*., 2001).

Obviously, the control of mosquito larvae has a less dramatic effect at any given level of coverage, and it is often more tasking nevertheless, the historically most significant effort ever attained in this respect were those of Brazil in the 1930s and early 1940s (Killeen *et.*

al., 2002b), and in Zambia copper mining region between 1929 and 1949 (WHO, 2008a; Chanda *et al*., 2008).

Another recent report by Chanda *et al*., (2008) described a comprehensive and highly successful IVM program that was implemented by the Zambian National Malaria Control Program. Following another major outbreak in malaria incidence from 121.5 cases per thousand people in 1976 to about 394 cases per thousand people in 2001 (Ministry of Health, Lusaka, 2006), the government of Zambia adopted a new National Malaria Treatment and Control Policy in 2002 that built on the earlier experience of 1929 and 1949 but in strict adherence to the WHO-IVM key strategic approaches and steps to vector control, and the implementation commenced in 2003 (Chanda *et al*., 2008). Over a relatively short time period, this program expanded coverage of vector control interventions and leveraged additional resources to build national capacity to the point where they successfully reduced malaria related morbidity and mortality as well as, improved the coverage and utilization of IRS and ITNs (Chanda *et al*., 2008).

The IVM approach continues to remain a strategy with great promise for disease control in Africa (Keisser *et al*., 2005; Utzinger *et al*., 2002; Utzinger *et al*., 2005). Castro *et al*., (2004) described a successful intervention in Dar es Salaam, Tanzania, from the late 1980's through the 1990's, with elements of IVM included in the strategy. A review on environmental management (EM) activities in peri-urban habitats, towns and areas of disturbed land, around Zambian Copper mines was carried out by Utzinger *et al*., (2001) and this also showed a significant reductions in malaria transmission over a three to five-year period, us-

ing a combination of drainage, filling larval habitats, and bed nets. Keisser *et al*., (2005) conducted a meta-analysis on EM studies globally and concluded that EM can have a significant impact on clinical malaria, if EM is appropriate to the eco-epidemiological setting. Environmental management was also used with available larvicides to control malaria in the coastal city of Mombasa (Wiseman, 1939). Schliessmann *et al*., (1973) used a combination of water drainage techniques and larviciding to reduce the number of malaria cases by 98% from 1969 to 1970 in a coastal flood plain of Haiti.

In India, over 95% reduction in malaria incidence over a four-year period for communities receiving a combination of water-source reduction activities and biological control in larval habitats was reported by Sharma and Sharma, (1989), and Sharma *et al*., (1986). Collectively, these studies suggested that multiple vector control strategies may be beneficial when used in combination.

One of the largest integrated disease control schemes developed in the 1980's was the Blue Nile Health Project (El-Gaddai, 1985). The 10-year project resulted in the development of better strategies for controlling schistosomiasis, malaria and diarrhoeal diseases in a major area of Sudan.

Sri Lanka has long linked vector control with agricultural development. In the 1970s and 1980s, the Mahaweli River hydroelectric and irrigation development project caused new foci of malaria transmission to emerge around Kandi. The hydrological changes and the relocation of people between malaria endemic and non-endemic areas as a result of resettlement due to the dam reservoirs, contributed to an upsurge in malaria (Wijesundera Mde, 1988)

and Japanese encephalitis (Peiris *et al*., 1993). This led to a series of integrated vector control strategies based on community participation, ITNs, and larval source management (Yasuoka *et al*., 2006).

The work in Mahaweli evolved in 2002 when Food and Agricultural Organization (FAO) provided a grant for the initiation of a project on "Integrated Vector and Pest Management" (Berg *et al*., 2006). An important innovation involved participatory education known as the "*Farmer Field Schools*", making the connections between health, vector borne disease control and agricultural productivity (Berg and Knols, 2006).

Eritrea uses national-level ITN distribution strategies, larval habitat management in malarious areas, and indoor residual spraying in areas with the appropriate eco-epidemiological characteristics. Although Eritrea has seen a marked drop in malaria parasite prevalence and disease, no rigorous evaluation of the IVM activities linked to health outcomes has been done (Shilulu *et al*., 2003).

"Community based" IVM programmes incorporating larval source management have recently been implemented in two different settings: in Rusinga Island, Western Kenya (Fillinger and Lindsay, 2006), and in Dar es Salaam, Tanzania (Castro *et al*., 2004; Fillinger *et al*., 2008). Earlier reviews of environmental management (Keisser *et al*., 2005; Utzinger *et al*., 2001) and larval control (Walker and Lynch, 2007) contain additional examples.

In Dar es Salaam (Tanzania), mosquito parasite rate reduced drastically within three intervention zones using an integrated approach of larviciding and insecticide treated bednets (Castro *et al*., 2004). It is pertinent to note that most of these outstanding successes were

achieved before the inception of modern technology and through an integrated programme that relied overwhelmingly upon larval control.

Efforts on IVM in Nigeria have focused more on adult mosquitoes. The Federal Government through the Federal Ministry of Health (FMH) initiated the use of indoor residual spraying (IRS) and distributed deltamethrin in July 2004 for trial runs on IRS, in addition to the distribution of Insecticide treated bednets (FMH, 2009). Few works carried out on integrated larval control included those from Igboora, Oyo state, where IVM plan consisted of filling and/or draining standing or slow flowing waters that may serve as *Anopheles* breeding sites. This action alone reduced malaria incidence by 10 to 12% in the study area (FMH, 2009). Again, Gilroy *et al*., (1945) reported decreases in malaria incidence and sporozoite prevalence rates in Nigeria using a variety of environmental management techniques, including source reduction and drainage.

There is a dearth of study on the use of larvivorus fish in concert with larvicides as components of integrated mosquito larval management owing to the challenges in establishing a compatible larvicide to the bio-control agent. A pilot project conducted in Goa, India, combined the use of the native fish *Aplocheilus blockii* in large breeding sites and Bti in smaller habitats which significantly reduced malaria transmission (Kumar *et al.*, 1998) but little was reported on the toxicological impact of the larvicide on the fish. Additional research is therefore important in this area for enhancement and integration into existing nationwide mosquito control programmes.

2.6. HISTOLOGICAL BIOMARKERS AS DIAGNOSTIC TOOLS

Histological analysis observes cell structures and assesses lesions that are not always visible to the naked-eye. Histological investigation therefore may be considered as biomarkers which are indicators of the impact of a xenobiotic compound on different levels of biological organizations (cell, organ, individual and population (Munoz *et al*., 1994).

2.6.1 Behavioural responses

Behavioural responses are effective indicators of contamination and reflect sub-lethal toxicity (Little, 1990; Drummond *et al*., 1986). Behavioral changes in animals due to exposure to chemicals usually indicate the effect of chemicals at organismal level and therefore used to describe the trends over time (Padmini *et al*., 2011).

Extensive studies have been carried out on the use of abnormal behavioural responses as diagnostic endpoints for determining the sub-lethal effect of chemicals as well as, their source and mode of action. Drummond *et al*., (1986) evaluated the use of behavioural and morphological changes in fish as diagnostic endpoints for screening and differentiating chemicals according to their mode of action after exposing 30-day–old fathead minnows *Pimephales promelas* to different chemicals. They observed unique morphological and behavioural signs of stress and then concluded that the selected abnormal responses are promising tool for predicting the mode of action of unknown xenobiotics. In another but similar study, Drummond and Russom (1990) further categorized chemicals corresponding to three general mode of action response syndromes: hyperactivity, hypoactivity and physical deformity. Each syndrome or sign of stress was indicative of a different mode of action. Halcombe *et al*., (1982) showed spinal deformities in fat head minnows *Pimephales promelas* and rain-

bow trout *Oncorhynchus mykiss* exposed to sub-lethal concentrations of chlorpyrifos. Investigations by Rice *et al*., (1997) showed that 30-day-old Japanese medaka *Oryzias latipes* exposed to various chemicals behaved differently from control by exhibiting symptoms ranging from loss of equilibrium to scoliosis in the caudal region (abnormal vertebral bending). They therefore concluded that the abnormal vertebral bending presumably led to heamorrhage and permanent disfigurement in the fish. Similarly, Jarvinen *et al*., (1988) recorded 50 % deformities which consisted of lateral bending in the spine, in fathead minnows after 15 h of 0.122 mgL^{-1} chlorpyrifos exposure. In more recent acute toxicity studies, Viran *et al*., (2003) reported loss of equilibrium, hanging vertically, rapid gill movement, gulping for air and prolonged motionless behaviour in guppies exposed to various concentrations of deltamethrin. Similarly, Mahmut *et al*., (2005) observed behavioural changes that were typical of neurotoxin toxicity: loss of equilibrium, erratic swimming and staying motionless at certain location generally at mid-water level for prolonged periods, in guppy exposed to chlorpyrifos methyl. Lawal and Samuel, (2010) examined another type of organophosphorus compound, Pirimiphos methyl (Actellic) on guppy and reported aggressive behavior, increased operculum movement and rapid gulping of water in the fish. They then concluded that actellic probably impaired respiratory function in the fish species which culminated into these stress symptoms.

2.6.2 Induction of micronuclei and nuclear abnormal cells

Micronuclei are small fragments of intra-cytoplasmic chromatin which arise from chromosome breaks or whole chromosomes, after the action of clastogenic substances or spindle-

poisons that do not migrate during anaphase (Gauthier *et al*., 1993; Cavas and Ergene-Gozukara, 2003b). Micronucleus (MN) is considered as the most suitable and effective method to use in fish when evaluating the genotoxic effects of xenobiotics because of its simplicity and ease of scoring (Al-Sabti and Meltcalfe, 1995; Vigfusson *et al*., 1983).

The significant induction of Nuclear abnormal cells (NA) including Polychromatic cells (PCE) and Binucleated cells (BN) in an organism are equally indicators of cytological toxicity from chemical exposures (Cavas and Ergene-Gozukara 2005a; Cavas, 2008). This effect is a consequence of structural aberration in the tissue as compared to induction of MN which is a parameter of functional aberration (Cavas and Ergene-Gozukara, 2003a). The evaluation of micronucleus and nuclear abnormal cells in organisms can be done using the Acridine orange and Giemsa staining protocols respectively.

Acridine orange (AO) is a nucleic acid selective metachromatic stain unlike Giemsa that is a non-selective conventional stain. Acridine orange interacts with DNA and RNA by intercalation or electrostatic attraction respectively (Darzynkiewicz, 1990). Under fluorescent microscope, AO maximum excitation shifts to 460 nm when bound with ribonucleic acid (RNA) and the maximum emission becomes red (650nm). Thus, a red-stained cytoplasm is a characteristic of an immature RNA- containing cell (PCE). The stain emits green light when excited only if bound to deoxyribonucleic acid (DNA), thus micronuclei and main nuclei appear as yellow-green fluorescence (Polard *et al*., 2011).

The AO staining technique, commonly used in mammalian micronucleus test was first modified for fish species by Ueada *et al*., (1992) and successfully used by Hayashi *et al*., (1998)

on fish erythrocyte and gill cells. Polard *et al*., (2011) compared the use of AO and giemsa stains in fish micronucleus assay and they concluded that the assay was more sensitive with AO stain because of the presence of ambiguous artefacts found with giemsa stain that led to false positive results. In the same study, they further evaluated the genotoxic potentials of water sampled in two hydrological contexts: basal flow and spring flood from the Save River in France. Thereafter, they discovered that the exposure of *Carrasius carassius* to spring flood water in an agricultural stream can induce mutagenicity in the fish compared to basal flow water (Polard *et al*., 2011).

The cytogenotoxic effects of various textile industrial effluents which consist of complex chemical mixtures have equally been studied on fish using giemsa and Acridine orange assays. Odeigah and Osanyipeju (1995) investigated the genotoxic effects of textile mill effluents on *Clarias lazera*, and reported a significant increase in the frequency of micronucleated erythrocytes in the fish. Another study was carried out on the gill and erythrocytic cells of the fish species *Oreochromis niloticus* exposed to different concentrations of textile dyeing effluents using the frequencies of nucleolar anomaly (Cavas and Ergene- Gozukara, 2003a). These authors discovered that MN frequencies in the fish gill cells were higher than those in erythrocytes and therefore made this conclusion in concordance with Hayashi *et al*., (1998) that the use of gill cells for MN test was more suitable, especially when cells are to be analyzed manually. Pacheco and Santos (2002) reported that PCE frequencies in peripheral blood of *Anguilla anguilla* decreased, while the frequencies of erythrocytic nuclear abnor-

malities increased as a result of Benzo[a]pyrene, dyhydroabietic acid and bleached kraft paper mill effluent treatments.

Heavy metal substances have also been used in this context. Al-Sabti *et al.*, (1994) demonstrated the relation between chromium and cytogenetic damage in Prussian carp fish *Carassius auratus gibelio* and discovered that similar values of micronuclei were induced at the field and laboratory study sites. Archipchuk and Garanko, (2005) carried out a comprehensive study on the fin, gill and erythrocytic cells of different fish species exposed to various heavy metals thereafter, concluded that cells that contacted directly with the test solution (fins and gills) revealed higher genotoxic potentials for the test substances than erythrocytic cells. Cavas, (2008) reported a genotoxic and cytotoxic effects on *Carassius auratus auratus* exposed to mercury and lead acetate compounds. Similarly, decreased PCE ratio in peripheral blood of fish exposed to polycyclic aromatic hydrocarbons (PAHs) contaminated areas was reported by Smith, (1990).

Antibiotic compounds and few pesticides have also been used to assess cytogenotoxicity in fish species. Cavas and Ergene- Gozukara, (2005b) investigated the genotoxic potential of metronidazole, an antibiotic-antiparasitic agent widely used in both clinical and veterinary areas, on *Oreochromis niloticus* and observed that micronucleus was significantly induced in the fish as well as, a decrease in the ratio of PCE to NCE frequencies in the peripheral blood thus indicating cytotoxic effect in the exposed fish species. Investigations by Campana *et al*., (1999) showed that the pyrethroid lambda-cyalothrin caused a significant induction of micronuclei in the erythrocyte of the fish *Cheirodon interruptus interruptus*.

2.6.3 Ultrastructural responses

Changes in the fine structures of a cell including mitochondria, nucleus, Golgi bodies and other cell inclusions, when exposed to chemicals or other substances are termed as ultra-structural responses (Bozzola and Russel, 1999). Many ultrastructural studies have been carried out to evaluate the stress impact on various organisms exposed to a range of compounds or confirm the functionality of a particular organ. Trinchet *et al*., (2007) investigated the effect of Microcystin, a toxic monocyclic heptapeptide produced by many cyanobacteria, on Japanese medaka, *Oryzias latipes*. The fish were exposed to the toxins for 30 days thereafter, the liver, intestines and gonads were evaluated for ultrastructural changes and the result showed tight junctions, severely damaged microvilli, lysed epithelial cells, pycnotic nuclei, mucin secretion and disorganization of desmosomes of the intestinal cells.

A study by Rangsayatorn *et al*., (2004) to assess the accumulation and toxicity of a heavy metal in *Puntius goniontus* fish that were fed with cadmium contaminated cyanobacterial cells using light and transmission electron microscopy showed that dietary cadmium caused ultrastructural damages in the cells of the various organs sampled and these included proliferation of vacuoles and lysosomes, swelling of mitochondria with loss of cristae as well as, vessiculation and dilation of rough endoplasmic reticulum. In another study, Barillet *et al*., (2010) assessed the histological damage on the gills, gonads and white muscles of Zebra fish *Danio rerio* exposed for 20 days to waterborne uranium compound and observed similar damages including abnormal localization of mitochondria within muscles, hyperplasia of the chloride cells and vacuolated nucleus in the fish gonads. Padmini and Rani, (2011) using

cytological structures, cell surface morphology and internal morphology as environmental stress monitors on the hepatocytes of Grey mullet *Mugil cephalus* living in industrial contaminated, and uncontaminated estuaries reported some ultrastructural responses in the fish species such as, distorted nuclear membrane with rearranged chromatin, irregular shaped organelles, mitochondrial swelling and loss of mitochondria with unusual shelf like cristae in the fish population from contaminated estuaries.

There is a dearth of information on the ultrastructural impact of pesticides to non-target aquatic species particularly fishes. Some studies that exist in this area include that of Ba-Omar *et al*., (2011) who investigated the effects of temephos on the gills of *Aphanius dispar* and reported a concentration dependent damage effect to the fish cells which included the presence of secretory vesicles in the pavement and chloride cells, numerous microvilli and cell death that became severe with the highest concentration of the organophosphorus compound. The observed cell death in their study was characterized by chromatin condensation, broken endoplasmic reticulum and degradation of almost all the cell membranes.

In a more recent study, Al-Ghanbousi *et al*., (2012) using the same fish species, reported the ultrastructural effect of deltamethrin on the fish gills to include swollen mitochondria cells with distorted internal cristae at the lowest concentration of the pesticide whereas, at the higher concentrations, deltamethrin induced hypertrophy of chloride cells with degenerations, large cytoplasmic vacuolation within the chloride cells, necrosis of pillar cells and blood cells as well as, the dilation of blood space.

The functionality of intestinal mucosa as a transport system has equally been studied using ultrastructural analysis. Fuglem *et al*., (2010) exposed the different segments of the intestinal mucosa of Atlantic salmon, *Salmo salar* to gold-BSA to identify antigen sampling cells in the fish. They discovered that gold- BSA uptake was restricted to very few dendritic-like cells and to a limited number of epithelial cells located in the mucosal folds in the second segment of the mid-intestine via some unique features that were found with the gold-BSA positive cells which displayed diverging and electron dense microvilli with channels intruding into the cytoplasm as against the control (gold-BSA negative cells).

To the best of our knowledge and as far as open literature is concerned, information on the cytogenotoxicity and ultrastructural effects of Chlorpyrifos, Actellic and Spinosad on the mosquito fish, *P. reticulata* is yet to be reported. The current research will therefore provide additional baseline data on the toxicological impact of the selected larvicides on the bio-control agent both at acute and sub-lethal toxicity levels aside the attempt to establish a candidate larvicide and the dosage solution required for a compatible integrated larval mosquito control practice.

CHAPTER THREE

MATERIALS AND METHODS

3.1. COLLECTION OF TEST ORGANISMS

Guppy and mosquito larvae species were sampled from different sites located on Figure 5 at the following sampling coordinates:

Sites	**Coordinates**
Harvey Road	N6.511069, 3.377159E
CMS Grammer School (A)	N6.533048, 3.388424E
CMS Grammer School (B)	N6.534327, 3.390012E
Zoological Garden	N6.51841, 3.400679E

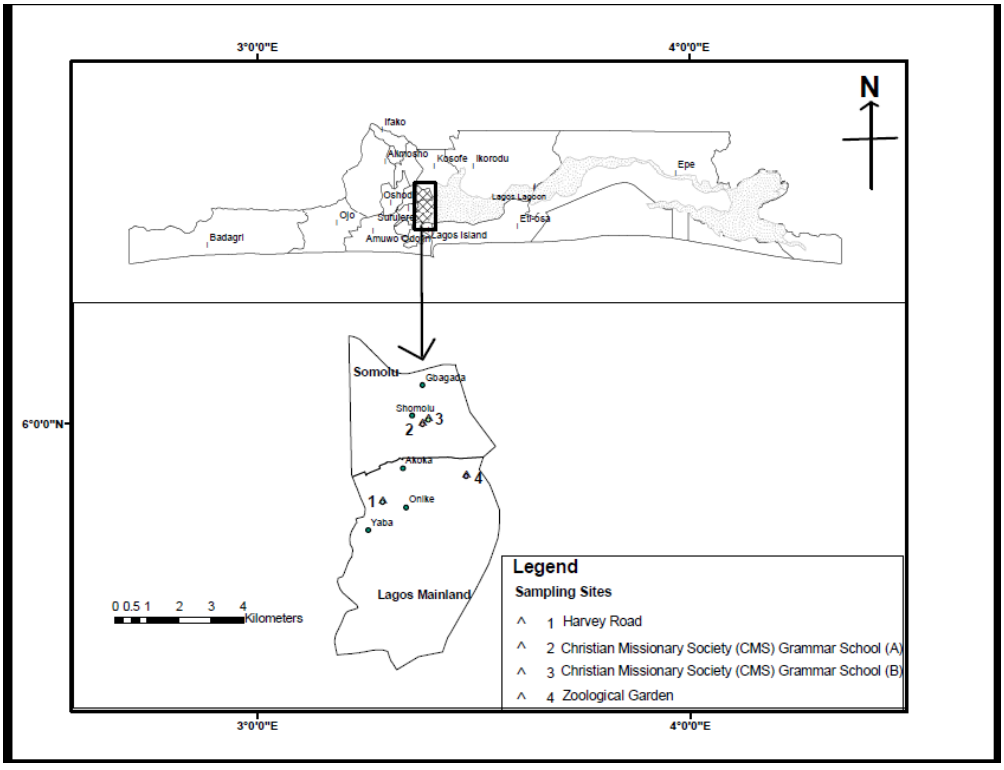


Figure 5: Collection sites of test organisms

3.1.1. Collection of mosquito species

Five scoops of *Culex* larvae were taken from the drainage at CMS Grammar School (A), Bariga, Lagos (Figure 5) between 7a.m and 9a.m and poured into a plastic bucket. Thereafter, it was taken to the University of Lagos Zoology laboratory for breeding. *Anopheles* larvae were collected from the University Biological Garden by setting up ten oviposition traps made out of plastic containers. The containers were placed at predetermined areas within the garden at 1.5m height. They were half filled with dechlorinated tap water and few dried leaves placed at the bottom of each container to give a dull background to attract gravid *Anopheles* females. An oviposition substrate (Whatman No 1 filter paper) was then lined vertically inside the containers where gravid females preferred to lay eggs just above the water level. Traps were monitored daily for the presence of mosquito larvae which were collected and then taken to the laboratory in transparent plastics containing dechlorinated tap water. Approximately 1,100 *Anopheles* and 2,300 *Culex* species were collected.

3.1.2. Collection of *Poecilia reticulata*

Poecillia reticulata were collected between 6.30 a.m and 9 a.m from drainages at CMS Grammar School, Bariga, Lagos (B), and Harvey Road, Yaba, Lagos (Figure 5) using fish net of mesh size 1.5mm in diameter. They were immediately transported to the Zoology laboratory in a transparent plastic bucket containing some drainage water.

3.2. BREEDING/REARING OF TEST ORGANISMS

3.2.1. Breeding of mosquito larvae species

Six mosquito cages of sizes 60 x 60 x 60cm constructed out of wooden material and nylon mesh were used to breed five generations of mosquitoes of *Culex* and *Anopheles* species respectively. Another cage of size 10 x 10 x 10cm with saw dust covered floor was built to house the laboratory rats purchased from Nigeria Institute of Medical Research (NIMR) at Edmond Crescent, Yaba. The saw dust was changed every 48 h and rats were fed with rat pellets and water placed in shallow cups within the cages. The rats were periodically introduced into the mosquito cages containing female adult species to supply blood meal for the development of their eggs.

Mosquito species were separated into their respective families (Appendix 1) using the keys of Oyerinde (1999). Larvae from Anopheline and Culicine families were then separately collected with nylon mesh and each poured into five transparent plastic containers of capacity 500ml, half filled with dechlorinated tap water in which a mixture of 1.50g mice pellets and 0.7g yeast were added on daily basis. Estimates of 50 larvae were introduced into each plastic container to avoid overcrowding. The media were changed every 48 h to avoid fungal growth from the feed and metabolic wastes. Larvae were then left to metamorphose into pupal stage. With the aid of a nylon mesh, pupae from each of the respective family were separately placed into 500 ml glass beaker half filled with dechlorinated tap water. Three beakers, each containing approximately 70 pupae from each family, were inserted into the mosquito cages to trap respective emerging adults (Plate 1).

Five adult mosquitoes from each family were frozen in plastic cups and taken to NIMR for confirmation of the species type. As mosquito breeding continued in the laboratory, glucose solution (10%) soaked in cotton wool were placed at the top of the cages and inside the cages to act as feed for the newly emerged adult mosquitoes. Males of both species were continually fed on 10% glucose solution while in addition to the glucose solution, the females were at specific time interval fed with the blood from the laboratory rats so as to allow for complete development of their eggs. Beakers lined with Whatman No 1 filter papers were placed in cages to allow for egg deposition and collection. The eggs were then stored in a dry place thereafter, introduced into a new beaker of dechlorinated tap water to hatch into larvae as at when needed. This procedure was repeated five times to produce the fifth generation mosquitoes. The 4^{th} instar larval stage from each species of the fifth generation of the mosquitoes was used for toxicity study as suggested by Don Pedro and Adegbite, (1985); Kimbi, (1989). Rearing condition of the mosquito in the laboratory were at $28^{o}C \pm 2^{o}C$ and 72% ± 2% RH.

Plate 1: Laboratory breeding of mosquito species

3.2.2 Rearing of *Poecilia reticulata*

Poecilia reticulata were gently released into a holding tank of capacity 200 L containing dechlorinated tap water at pH 7. The fish were reared under laboratory condition of 28^{o}C±0.8^{o}C, 72% ± 2% RH and 12 h light and 12 h dark regime. The tank was drained then washed and refilled with fresh dechlorinated tap water twice weekly to prevent the accumulation of fish metabolic wastes. After 8days of acclimatization period, selected brood stocks were transferred into 5 litres plastic containers to obtain offspring. After 3-4weeks period of completion of a cycle of reproduction, 2 day old juveniles were separated from adults and introduced into 2 litres well-aerated dechlorinated tap water where they were allowed to mature into adult sizes of mean length 3.5±0.2cm.

3.3. SOURCES OF LARVICIDES

Spinosad with active ingredient 1.25g/kg consisting of Spinosyns A (CAS: 131929-60-7) and D (CAS: 131929-63-0) was obtained as spintor dust from Nigeria Stored Product Research Institute, Yaba (NSPRI) while commercial formulations of Pirimiphos methyl sold as Actellic 25EC (Emulsifiable concentrate) (CAS: 29232-93-7) containing 250 gL^{-1} active ingredient, and Chlorpyrifos 48EC, CAS: 3383-98-8 containing 480 gL^{-1} active ingredient were purchased from Sygenta Nigeria Limited, Lagos, and Afcott Nigeria Plc. Lagos, respectively.

3.4. FEEDING DENSITY STUDIES OF *POECILIA RETICULATA* ON MOSQUITO LARVAE

Culex quinquefasciatus and *Anopheles gambiae* larvae at increasing densities of ten were separately introduced to *P. reticulata* of varying densities with each placed in plastic

container of 1 L capacity, half filled with dechlorinated tap water. The reactions of the mosquito species to predation by the fish were closely observed. The following predator-prey densities in ten replicates were used:

Fish density	**Larvae density/0.5 L**
1	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100
2	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100
4	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100
6	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100
8	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100
10	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100

After 48 h, which was preceded by another 24 h starvation period, additional number of mosquito larvae in multiples of 50 were supplied to the fish population where satiation was not attained as follows:

Fish density	**Larvae density/0.5 L**
6	150, 200, 250, 300 and 350
8	150, 200, 250, 300 and 350
10	150, 200, 250, 300 and 350

3.5. PREY PREFERENCE STUDIES OF *POECILIA RETICULATA*

Some drainage water collected from the natural breeding site of *P. reticulata* was analyzed for planktonic load to determine alternate food materials available to the fish. Thirty each of 3rd and 4th instars of mosquito larvae, *Chironomid sp*. and *Eudrilus eugeniae* (earthworms),

were introduced at 6 h, 12 h, and 24 h into plastic containers half filled with dechlorinated tap water with *P. reticulata* of same size range at densities 1, 4, 6, 8 and 10 in three replicates to investigate the fish prey preference.

3.6. ACUTE TOXICITY STUDIES ON MOSQUITO LARVAE AND *POECILIA RETICULATA*

3.6.1. Preparation of test concentrations

Stock solutions of spinosad, actellic and chlorpyrifos at concentrations of 1.25mgL^{-1}, 250mgL^{-1} and 480mgL^{-1} respectively were prepared using dechlorinated tap water as solvent. Serial dilutions were then made from each stock for acute toxicity studies against *P. reticulata* and mosquito larvae species respectively.

3.6.2. Physico-chemistry of test media

Prior to the start of experiment, the physico-chemical characteristics (pH, dissolved oxygen, conductivity, temperature and salinity) of the test media and dechlorinated tap water (control) were analyzed with a pH meter (©Mettler Toledo AG), DO meter (©Mettler Toledo AG), Conductivity meter (©Mettler Toledo AG), Stem Glass Thermometer (Uniscope,) and Master Refractrometer (Atago, Japan), respectively.

3.6.3. Acute toxicity bioassay on mosquito species

Mosquitoes were not fed 24 h prior to bioassay to eliminate the possible effects from differential feeding amongst the organisms. Active 4th instar mosquito larvae of *Anopheles* and *Culex* species in three replicates of 50 each were randomly selected and placed in bioassay containers of capacity 1litre half filled with treated and untreated media (control). The

following concentrations that were extrapolated from preliminary toxicity study against mosquito species were used for the static bioassay.

Larvicide	**Concentrations (μgL^{-1})**
Spinosad:	0, 25, 50, 75, 100, 125,150
Actellic:	0, 15, 25, 50, 75, 100, 125
Chlorpyrifos:	0, 0.24, 0.48, 0.72, 0.96, 1.20, 1.44.

A larva was classified as dead if it failed to move when gently probed with the edge of a glass rod.

3.6.4. Acute toxicity bioassay on *Poecilia reticulata*

Poecilia reticulata were not fed 24 h prior to bioassay. Three replicates of 25 fish of mean size 3.5±0.2cm were randomly selected and placed in bioassay containers of 1litre capacity half filled with treated and untreated media as control. Test concentrations which were extrapolated from preliminary bioassays on guppy were used as follows:

Larvicide	**Concentrations (μgL^{-1})**
Spinosad	0, 20, 40, 100, 250, 500, 1500
Actellic	0, 250, 500, 750, 1000, 1250, 1500
Chlorpyrifos	0, 2.40, 3.60, 4.80, 7.20, 9.60, 12.0

A fish was classified as dead if it failed to move when gently probed with the edge of a glass rod.

3.6.5. Quantal response (Mortality)

Static bioassays were assessed by taking mortality counts at definite time intervals of 6 h, 12 h and 24 h. The maximum time corresponded to the current WHO recommended period for toxicity tests on insecticidal compound (WHO, 2007).

3.7. SUB-LETHAL TOXICITY STUDIES ON *POECILIA RETICULATA*

3.7.1. Selection of test concentrations

Sub-lethal fractions of 24 hLC_{50} values for Actellic on *Poecilia reticulata* ($1/29^{th}$, $1/12^{th}$, and $1/3^{rd}$) that were within the range of concentrations that killed 40%, 75% and 98% of the more resistant *Culex quinquefasciatus* larvae but caused no mortality in the fish during acute toxicity study in **Section 3.6** were computed.

3.7.2. Induction of micronuclei and nuclear abnormal cell in *Poecilia reticulata* with Giemsa assay

Poecilia reticulata were not fed 24 h before testing and the static renewal test technique was adopted where the test media was renewed at the same concentration once every 48 h (Wester and Canton, 1992). Sixty three fish of mean length 3.5±0.2cm were randomly selected and divided into 3 groups (21fish/group). Each group was exposed to the larvicides for 28 consecutive days. The test concentrations used were as follows:

Actellic: 0 μgL^{-1}, 24μgL^{-1}, 58μgL^{-1} and 230μgL^{-1}

Spinosad: 0 μgL^{-1}, 60 μgL^{-1}, 123 μgL^{-1} and 361 μgL^{-1}.

Fish were checked every 24 h and dead fish were removed immediately. From each replicate and control, 3 fish were randomly sampled for structural aberrations including micronucleus

at days 1, 3, 7, 14, 21 and 28 respectively using the conventional Giemsa protocol as described by Campana *et al*., (1999). Gill cells were collected from gill arches of each fish and smeared on three clean slides. The cells were then fixed in absolute ethanol for 20 minutes and air dried. After 24 h, each slide was stained in May-Grunewald for 6 minutes and 15% Giemsa solution for 10 minutes. Stained slides were rinsed thoroughly with distilled water and left to air dry. Slides were randomly selected and coded. From each slide, the frequencies of Micronucleated cells, Binucleated cells, Poly chromatic or immature cells and Normochromatic or mature cells were determined for three thousand (3000) cells at 63x/1.4 oil immersion (Zeiss Axio Imager Microscope). PCEs were identified as young cells without visible cytoplasmic boundary (Cavas, 2008), BN as cells with two nuclei of relatively equal size bounded as in mature cells or unbounded as in immature cells (Cavas and Ergene- Gozukara 2003a; Cavas and Ergene-Gozukara 2005a), and Normo-chromatic erythrocytes as normal cells with distinct cytoplasmic boundary (Cavas and Ergene-Gozukara, 2005b). The criteria for identification of micronuclei in the fish were as follows: a) MN must be smaller than one-third of the main nuclei; b) MN must be clearly separated from the main nuclei; c) MN must be on the same plane of focus and have the same colour of stain as the main nucleus (Cavas and Ergene-Gozukara, 2005a).

3.7.3. Behavioural study on *Poecilia reticulata*

Lower concentrations of the larvicides which were within the range of concentrations that killed 30%, 50% and 70% of *C. quinquefasciatus* larvae but did not cause mortality in *P. reticulata* were selected from the Probit values obtained from earlier 24 h acute toxicity tests carried out in **Section 3.6.**The following concentrations were used:

Spinosad: 0 μgL^{-1}, 49 μgL^{-1}, 73 μgL^{-1}, 110 μgL^{-1}

Actellic: 0 μgL^{-1}, 18 μgL^{-1}, 30 μgL^{-1}, 50 μgL^{-1}

Chlorpyrifos: 0 μgL^{-1}, 0.4μgL^{-1}, 0.6μgL^{-1} and 0.8μgL^{-1}.

Heterogeneous sexes of fish consisting of 18 females (gravid and non-gravid), and 9 males, in three (3) replicates for each concentration of larvicide and control respectively were exposed for 28days under static renewal bioassay, and then evaluated for sub-lethal effects using behavioural changes, micronucleus and nuclear abnormal cell induction, and ultra-structural changes as biomarkers. Gravid females were only placed in the highest concentrations of the respective larvicides as well as, in the untreated dechlorinated tap water that served as control. Behavioral changes in guppy were critically monitored at every 24 h interval and responses were recorded if they differed from the control and occurred in ≥10% of the fish within each test chamber (Rice *et al*., 1997).

3.7.4. Induction of micronuclei in *Poecilia reticulata* with acridine orange assay

This study was carried out at the Biology Department in Central Michigan University (CMU), USA (Plate 2). The highest and the lowest concentrations of the larvicides from **Sub section 3.7.3** were selected as follows:

Spinosad: 0 μgL^{-1}, 49 μgL^{-1}, 110 μgL^{-1}

Actellic: 0 μgL^{-1}, 18 μgL^{-1}, 50 μgL^{-1}

Chlorpyrifos: 0 μgL^{-1}, 0.4μgL^{-1}, 0.8μgL^{-1}

At Days 3 and 14 respectively, 3 fish were randomly selected from each replicate for treatment, negative and positive control groups respectively and immediately dissected to

remove gill arches. These days corresponded to the days of the highest induction of micronuclei following preliminary cytological studies (**Sub-Section 3.7.2)**. The processing of gill cells was according to Cavas (2008) as adapted from Ueda *et al*., (1992). Gill cells were smeared on clean slides and fixed in three successive changes of methanol-acetic acid solution of ratio 1:3v/v. After 24 h, phosphate buffered solution (pH 7.4) containing AO at a concentration of 0.003% was dropped on each slide and then covered with cover slips. Three slides were prepared from each randomly selected fish in each replicate and control. Prepared slides were viewed for the presence of micronuclei with 63x/1.4 oil immersion under Olympus BX51 microscope. One thousand five hundred (1500) cells were scored from each slide. Micronuclei were detected as exhibiting yellow green fluorescence under blue excitation (Hayashi *et al*., 1990) using FITC barrier filter. Micronuclei were described according to Al-Sabti and Metcalfe (1999) and Cavas, (2008).

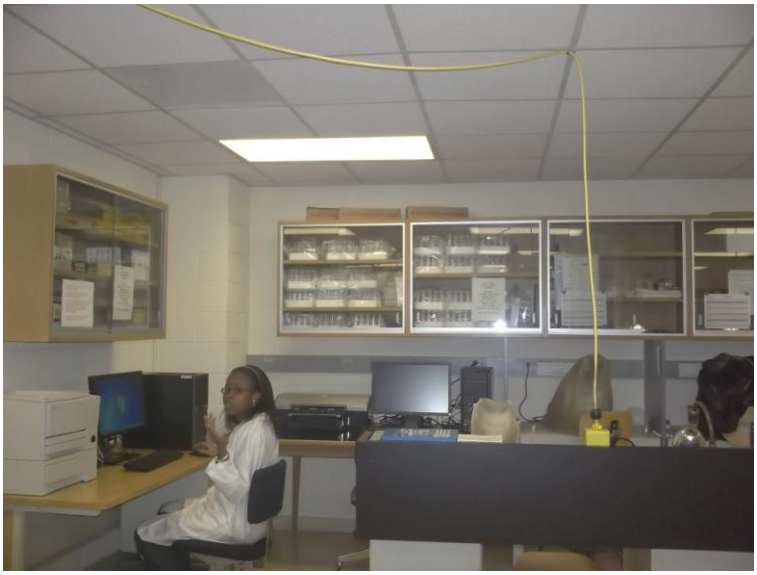


Plate 2: Microscopy facility at CMU.

3.7.5. Ultrastructural study on *Poecilia reticulata*

This study was carried out at the Microscopy facility in Biology Department of the Central Michigan University, USA (Plate 2) and test concentrations used were as in **Sub section 3.7.3.** At Day 28 of exposure period, 3 fish were randomly selected from each replicate and control (untreated dechlorinated tap water). They were dissected to remove intestinal tissues and then immediately fixed with 1.25% Glutaraldehyde (EMS, USA) in 0.10M phosphate buffered solution, pH 7.4 at 4°C for 1h in the dark. Tissues were then rinsed 3 times in phosphate buffer solution pH 7.4 with each rinse lasting for 15minutes in the hood. Tissues remained in the buffer solution until further processing.

In buffer solution, tissues were again rinsed in a newly prepared phosphate buffer solution at the same pH of 7.4 for 1h and then post fixed in 2% osmium tetroxide for 2 h under the hood. Post fixing was followed by the rinsing of the tissue two times in distilled water for 5minutes each. This was then followed by an ascending series of dehydration of graded alcohol (25%, 50%, 75%, 95% and 100%). After 24 h, tissues were infiltered at ratios 2:1; 1:2 (ethanol: plastic) on rotator; 100% plastic on rotator and another 100% plastic on rotator. Each series of infiltration lasted for over 8 h. Tissues were then embedded in siliconized rubber mould with epoxy resin (Epon 812) and placed in the oven at 60°C overnight. After 24 h, samples were removed and allowed to cool. Hardness was checked followed by trimming of the blocks for sectioning (Plate 3a).

Thick sections (1µm) were cut with glass knife and stained with toluidine blue dye. The sections were then examined under light microscopy to select areas for fine structural study and

photomicrography. Ultrathin sections (80 nm) were cut through M2V Ultramicrotome (Sorvalle) using glass knife with A-face. The ultrathin sections were taken on copper grid and stained with 2% Uranyl acetate and Reynold's lead citrate solution for 30 minutes and 3 minutes respectively. The samples were imaged at 80 kV with Philips CM-10 Transmission Electron Microscope (Plate 3b).

Plate 3a: Cutting of ultrathin sections

Plate 3b: Philips CM-10 Transmission Electron Microscope

3.8. THE INTEGRATION OF LARVICIDES AND GUPPY AGAINST *CULEX QUINQUEFASCIATUS* LARVAE.

Culex quinquefasciatus larvae were used for this study because it was the more resistant mosquito larvae species following the acute toxicity study that was earlier carried out in **Sub-section 3.6.3**. *Poecilia reticulata* and *C. quinquefasciatus* in ratios: 1:35; 5:70 and 10:350 were exposed for 24 h at the already established concentrations (**Sub-Section 3.7.3)** as follows:

Spinosad: 0 μgL^{-1}, 49 μgL^{-1}, 73 μgL^{-1}, 110 μgL^{-1}

Actellic: 0 μgL^{-1}, 18 μgL^{-1}, 30 μgL^{-1}, 50 μgL^{-1}

Chlorpyrifos: 0 μgL^{-1}, 0.4μgL^{-1}, 0.6μgL^{-1} and 0.8μgL^{-1}.

Each treatment and control was replicated six times in containers of capacities 0.5 L, 3 L and 5 L respectively. The number of larvae consumed or dead was counted after the 24 h exposure period. The impact of the integrated approach on mosquito larvae population was assessed.

3.9. STATISTICS

3.9.1. Feeding density study

Two way factorial analyses of variance (ANOVA) and comparison of means by Duncan's multiple range test (DMRT) were used to test for significant differences in the data obtained.

3.9.2. Prey preference study

Planktonic load was analyzed with *Mangalef* and *Shannon- Weaner* diversity indices for species richness and evenness respectively (Ogbeibu, 2005), while Manly's selectivity index as applied by Krebs (1999) and Rehage *et al*., (2005) was used to determine *P. reticulata* prey choice using the equation:

$S_i = w_i/\Sigma w_i$, where $w_i = c_i/a_i$; w_i = prey preference for prey type i;

c_i = proportion of preys consumed, a_i = proportion of the prey available;

S_i = Equivalent to *Manly's* α (selectivity index)

3.9.3. Acute toxicity study

The dose mortality response of the 24 h toxicity test was analyzed by Probits after Finney (1971). The standard safety margin (SSM) as applied by Foster (1939) was used to determine the safety margin of the test larvicides on the test animals.

3.9.4. Sub-lethal toxicity study

The Student T-test (paired sample) from SPSS Version 15.0 for Windows was used to analyze significant differences in the frequency of micronucleus and nuclear abnormal cells in treated and control media. The ratio of PCE to NCE cells was calculated according to Pacheco and Santos, (2002) and expressed in (%).

$$\text{PCE frequency (\%)} = \frac{\text{No PCEs}}{\text{No. PCEs + NCEs}} \times 100$$

3.9.5. Integrated larval control study

Student T-test (paired sample) from SPSS Version 15.0 for Windows was used to assess the effectiveness of integrated approach at $P<0.05$ significant level.

CHAPTER FOUR

RESULTS

4.1. PREDATOR PREY DENSITY OF *POECILIA RETICULATA* ON MOSQUITO LARVAE

The number of mosquito larvae consumed by *P. reticulata* varied significantly with predator-prey density and time interval (Table 1; Appendix 2) and the highest significant level was attained at predator density of 10 (P-value = 1.02E-72). Duncan post- hoc test revealed significant differences ($P<0.05$) in the time of application at all the predator densities except 1 and 10 (Table 2). The optimal consumption of mosquito larvae by one fish in a 24 h feeding bout was attained at predator-prey density of 1fish to 30 mosquito larvae (Figure 6a). Peak feeding points in guppy were attained at predator densities of 1 and 2 followed by a decline in the number of mosquitoes consumed even with increasing prey densities however, at predator densities of 6, 8 and 10, consumption of larvae was observed as an exponential increase (Figures 6a & 6b). There was a slight decline in the feeding growth curve at predator density of 4 compared to predator densities of 6, 8 and 10 that showed distinct exponential increase in feeding (Figure 6a).

At the densities where fish satiation was attained, it was observed that prior to the resumption of feeding in the fish, most of the mosquito larvae had metamorphosed into pupal or adult stages.

TABLE 1: Effect of Predator-Prey Density and Time Interval on Feeding in *Poecilia reticulata* (n = 10 observations per group)

Source of Variation	MS	P-value
1:10-100		
Predator-prey density	285.5407	5.67E-18
Time interval	559.4444	1.94E-16
Error	13.275	
2:10-100		
Predator-prey density	847.3111	2.01069E-34
Time interval	1721.433	3.53681E-32
Error	11.93333	
4:10-100		
Predator-prey density	4129.927	6.21E-39
Time interval	10409.28	7.61E-40
Error	43.5	
6:10-100		
Predator-prey density	5407.07	1.7E-49
Time interval	8694.6	4.61E-43
Error	29.625	
8: 10-100		
Predator-prey density	6204.707	1.37761E-57
Time interval	6089.967	6.26304E-43
Error	20.925	
10:10-100		
Predator-prey density	8951.052	1.02E-72
Time interval	4077.367	7.03E-45
Error	12.4	
Total		

P< 0.05

MS = Mean of Square

Table 2: Duncan Post Hoc Test on the differences with Time of Feeding in *Poecilia reticulata*

	PREDATOR DENSITY (Pop. of fish/0.5L water) (MEAN ± S.D)					
HOUR	**1**	**2**	**4**	**6**	**8**	**10**
6	2.66 ± 2.31 (a)	3.0 ± 1.73 (a)	30.00 ± 10.15 (a)	28.33 ± 5.69 (a)	39.67 ± 6.50 (a)	92.67 ± 12.70 (a)
12	4.67 ± 3.51 (a)	5.67 ± 2.87 (a)	51.0 ± 10.00 (b)	63.33 ± 25.50 (b)	78.00 ± 5.00 (b)	100.00 ± 00 (a)
18	7.33 ± 5.51 (a)	12.33 ± 3.51 (b)	75.67 ± 11.93 (c)	100.00 ± 00 (c)	100.00 ± 00 (c)	100.00 ± 00 (a)
24	10.67 ± 7.51 (a)	18.67 ± 4.73 (b)	96.00 ± 6.93 (d)	100.00 ± 00 (c)	100.00 ± 00 (c)	100.00 ± 00 (a)

Means with the same letter(s) in a column were not significantly different at

P<0.05

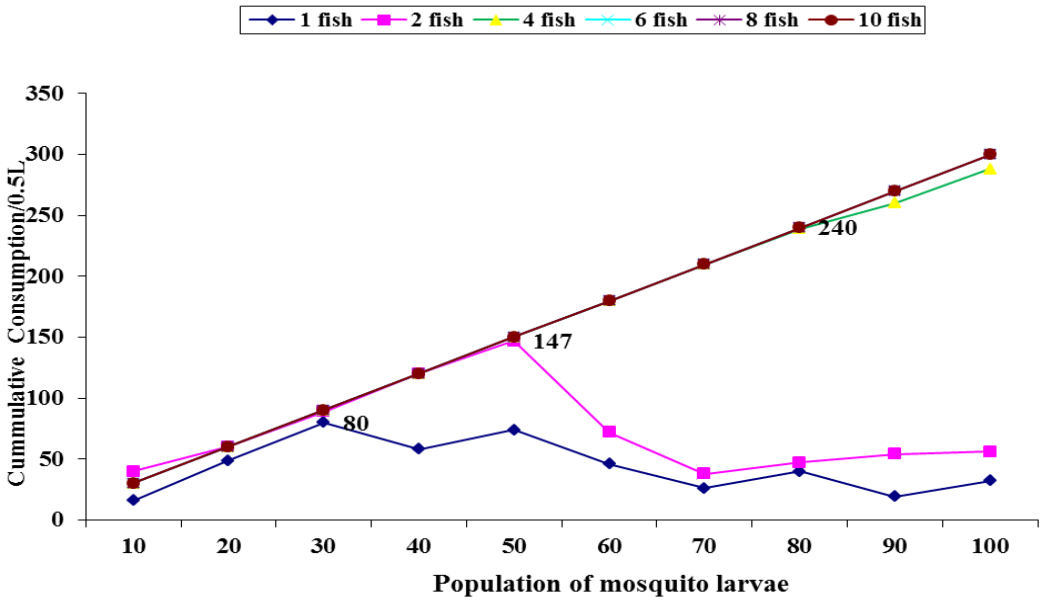


Figure 6a: Feeding Pattern of *Poecilia reticulata* at 24 h

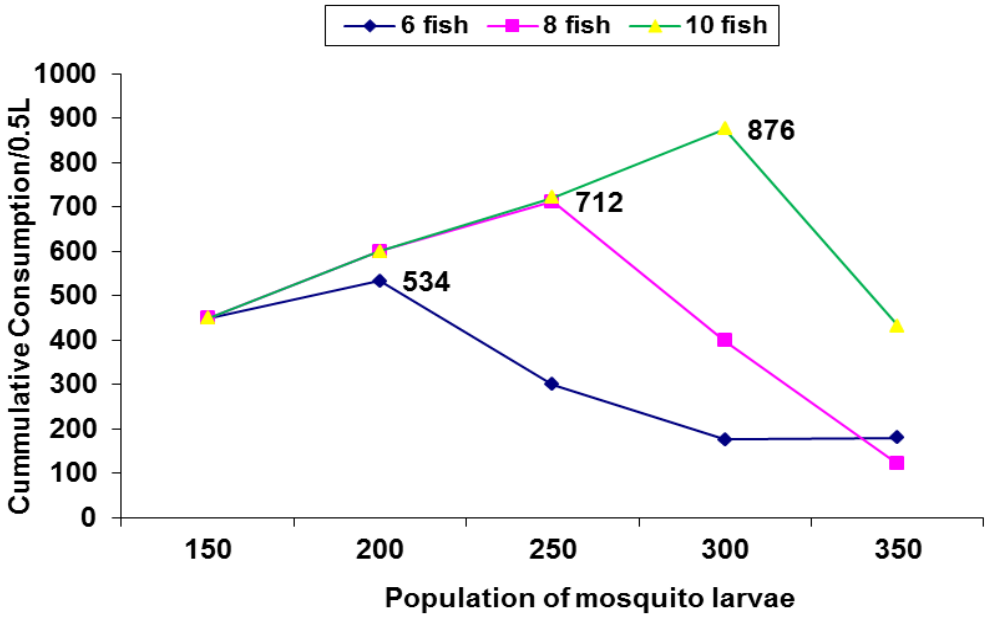


Figure 6b: Feeding Pattern of *Poecilia reticulata* after 24 h at higher densities.

4.2. PREY PREFERENCE OF *POECILIA RETICULATA*

The planktonic analysis revealed that the fish's natural environment was fairly rich in biodiversity evidenced by the values obtained from the biodiversity indices (Table 3). The class insect was the largest animal population of which chironomid was the most abundant species with a population of 17.59% while *Eudrillus sp*. (earthworm) had the lowest population of 1.59% (Table 3). *Euglena sp*. and *Oscillatoria agardhi* Gom. were amongst other organisms that were abundant in guppy natural environment.

The prey consumption by *P. reticulata* varied significantly with predator density and prey types (Table 4). The trend revealed an increase in consumption of prey with increase in *P. reticulata* density except for earthworms. The feeding preference of *P.reticulata* at all the predator densities used in this study remained same with *Chironomid* larvae being the most preferred prey type and earthworms the least preferred as revealed by the computed *Manly's* selectivity indices (S) on Figure 7. The feeding interaction however revealed that at no point in time did the fish seize to prey on the mosquito larvae even when the most preferred prey species (*Chironomid*) was present (Figure 8).

Observation on the predatory behavior of *P. reticulata* showed that the fish was more efficient when paired and preferred to chase after the preys before consuming them except in the case of earthworms where the mode of prey capture was defensive (Table 5). The feeding behaviour of the female fish towards mosquito larvae species was different from the male ones in that the females ate the mosquitoes after pursuing and capturing them while the males would pursue, capture and abandon the prey.

There was an evidence of high competition for *Chironomus* and mosquito larvae species which was not found with the mosquito pupa and earthworm. *Chironomus sp*. was the most consumed prey type while mosquito pupa and earthworms were not eaten at all within 1minute time interval.

Table 3: Planktonic load of *Poecilia reticulata's* natural habitat

Organisms	Number of Individuals	% composition of Individual species
CLASS BACILLARIOPHYCEAE		
Amphora ovalis (Kutz)	4	0.91%
Navicula forcipata Greville	6	1.36%
Navicula radiosa Kutz	8	1.82%
Navicula sp	6	1.36%
Nitzschia palea (Kutz) W.Sm	21	4.77%
Nitzschia sublinearis Hustedt	4	4.77%
Pinnularia sp	4	4.77%
Pinnularia subcapitata Gregson	12	2.73%
CLASS CYANOPHYCEAE		
Oscillatoria agardhii Gom.	65	14.77%
Oscillatoria chlorina Kutz.	6	1.36%
Oscillatoria subuliformis Gom.	7	1.59%
Phormidium crouani Gom.	5	1.14%
Phormidium sp	4	0.91%
Phormidium tenue (Meneghini) Gomont	5	1.14%
Spirulina sp	5	1.14%
Trichodesmium sp	25	5.68%
CLASS CHLOROPHYCEAE		
Ankistrodesmus sp	6	1.36%
Closterium sp	4	0.91%
Microspora floccosa (Vauch.) Vagerhein	8	1.82%
Mougeotia sp	5	1.14%
CLASS EUGLENOPHYCEAE		
Euglena cordata Hubner	7	1.59%
Euglena proxima Dang	3	0.68%
Euglena sp	76	17.27%
Euglena spirogyra var. *fusiformis* Deflandre	12	2.73%
Lepocinclis sp	5	1.14%
*Phacus*sp	21	4.77%
CLASS INSECTA		
Mosquito larvae	13	2.96%
Chironomid larvae	79	17.95%
CLASS BDELLOIDA		
Rotifers	7	1.59%
CLASS OLIGOCHAETES		
Earthworms	7	1.59%
Total number of species	30	
Total number of individuals	440	
Margalef	4.764	
Shannon-Weaner (H^1)	2.794	
Species Evenness (j)	0.8214	

Table 4: Effect of Predator Density and Prey Types on the feeding potential of *Poecilia reticulata*

(n=3 replicates per sample size)

Source of Variation	SS	Df	MS	F	P-value	F crit
Density	371.9111	4	92.97778	9.318486	5.10633E-05	2.689628
Prey types	890.5333	2	445.2667	44.62584	1.02294E-09	3.31583
Interaction	203.0222	8	25.37778	2.54343	0.030321481	2.266163
Error	299.3333	30	9.977778			
Total	1764.8	44				

P<0.05

SS = Sum of Squares; df = degree of freedom; MS = Mean of Square

Table 5: Predatory Behaviour of *Poecilia reticulata*

Criteria	Mosquito larvae	Mosquito pupa	Chironomid larvae	Earthworms
Mode of prey capture	♀Pursue,capture & eat ♂Pursue, attack and abandon	♀♂Pursue, attack and abandon	♀♂Pursue, capture & eat	Defensive
Pattern of consumption	♀Swallowing	Abandon	♀♂Swallowing	Abandon
Estimated time interval between each prey consumed/killed	Minute	2 Hours	Second	Several Hours
Spatial orientation between 1 min. time interval	1 fish ate 1 mosquito larvae in 1 minute	No pupa eaten within 1 minute	1 fish ate 2 chironomus larvae in 1 minute	No worm eaten within 1minute
Foraging pattern	Low when single	Absent when single	Low when single	Absent when single
Competition	Very high	Low	Very high	Absent

♂ Male fish

♀ Female fish

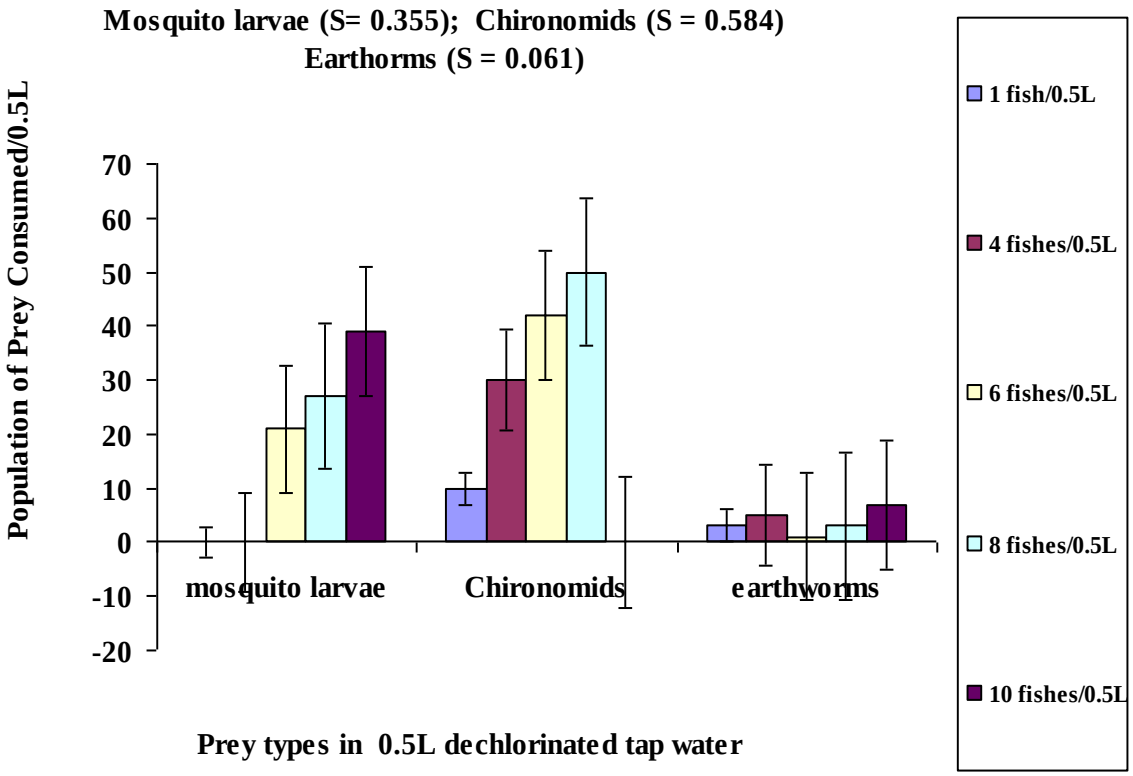


Figure 7: ***Poecilia reticulata*** **prey preference at 6 h**

S = ***Manly's*** **Selectivity Index**

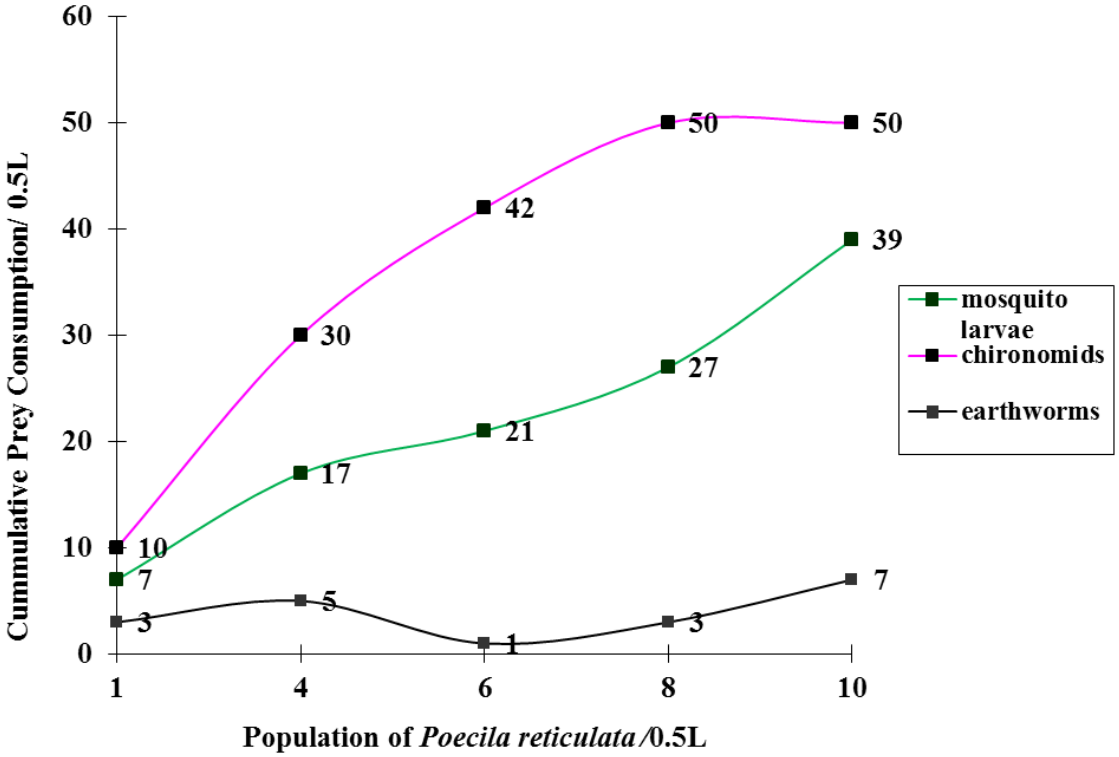


Figure 8: *Poecilia reticulata*-prey feeding interaction at 6 h

4.3. MEAN LETHAL CONCENTRATION OF LARVICIDES ON MOSQUITO LARVAE AND *POECILIA RETICULATA*

4.3.1. Physicochemistry of Test media

The physicochemical parameters of the test media were fairly stable when compared with the control except with salinity which was higher in the test media than in the control and for the dissolved oxygen that was lower in the control than in the spinosad treatment alone (Table 6).

4.3.2. Relative Acute toxicity and Susceptibility of larvicides to test organisms

Table 7 shows that chlorpyrifos was consistently the most toxic compound and the non-target *P. reticulata* was at equal risk with an LC_{50} value estimated at 4.23µgL^{-1} (3.99 – 4.46). In comparison, spinosad compound exerted no lethal toxicity on *P. reticulata* thereby making it impossible to calculate its mean lethal concentration on the fish species however, it remained appreciably lethal to the more tolerant target species (*C. quinquefasciatus*). There was no fish mortality recorded in the control throughout the experimental period.

Culex quinquefasciatus was the more tolerant target organism having a 1.5 times, 1.4 times and 1.2 times less susceptibility to actellic, chlorpyrifos and spinosad respectively when compared to *An. gambiae* (Table 7). *Poecilia reticulata* was 34 times less susceptible to actellic, and 11 times less susceptible to chlorpyrifos than Anopheles mosquitoes, but did not show any susceptibility to spinosad treatment (Table 7). The fish had low tolerance to the organophosphorus larvicides compared to spinosad. There were significant increases

($P<0.05$) in the mortality of test organisms with increasing concentration (Appendices 3-5) however, the effect with time of exposure was not significant at $P = 0.05$ (Appendices 6-8).

4.3.3. Safety margin

Spinosad in comparison to actellic and chlorpyrifos was the safest larvicide on the non-target guppy fish with a 97 fold safety margin between the more resistant *Culex* mosquito larvae and the fish species (Figure 9)**.**

4.3.4. Biological Activity of larvicides

Figures 10a and 10b; Appendices 9 - 11 illustrate a loss of biological activity by each larvicide on the test organisms ($P>0.05$). Chlorpyrifos was the fastest larvicide to lose its biological activity on the target mosquito larvae species when compared to spinosad and actellic. The loss of biological activity by actellic however, became constant after 6h while spinosad gradually lost its activity on culex larvae but not as fast as with chlorpyrifos (Figure 10a). With respect to the non-target guppy fish, there was a marked consistent decline in the activity of spinosad unlike with actellic and chlorpyrifos where loss of activity became constant on the fish after 6 h exposure period (Figure 10b).

Table 6: Mean physicochemistry of test media

Parameters	Control	Spinosad	Actellic	Chlorpyrifos
pH	6.80	6.75	6.81	6.80
Salinity	0.00‰	2.5‰	2.5‰	2.5‰
Conductivity	0.09 mgL^{-1}	0.09 mgL^{-1}	0.10 mgL^{-1}	0.11mgL^{-1}
Dissolved Oxygen (DO)	4.90 mgL^{-1}	5.00 mgL^{-1}	4.20 mgL^{-1}	4.20 mgL^{-1}
Temperature	23.0ºC	23.1ºC	23.1ºC	23.1ºC

Table 7: Relative Acute Toxicity and Susceptibility of larvicides to test organisms

Species/ Insecticide (μgL^{-1})	24 h LC_{50} (95% CL)	Slope ± S.E	DF	Probit-line	TF	SF
Culex						
Chlorpyrifos	0.56 (0.75 – 0.36)	2.95± 0.19	4	5.74+2.95X	1	1.4
Actellic	30.47 (44.39-16.18)	2.37 ± 0.15	4	1.50+ 2.37X	54.4	1.5
Spinosad	73.06 (93.02 – 55.29)	2.96 ± 0.20	4	-0.52 + 2.96X	130.4	1.2
Anopheles						
Chlorpyrifos	0.40 (0.42 – 0.37)	4.90 ± 0.30	4	6.96 + 4.90X	1	1
Actellic	20.44 (30.40 – 8.57)	2.80± 0.29	4	1.33 + 2.80X	51.1	1
Spinosad	59.34 (104.39 – 13.44)	3.02 ± 0.20	4	-0.35 + 3.02X	148.3	1
P.reticulata						
Chlorpyrifos	4.23 (4.46 – 3.99)	4.41 ± 0.26	4	2.24 + 4.41X	1	11
Actellic	697.30 (935.79– 477.02)	3.32 ± 0.21	4	-4.43 + 3.32X	164.8	34
Spinosad	0	0	0	0	0	0

0= Indeterminate
95% CL= 95% Confidence Limit in parenthesis;
DF= Degree of Freedom;
SE= Standard Error

TF= Toxicity Factor= $\frac{\text{24h } LC_{50} \text{ value of other Test Insecticides}}{\text{24h } LC_{50} \text{ value of most Toxic (Chlorpyrifos)}}$

SF= Susceptibility Factor= $\frac{\text{24h } LC_{50} \text{ value of other Test Organisms}}{\text{24h } LC_{50} \text{ value of most sensitive Test Organism}}$

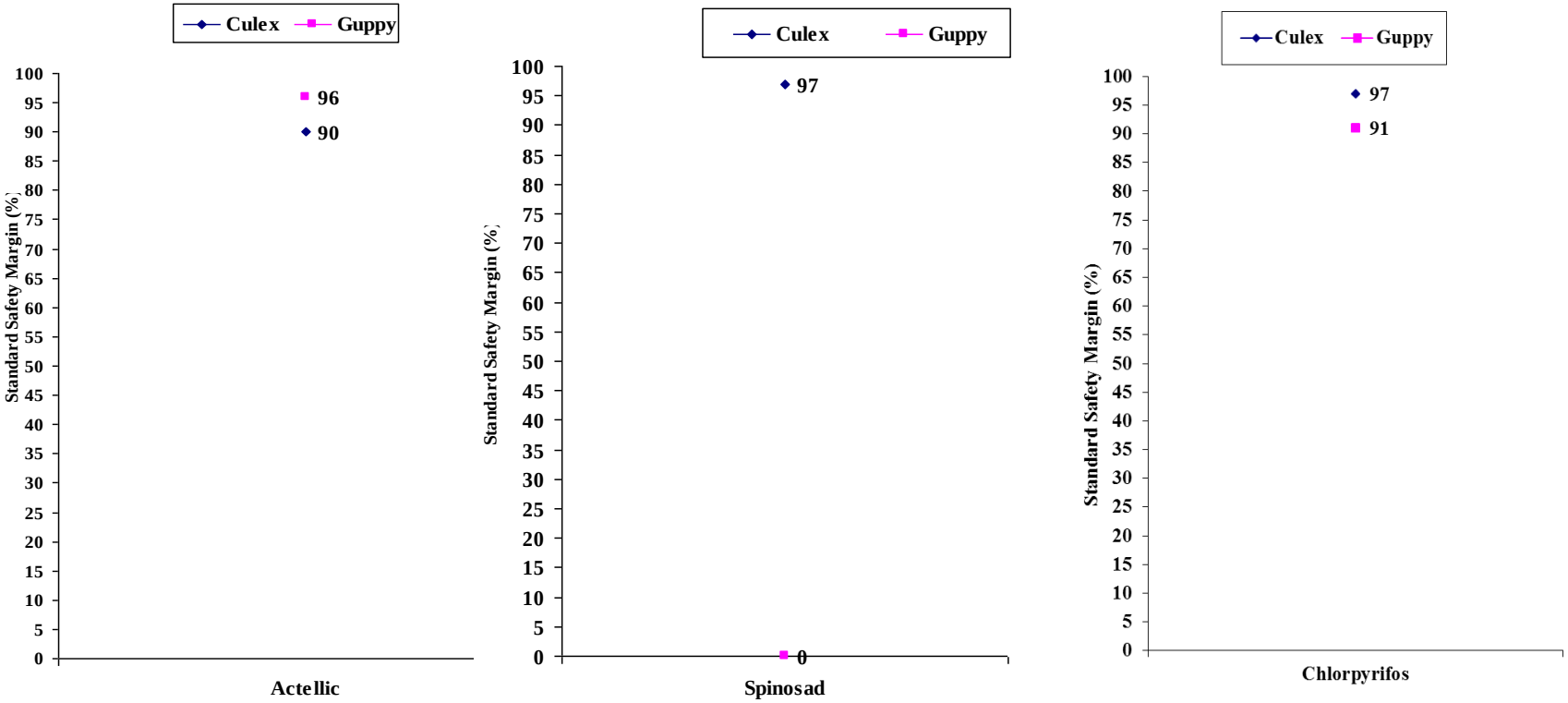


Figure 9: Standard Safety Margins between *Culex quinquefasciatus* and *Poecilia reticulata* (guppy)
Standard Safety Margins (SSM) = the zone between the surely Effective dose (ED99) and the lowest lethal dose (LD1) expressed as percentage of the ED99 (Foster, 1939)

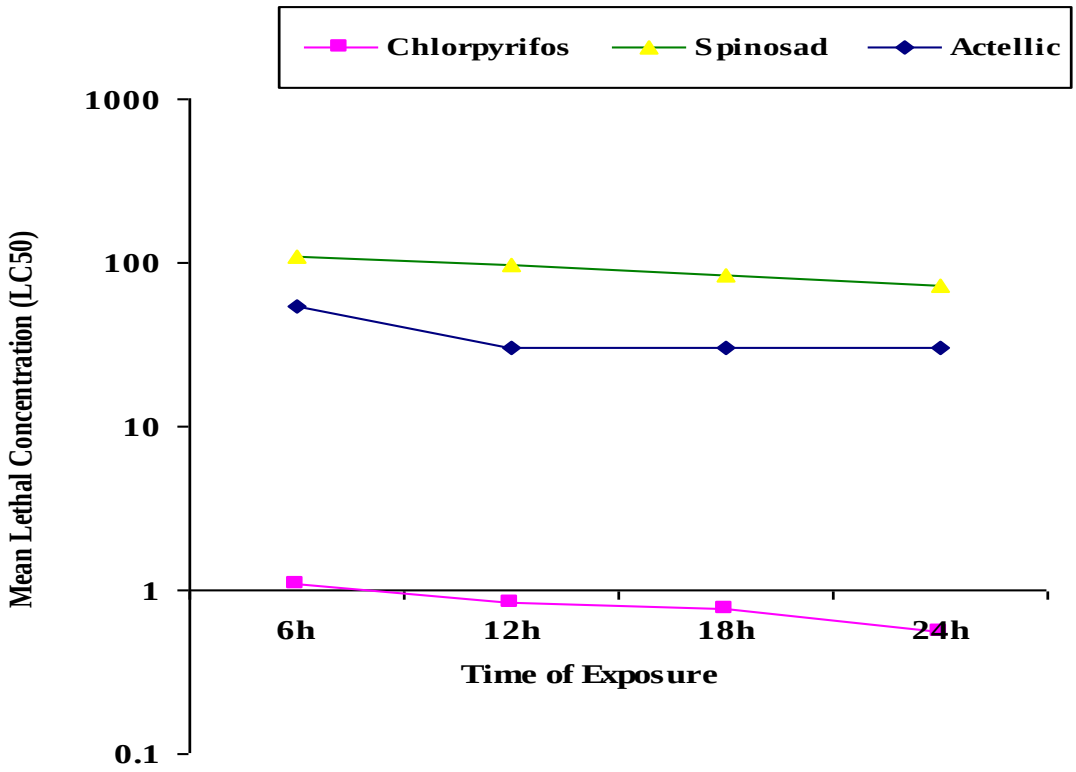


Figure 10a: Pattern of Biological Activity of larvicides on *Culex quinquefasciatus*

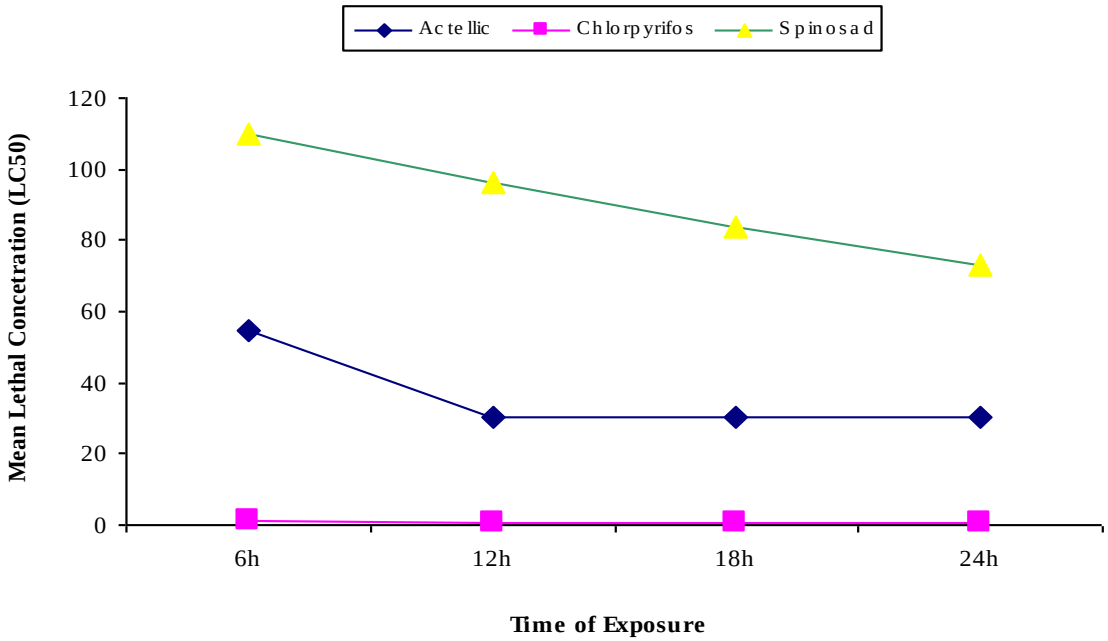


Figure 10b: Pattern of Biological Activity of larvicides on *Poecilia reticulata*

4.4. SUB-LETHAL TOXICITY ON *POECILIA RETICULATA*

4.4.1. Induction of micronuclei and nuclear abnormal cells in *P. reticulata* with Giemsa assay

The cells of *P. reticulata* have centrally placed round nuclei and a sizeable cytoplasm (Plates 4-7). The size as well as the location of the micronucleus within the cytoplasm varied from cell to cell and the shape was oval in almost all the cells. The nucleus has a well-defined boundary distinctly larger than the MN fragments which facilitated its ease of identification. Single MN was generally seen in most affected cells but there were incidences of MN occurring with other nuclear abnormal cells including PCE cells (Plate 4).

The frequency of MN in the treated cells showed distinct dependence on concentration (Table 8). At the highest concentrations of spinosad and actellic respectively, there were corresponding increases in MN frequencies. There was no induction of MN in the control and the differences in the increase of MN between the treatments and control groups were highly significant at $P<0.01$ and $P<0.001$ (Table 8). In comparison, treatment with spinosad induced a higher number of MN in the fish gill cells than actellic even at its lowest concentration (Table 8).

The frequency of nuclear abnormal cells other than MN in the exposed gill cells of *P. reticulata* was not significantly different from control ($P>0.05$) as shown on Table 9. Of the two types of Nuclear abnormal cells analyzed, Polychromatic erythrocytes (PCE) or immature cells, were faster to manifest than Binucleated cells (BN) and spinosad treatment contributed more significantly to this response ($P<0.05$; $P<0.01$). A significant induction of PCE cells

was only found at the lowest concentration of actellic as shown in Table 9 ($P<0.05$). The repression of normo-chromatic cells (mature cells) in the treatments was not significantly different from control as shown in Appendix 12 ($P>0.05$).

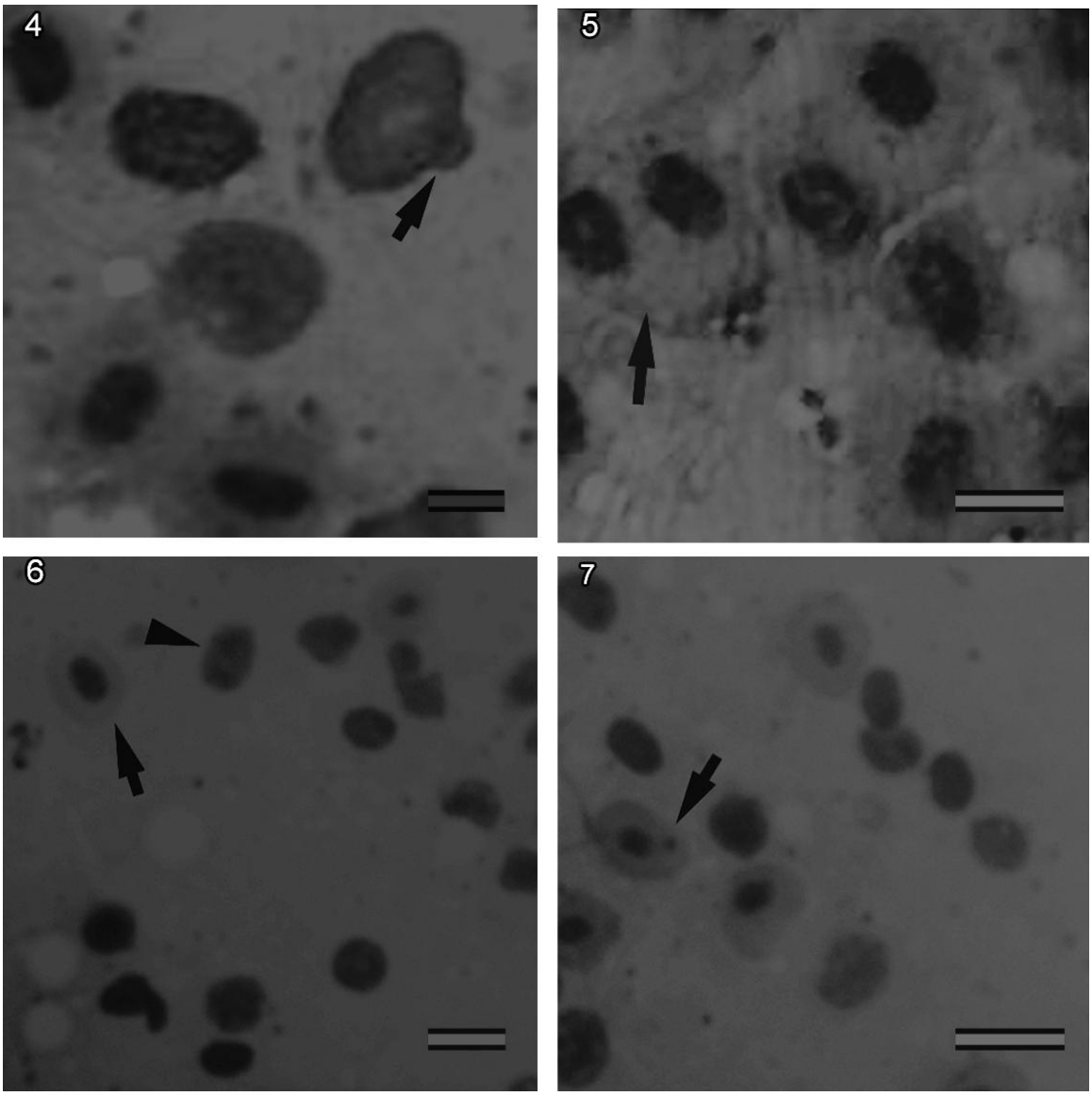
The % ratio of PCE to NCE increased significantly ($P<0.05$) in the gill epithelial cells of *P. reticulata* with increasing concentration and time of exposure as shown in Figures 11a and 11b as well as, in Appendices 13 & 14.

There were variations in the pattern of induction of MN, NA and NCE cells by the larvicides as shown in Figures 12a, 12b, 13a, 13b, 14a, and 14b respectively. With respect to MN, the difference in the elevated response at the highest concentration of spinosad peaked at Day 3 while at its lowest concentration peak effect from the larvicide was attained at Day 14 (Figure 12a). In contrast, the highest concentration of actellic gradually increased with peak effect of MN induction observed on Day 28 while damage from its lowest concentration was most pronounced at Day 14 (Figure 12b).

The induction of nuclear abnormal cells at the highest concentration of spinosad peaked at Day 1 and decreased thereafter however, at its lowest concentration, there was an initial increase in the induction of NA cells that peaked at day 14 (Figure 13a). Considering actellic treatment therefore, the induction of nuclear abnormal cell was greatest at Day 14 at the highest concentration of the larvicide while at its lowest concentrations, NA induction decreased after Day 3 (Figure 13b).

The formation of mature cells (NCE) was greatly repressed at Day 7 under the highest concentration of spinosad while at its lowest concentration the repressive effect peaked at Day 21 (Figure 14a). Peak repression of NCE cells by the highest concentration of actellic was attained at Day 14 while at its lowest concentration NCE repression was intermittent and corresponded with the period of change of bioassay (Figure 14b).

INDUCTION OF MICRONUCLEI AND NUCLEAR ABNORMAL CELLS IN *POECILIA RETICULATA* WITH GIEMSA ASSAY



Plates 4-7: Guppy gill cell stained with 15% giemsa and observed under Zeiss Axio Imager microscope at 63x/1.4 oil immersion. **4**: Micronucleated polychromatic cell (arrow). **5**: Binucleated normo-chromatic cell (arrow). **6**: Binucleated polychromatic cell (arrowhead); Normal cell with distinct cytoplasm (arrow). **7:** Micronucleated Normal cell (arrow). Scale bars = 10μm each.

Table 8: Frequency of Micronuclei cells in *Poecilia reticulata*

Treatment	Conc (µgL^{-1})	Tot Av. Cells analyzed (N x 3000)	MN (Mean ‰ ± SE)
Control	0	54,000	0 ± 0.000
Actellic	24	54,000	0.012 ±0.002^{oo}
	58	54,000	0.012 ± 0.002^{oo}
	230	54,000	0.014 ± 0.002^{ooo}
Spinosad	60	54,000	0.018 ± 0.003^{ooo}
	123	54,000	0.002 ±0.004^{oo}
	361	54,000	0.002 ± 0.001^{ooo}

N =Sampling time x Nos of replicates (3)
^{ooo}P<0.001
^{oo}P<0.01
^{o}P<0.05

Table 9: Frequencies of Nuclear Abnormal Cells in *Poecilia reticulata* (Mean‰ ± SE)

Treatment	Conc (μgL^{-1})	Tot. Av. Cells (N x 3000)	PCE	BN	Tot. NA cells (PCE + BN)	NCE
Control	0	54,000	1.054± 0.193	1.187 ± 0.105^{ns}	2.323± 0.239	2.122
Actellic	24	54,000	1.452 ± 0.0270^{o}	1.142 ± 0.148^{ns}	2.594 ± 0.238^{ns}	0.626±0.110
	58	54.000	1.548 ± 0.124^{ns}	1.401 ± 0.202^{ns}	2.949 ± 0.206^{ns}	0.537±0.105
	230	54,000	1.795 ± 0.235^{ns}	1.213 ± 0.151^{ns}	3.012 ± 0.124^{ns}	0.187±0.053
Spinosad	60	54,000	1.631 ± 0.292^{o}	1.053 ± 0.121^{ns}	2.684 ± 0.296^{ns}	0.666 ± 0.170^{ooo}
	123	54,000	1.844 ± 0.186^{o}	0.894 ± 0.122^{ns}	2.738 ± 0.264^{ns}	0.634 ± 0.061^{ooo}
	361	54,000	2.079 ± 0.305^{oo}	0.913 ± 0.089^{ns}	2.992 ± 0.245^{ns}	0.629 ± 0.151^{ooo}

N =Sampling time x Nos of replicates (3)

BN (binucleated cell); PCE (polychromatic cells); NA (Nuclear abnormal cells); MN (micronucleus)

^{o} $P<0.05$; ^{oo} $P<0.01$; ^{ooo} $P< 0.001$; ^{ns} $P>0.05$

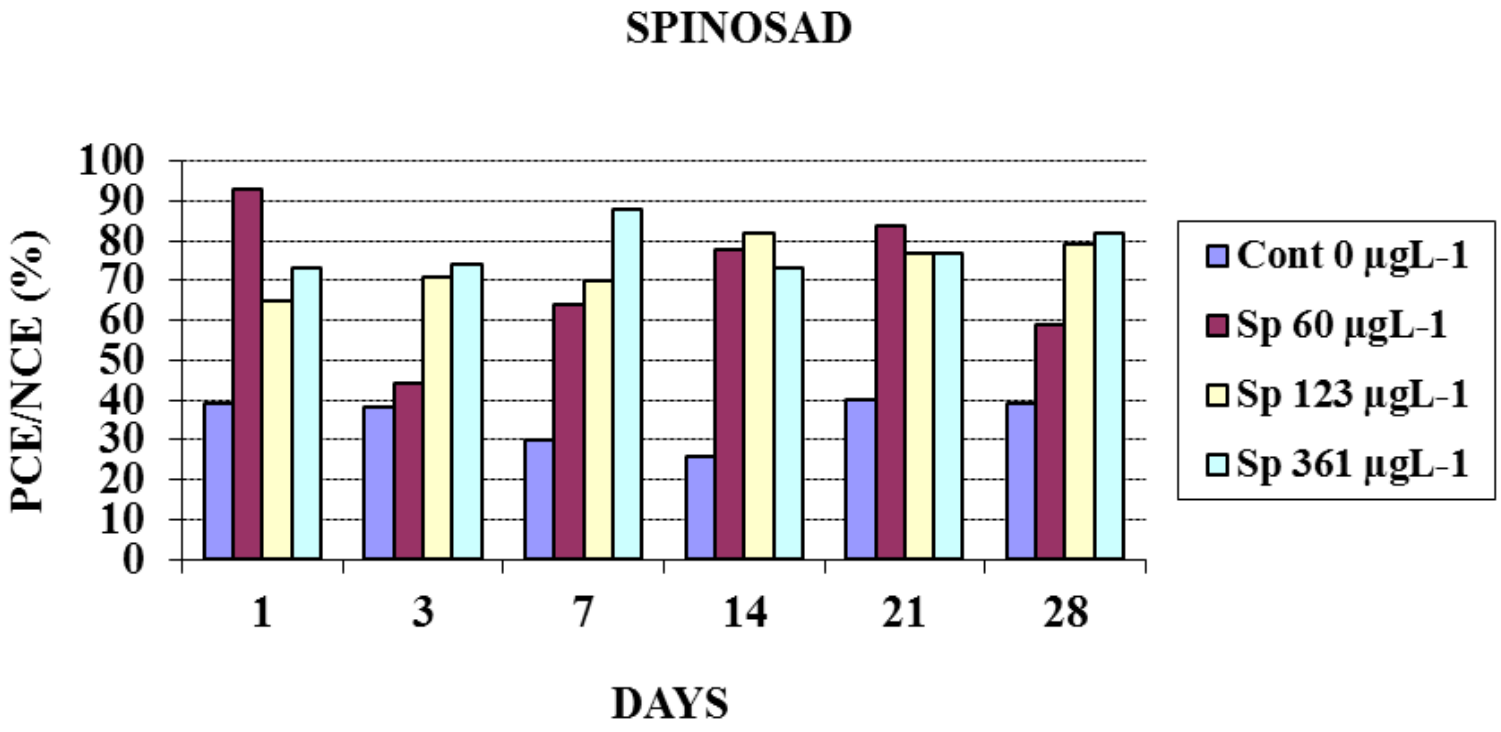


Figure 11a: % PCE/NCE ratio in gill erythrocytes of *Poecilia reticulata* exposed to Spinosad

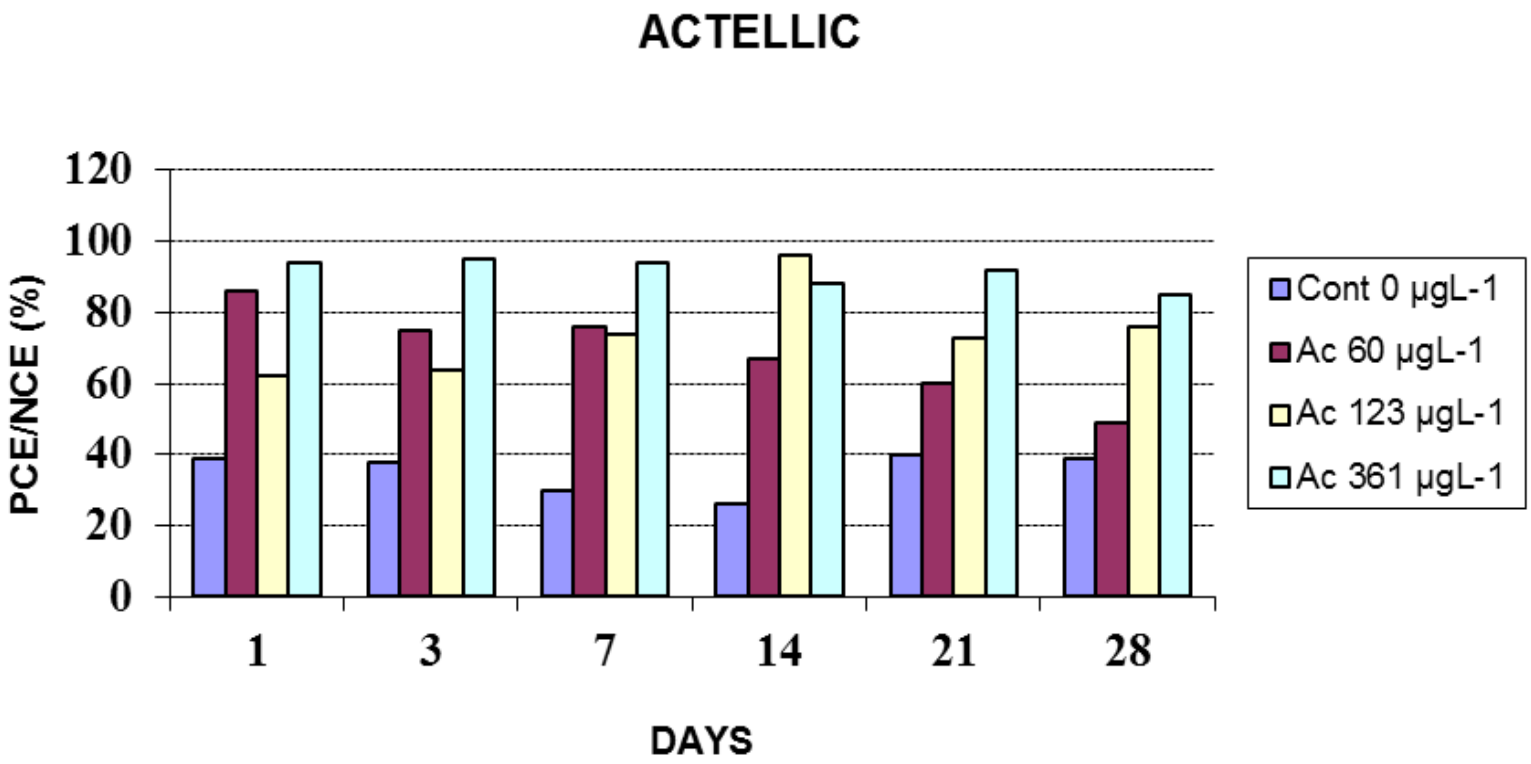


Figure 11b: % PCE/NCE ratio in gill erythrocytes of *Poecilia reticulata* exposed to Actellic

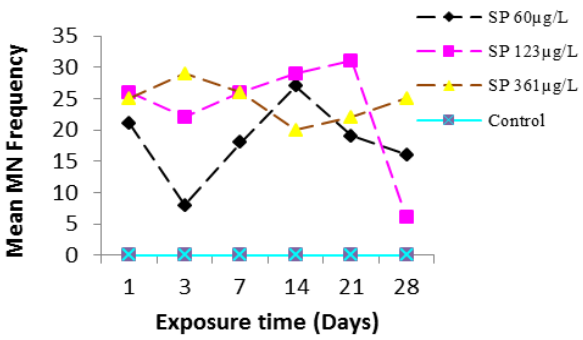


Figure 12a: Pattern of Micronucleus induction by Spinosad

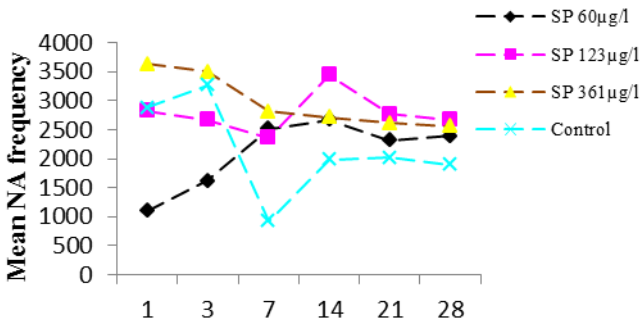


Figure 13a: Pattern of Nuclear Abnormal cell induction by Spinosad

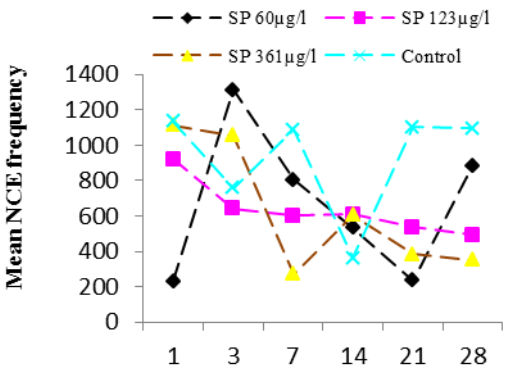


Figure 14a: Pattern of Normo-chromatic cell induction by Spinosad

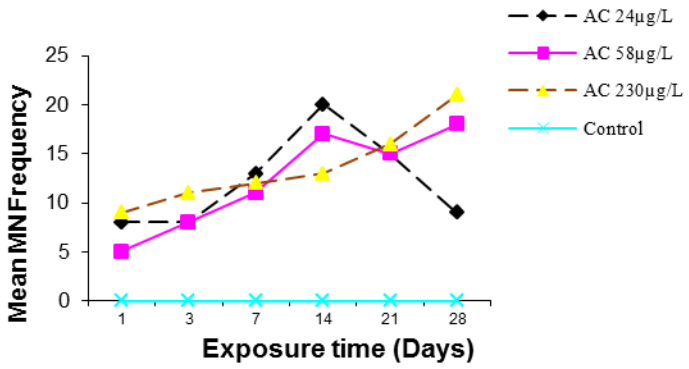


Figure 12b: Pattern of Micronucleus induction by Actellic

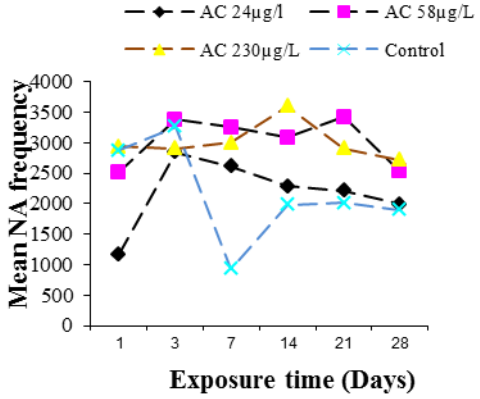


Figure 13b: Pattern of Nuclear Abnormal cell induction by Actellic

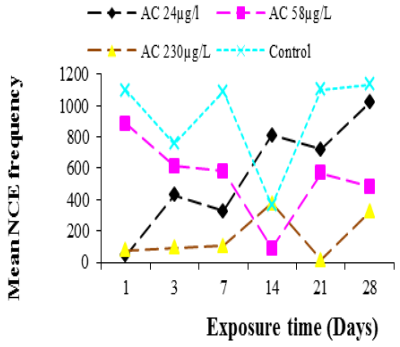


Figure 14b: Pattern of Normo-chromatic cell induction by Actellic

4.4.2. Behavioural responses in *Poecilia reticulata*

Chlorpyrifos exerted long lasting impact on the fish physiology that manifested as behavioural changes including reduction in feeding, heamorrhage and hypoactivity unlike actellic and spinosad that were well tolerated by the fish except at their highest concentrations (Table 10). The behavioural responses observed in *P. reticulata* varied with the type and concentration of larvicides but reduction in feeding was a response that was common in the exposed fish particularly at the highest concentrations of the larvicides. Heamorrhage of the operculum and fish mortality occurred mostly at the highest concentration of chlorpyrifos treatment (Table 10). Hatching of embryo was observed in the three larvicides and control but its occurrence varied with time of fish exposure. The hatching of fries occurred at Day 3 in actellic and spinosad media respectively and at Day 17 in chlorpyrifos medium. These events were earlier than in the control where hatching occurred at Day 21 (Table 10).

4.4.3. Induction of micronuclei with Acridine Orange Assay

Micronuclei were induced in the control and treated media at concentrations lower than those used for Giemsa assay (Table 11). The frequencies of MN by the three larvicides were not significantly different from the negative control ($P>0.05$) but in the benzene treatment (positive control), there was a significant difference in the frequency of MN when compared with the negative control. The patterns of induction of micronuclei in the exposed fish cells are well illustrated in Plate 8 and Plate 9. Micronucleus was found in mature cells with well bound cytoplasm (Plate 8) and immature cells without cytoplasmic membrane (Plate 9).

4.4.4. Ultrastructural responses in *Poecilia reticulata*

Cell organelles were intact in the control group as in mitochondria with distinct cristae and well defined matrixes. In addition, the cytoplasm and nuclear membrane were with well-defined nucleus and one eccentric nucleolus (Plates 10, 13, 16, 19, 22 and 25). In all the treatment cells except with spinosad at 49 μgL^{-1} (Plate 11 & Plate 14), there were marked damages that became more severe with increasing concentration of the larvicides. These damages included the presence of pycnotic nuclei and rupture of lysosome (Plate 12); the presence of electron dense cytoplasm (Plate 15); disintegration of cytoplasm and cytoplasmic inclusions (Plates 12, 15, 17, 18, 23, 24, 26 and 27); loss of grey areas in the cell membrane particularly in the organophosphorus treatments (17, 18, 20, 21 and 27); shriveled cytoplasm with dead nuclei especially with chlorpyrifos treatment (Plates 17, 18, 23, 24 and 27). Necrosis of nucleus and mitochondria cells with fewer cristae when compared with the control were also common in the organophosphorus treatments (Plates 18, 20, 21, 26, and 27).

Table 10: Behavioral responses in *Poecilia reticulata* exposed to lowered concentrations of larvicides

Days	Behavioural symptoms/ Morphological symptoms	Control	Chlorpyrifos (µg/L)			Actellic (µg/L)			Spinosad (µg/L)		
			0.4	0.6	0.8	18	30	50	49	73	110
1	Hyperactivity										
	Abnormal lateral flexure										
3	Hatching							[c]X			[c]X
	Hypoactivity										X
7	Haemorrhage				[b]X						
14	Abnormal lateral flexure			X							
	Loss of equilibrium			X	X						
	Mortality			X	X						
	Pectoral fin forward			X	X						
	Haemorrhage		[a,b]X	[a,b]X	[a,b]X						
	Hypoactivity			X	X			X			
	Reduced feeding		X	X	X						X
	Scoliosis				X						
15	Loss of equilibrium				X						
	Reduced feeding			X	X			X			
	Pectoral fin forward			X	X						
	Hypoactivity			X	X			X			
16	Scoliosis			X							
	Loss of equilibrium				X						
	Haemorrhage				[a,b]X						
	Pectoral fin forward				X						
	Hypoactivity				X						
	Reduced feeding				X						X
	Lordosis				X						
	Mortality				X						
17	Mortality		X	X	X						
	Hatching				[c]X						
	Lordosis				X						
20	Mortality			[a]X	[d]X						
	Lordosis			X	X						
21	Reduced feeding			X	X						
	Hatching	[c]X									
22	Mortality			[a]X	X						
	Reduced feeding			X	X						
23	Reduced feeding			X	X						
	Mortality			[a]X							
24	Reduced feeding			X	X			X			
	Mortality				X						
27	Reduced feeding			X	X						
	Mortality			[a]X	X						

[a]Male fish; [b]Operculum region; [c]Gravid Female; [d]Fries

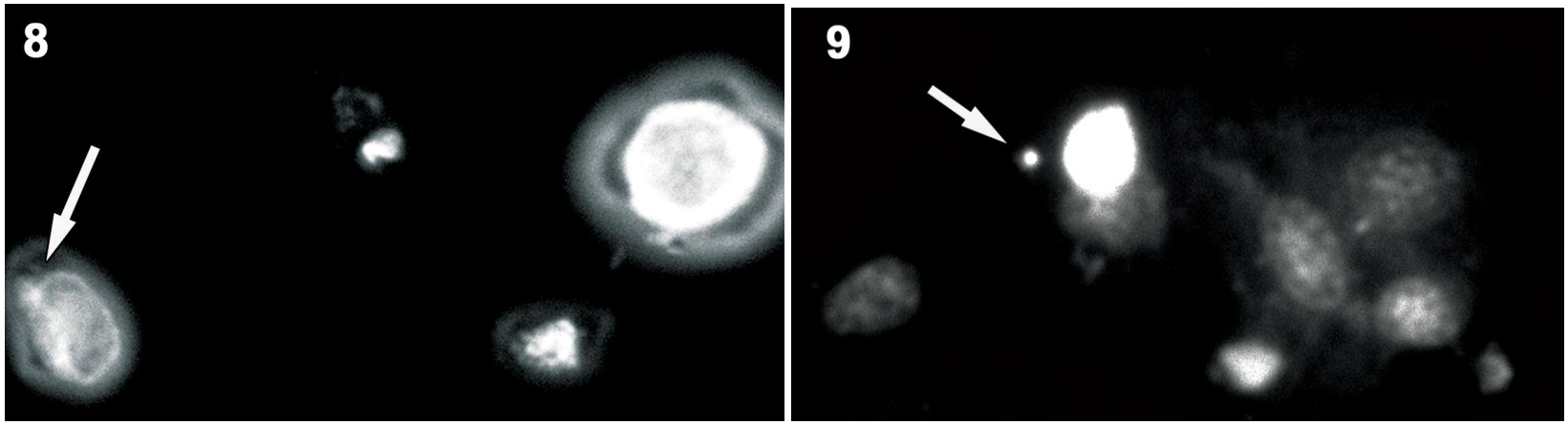
NB: Responses were recorded if they differed from control and occurred in ≥10% of the fish within each test chamber.

Table 11: Frequency of Micronuclei in *Poecilia reticulata* at lowered concentrations

Day	Treatment	Concentration (μgL^{-1})	Micronucleus (Mean ± SE)
3	Negative control	0	0.33 ± 0.33
	Positive control	0.8	5.00 ± 1.00^{o}
	Actellic	18	0.00 ± 0.00^{ns}
		50	1.00 ± 0.58^{ns}
	Chlorpyrifos	0.4	1.00 ± 1.00^{ns}
		0.8	2.67 ± 1.53^{ns}
	Spinosad	49	0.00 ± 0.00^{ns}
		110	1.67 ± 0.88^{ns}
14	Negative control	0	0.00 ± 0.00
	Positive control	0.8	5.33 ± 1.86^{o}
	Actellic	18	0.00 ± 0.00^{ns}
		50	0.33 ± 0.33^{ns}
	Chlorpyrifos	0.4	0.67 ± 0.67^{ns}
		0.8	1.33 ± 0.67^{ns}
	Spinosad	49	0.00 ± 0.00^{ns}
		110	0.33 ± 0.33^{ns}

$^{o}P<0.05$
ns = not significant at P=0.05

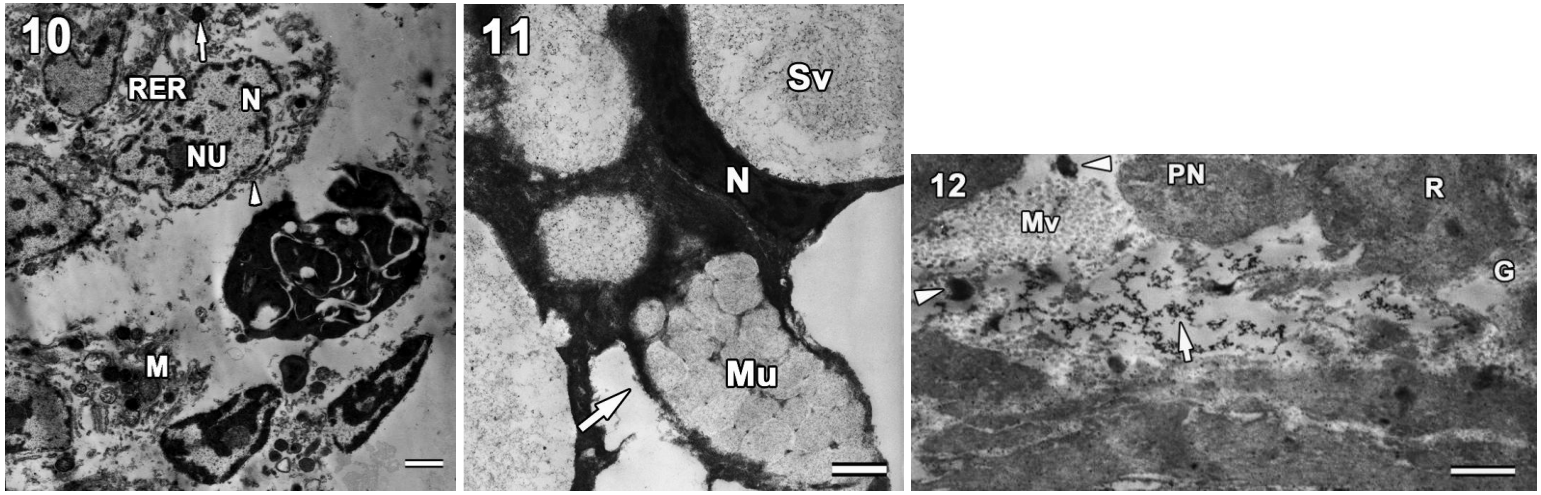
INDUCTION OF MICRONUCLEI CELLS IN *POECILIA RETICULATA* USING ACRIDINE ORANGE ASSAY



Plates 8 & 9: Acridine orange stained gill cells *of P. reticulata* observed with BX51 flourview microscope at 63x/1.4 oil immersion **(8):** Mature cell (NCE) with micronucleus (arrow). **(9**): Immature (PCE) cell with micronucleus (arrow).

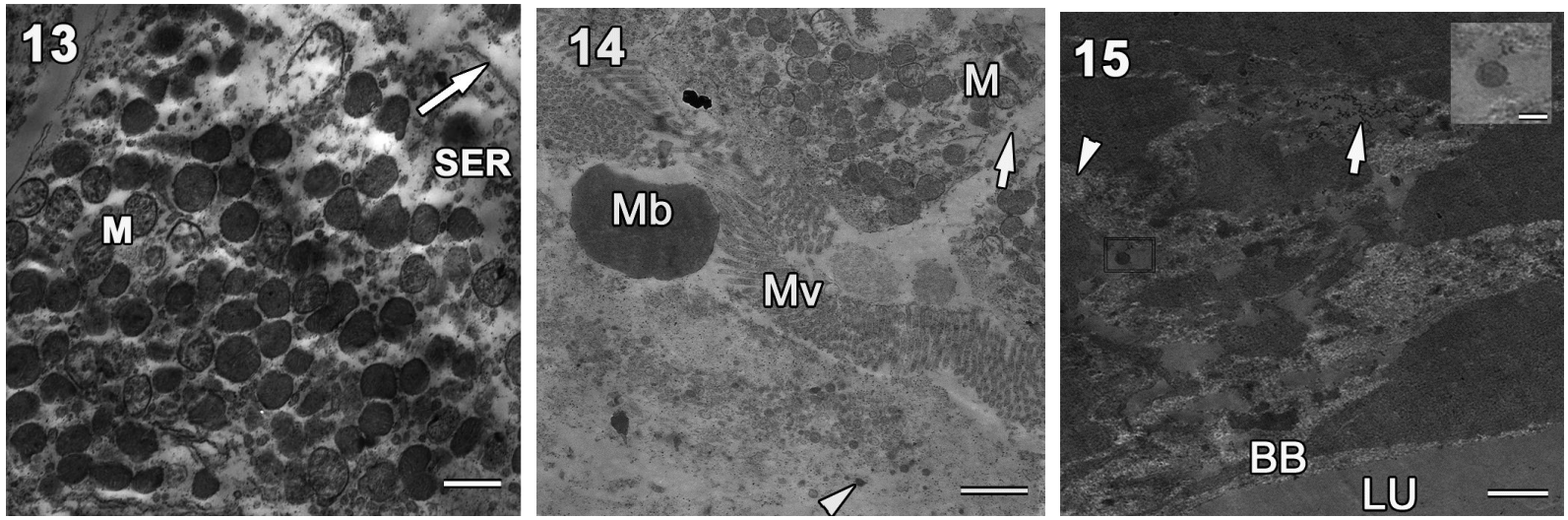
TEM OF *POECILIA RETICULATA* CELLS WITH REPECT TO TREATMENTS AND CONCENTRATIONS

4.4.4.1. Guppy Nuclei cells exposed to Spinosad



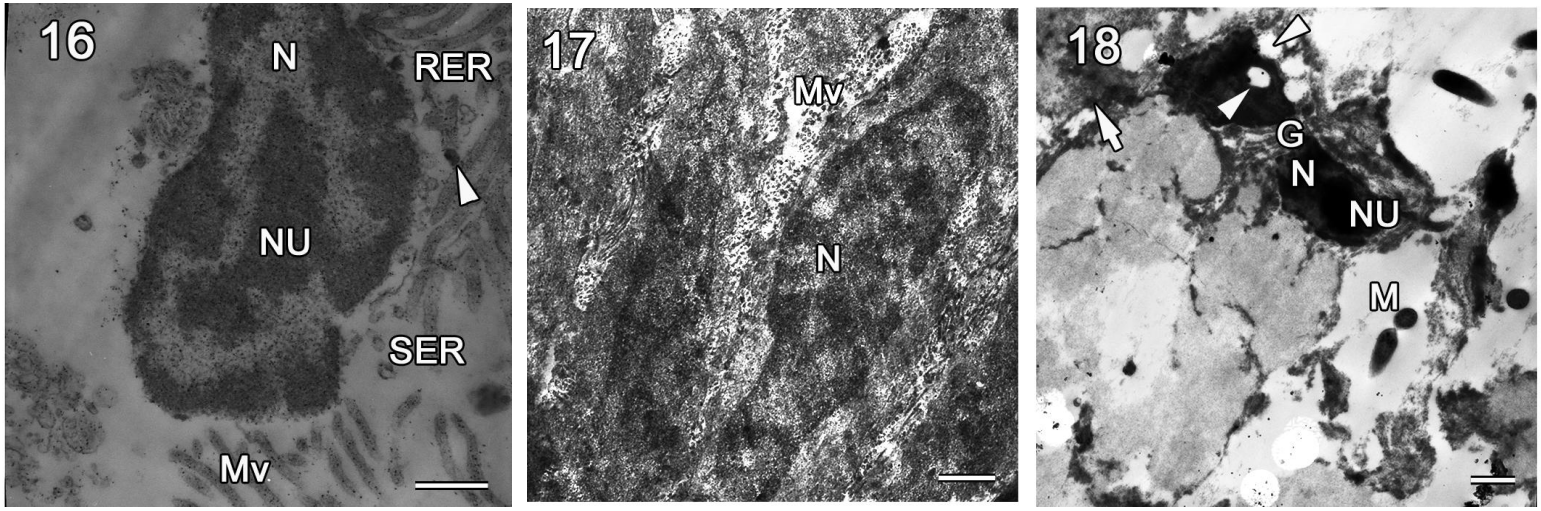
Plates 10-12: TEM of *Poecilia reticulata* showing the nuclear cells of control fish and those exposed to Spinosad larvicide. (**10)**: Control fish with intact Nucleus (N); Nucleolus (NU). Mitochondria (M); Lysosome (arrow); Rough endoplasmic reticulum (RER); Smooth endoplasmic reticulum (arrow head), **scale bar = 1µm**. (**11)**: At 49µgL^{-1} no marked damage observed. Nucleus (N); Secretory vesicles (Sv), Mucin (Mu), **scale bar = 1µm**. **(12):** At higher concentration of 110µgL^{-1} severe damage was seen, Pycnotic nucleus (PN), signs of rupture in lysosome (arrow heads), disintegration of cytoplasmic inclusions (arrow), Golgi body (G), Microvilli (Mv), Ribosomes (R), **scale bar = 0.5µm**.

4.4.4.2. Guppy Mitochondria cells exposed to Spinosad



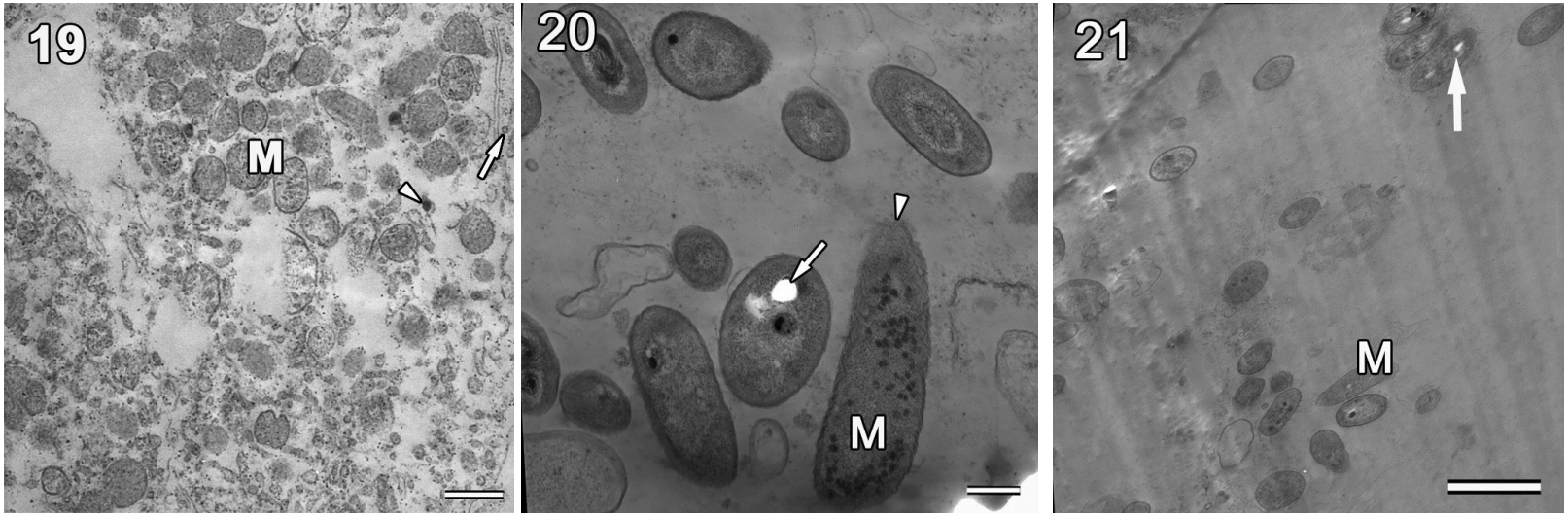
Plates 13-15: TEM of mitochondrial cells of control fish and exposed fish under spinosad treatment. **13**: Control fish showing Mitochondria (M), Smooth endoplasmic reticulum (SER); Rough endoplasmic reticulum (arrow), **scale bar =0.5µm**. **14**: At 49µgL^{-1}no marked difference from control. Intact cristae in Mitochondria (M), Microbodies (Mb), Microvilli (Mv), Smooth endoplasmic reticulum (arrow), Lysosome (arrow head), **scale bar = 1µm**. **15:** At the highest concentration of 110µgL^{-1}, there was marked degradation of the electron dense cytoplasm (arrow), mitochondrion with few cristae (inset), Brush border (BB), Lumen (LU), Microvilli (arrow head), **scale bars = 1µm** and 0.25µm respectively.

4.4.4.3. Guppy Nuclei cells exposed to Actellic



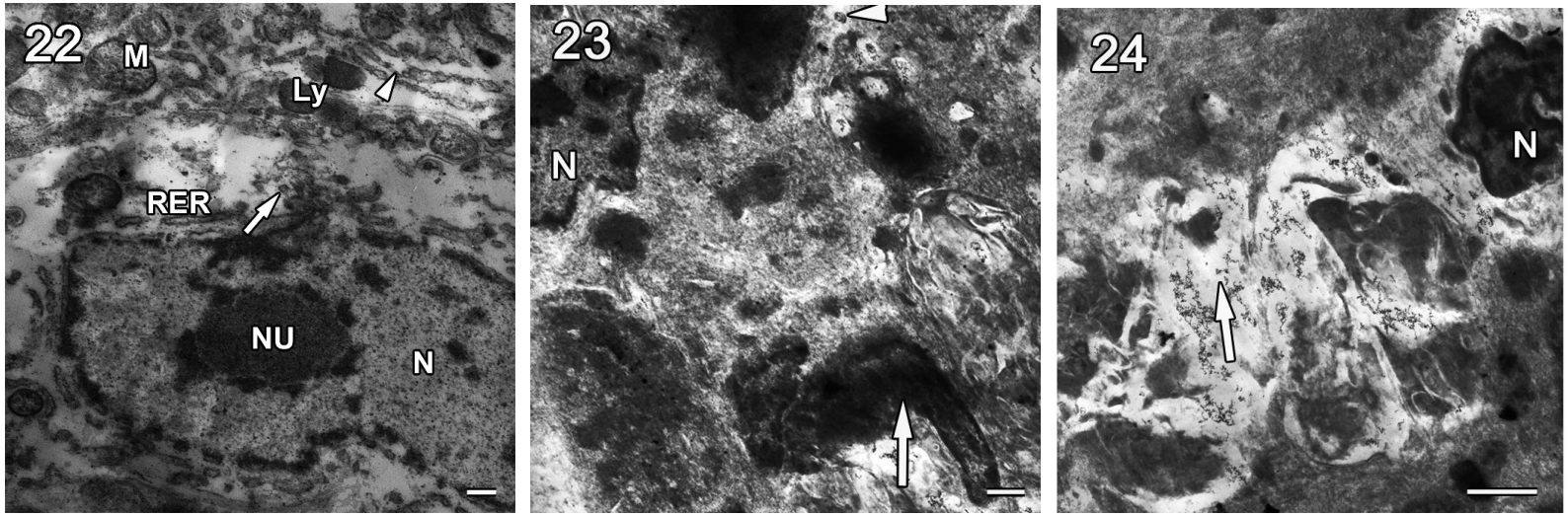
Plates 16-18: TEM of nuclear cells of control and exposed *Poecilia reticulata* under Actellic treatment.**16**: Control fish showing intact Nucleus (N) with a single eccentric Nucleolus (NU); Rough Endoplasmic Reticulum (RER); Lysosome (arrow head); Microvilli (Mv), **scale bar 250nm**. **17:** At lower concentration of 18µgL^{-1} there were marked damages, Nucleus (N). Microvilli (Mv), **scale bar 0.5µm**. **18**: At higher concentration of 50µgL^{-1}, necrosis in the Nucleus (arrow head), shriveled Nuclei (N) and Nucleolus (NU), Golgi body (G), Mitochondrion (M), Lipid (arrow) **scale bar 1µm**.

4.4.4.4. Guppy Mitochondria cells exposed to Actellic



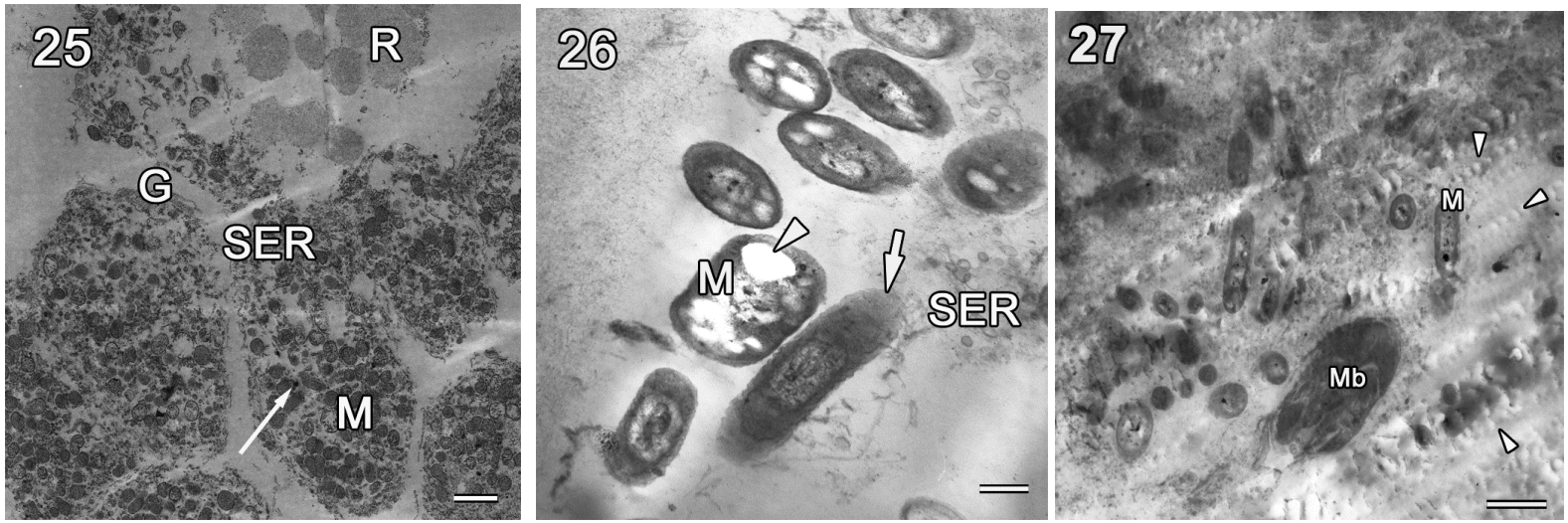
Plates 19-21: TEM of mitochondria cells of control and exposed *P. reticulata* under Actellic treatment.**19**: Control fish with distinct cristae in mitochondria (M); Smooth endoplasmic reticulum (arrow); Lysosome (arrow head); **scale bar = 0.5µm**.**20:** At 18µgL^{-1} features included swollen mitochondria (M) with gradual loss of cristae, signs of rupture in mitochondria membrane (arrow head), lesion on mitochondria (arrow) **scale bar = 0.25µm**. **21**: At 50µgL^{-1}, there was shrunken cell membrane characterized by increased lesions on mitochondria cells (arrow); loss of cristae in mitochondria (M), **scale bar = 1µm**.

4.4.4.5. Guppy nuclei cells exposed to chlorpyrifos



Plates 22-24: TEM of nuclear cells of control and exposed *P. reticulata* under Chlorpyrifos treatment. **22**: control fish with intact cytoplasmic and nuclear membrane. Well centralized nucleolus (NU) in nucleus (N); Distinct cristae in mitochondria (M); Smooth endoplasmic reticulum (arrow); Rough endoplasmic reticulum (RER); lysosome (Ly); Golgi body (arrow head),Scale bar 250nm. **23**: At 0.4μgL^{-1} features included marked damage of cytoplasmic membrane and nucleic components (arrow), shriveled and dying nuclei (N), scale bar 1μm **24:** Severe distortion of the cytoplasmic membrane was seen at higher concentration of 0.8μgL^{-1} characterized by a total damage of the nucleus (N), loss of grey area in cytosol and accompanying organelles (arrow). Scale bar 0.5μm.

4.4.4.6. Guppy Mitochondria cells exposed to chlorpyrifos



Plates 25-27: TEM of mitochondria cells for control and exposed *Poecilia reticulata* under Chlorpyrifos treatment.**25:** control fish with intact organelles. Mitochondria (M); Smooth endoplasmic reticulum (SER); Lysosome (arrow); Golgi body (G), Ribosomes (R), **scale bar = 1µm**. **26:** At 0.4µgL^{-1}, there was marked necrosis on the mitochondria (arrow head) with sign of rupture (arrow) **scale bar = 0.25µm**. **27**: At 0.8µgL^{-1} there was general shrinkage of cell membrane (arrow), Mitochondria (M), Microbody (Mb), Cell proliferation (arrow heads) **scale bar =1µm**.

4.5 INTEGRATING *POECILIA RETICULATA* AND LARVICIDES AGAINST *CULEX* MOSQUITOES.

At predator-prey density of 1 fish to 35 culex mosquitoes, the consumption of *C. quinquefasciatus* by exposed *P. reticulata* significantly decreased ($P<0.05$) mostly at the higher concentrations of actellic and chlorpyrifos whereas feeding in the fish significantly increased ($P<0.05$) in all the concentrations of spinosad treatment when compared with control (Figure 15; Appendix 15).

Figure 16 and Appendix 16 show that the consumption of culex larvae by guppy at predator-prey density of 5:70, resulted in a significant decrease with chlorpyrifos treatment ($P<0.05$) but increased significantly at the lowest concentration of actellic and in all the concentrations of spinosad.

At the highest predator-prey density of 10:350, the consumption of *Culex* mosquitoes generally increased in the presence of the three types of larvicides however, this was significant ($P<0.05$) only with the lowest concentration of actellic and in all spinosad concentrations (Figure 17; Appendix 17).

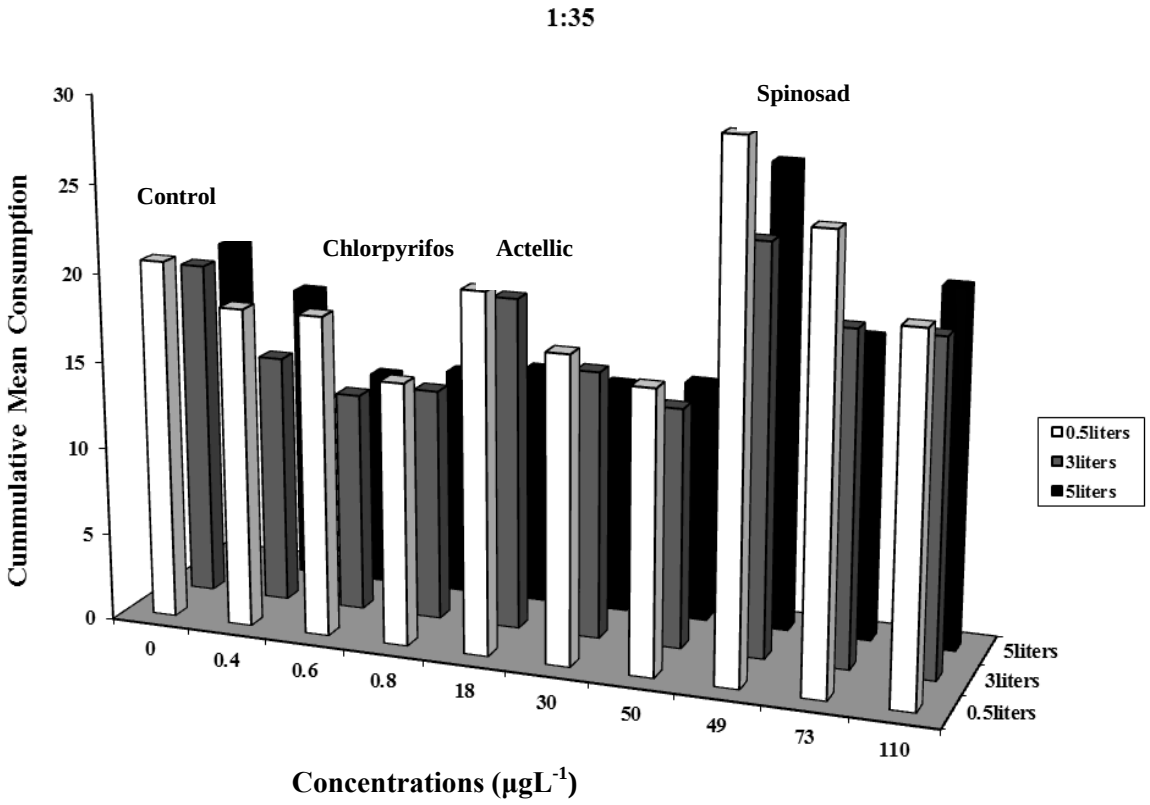


Figure 15: Feeding pattern of *Poecilia reticulata* at 1:35 predator-prey density

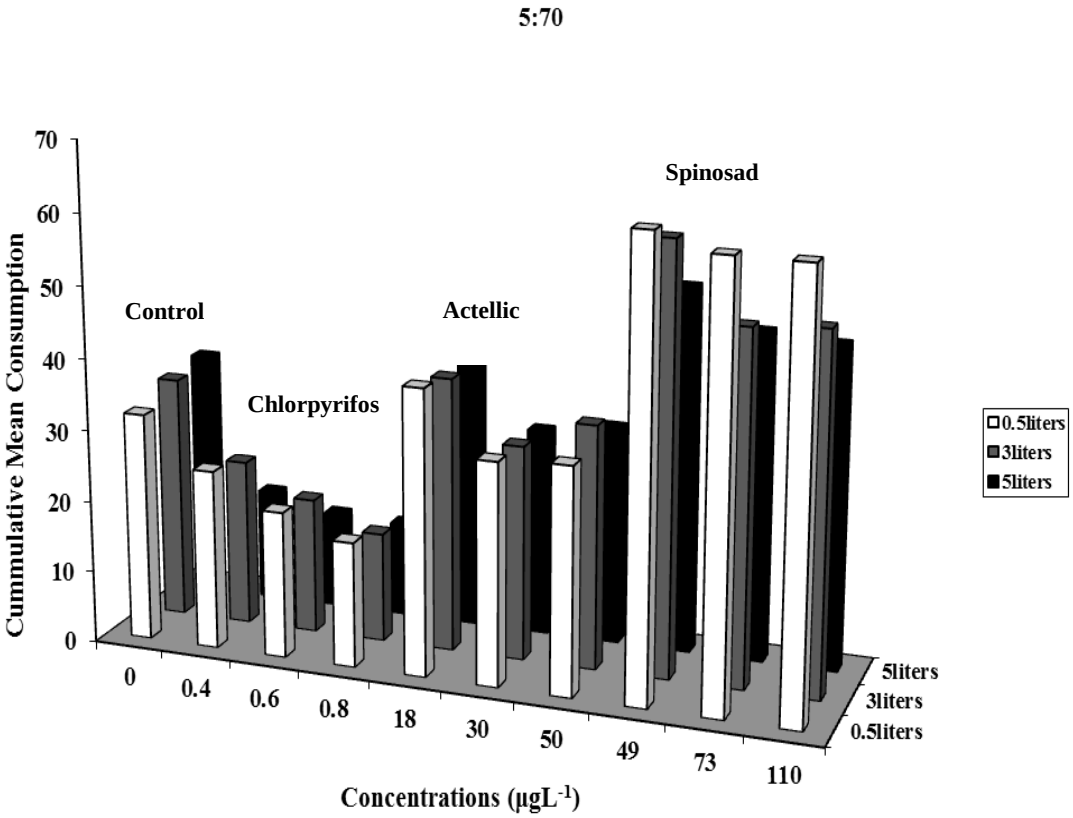


Figure 16: Feeding pattern of *Poecilia reticulata* at 5:70 predator-prey density

10:350

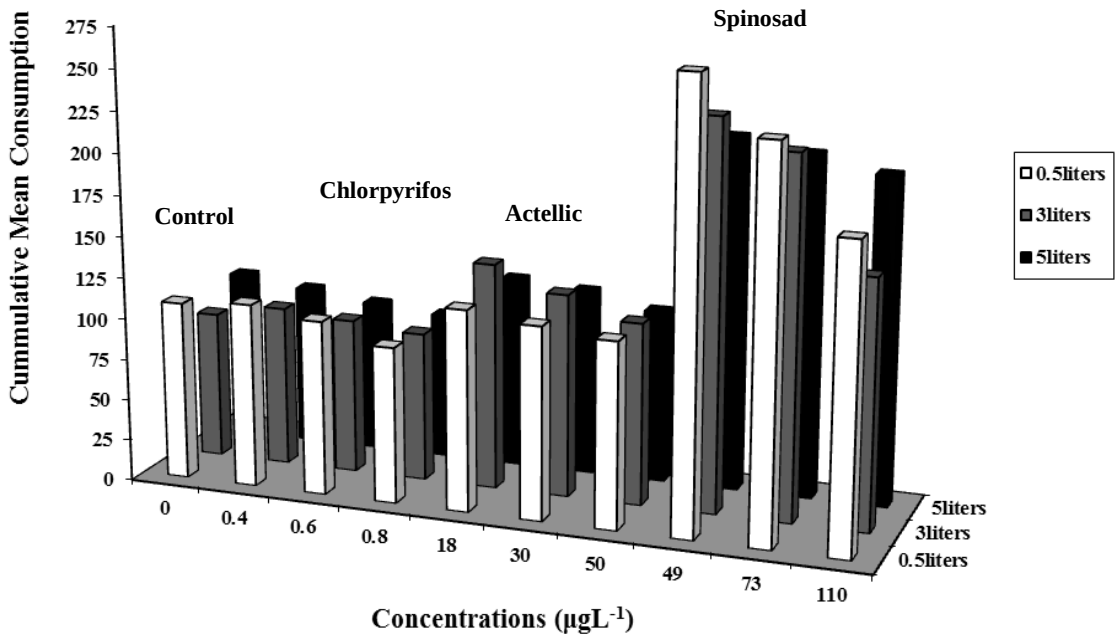


Figure 17: Feeding pattern of *Poecilia reticulata* at 10:350 predator-prey density.

CHAPTER FIVE

DISCUSSION

5.1. PREDATOR PREY DENSITY OF *POECILIA RETICULATA* ON MOSQUITO LARVAE

The ability of guppies to consume mosquito larvae is well documented. In this study, one guppy was able to consume 30 mosquito larvae species within 24 h similar to reports from earlier researchers. Seng *et al*., (2008), reported that a guppy ate an average of 102 larvae per day in a community based intervention study at Cambodia. In Bangladesh, Elias *et al.*, (1995), reported that a guppy ate an approximate of 41 *Culex sp.* larvae per day. In another laboratory study, Dua *et al*., 2007, reported the mean consumption of *C. quinquefasciatus* larvae per day by one guppy to be 23.8 ± 3.5. The feeding estimate obtained in the present study will assist in the extrapolation of the fish population to apply in the control mosquito larvae in a particular aquatic medium, and during fish re-stocking.

The consumption of mosquito larvae by *P. reticulata* increased with increasing predator-prey density but at certain predator densities, there were either a decline in feeding with increasing population of mosquito larvae or an exponential growth in feeding. The decline in feeding that was observed at predator densities of 1 and 2 indicated that the fish had attained satiation while the exponential growth in feeding at higher densities implied that the fish species had not been satiated. The establishment of the various points at which a set of population of guppy attained satiation within a 24 h feeding

bout shows the need for a right feeding estimate between guppy and mosquito larvae prior to embarking on a mosquito larval control programme particularly that involving the use of fish as a biological control component. It is of utmost importance to consider the predator-prey feeding relationship between guppy and mosquito larvae species so as to prevent the underutilization of the fish larvivorus potentials or avoid creating a situation where the fish species are being overwhelmed by the presence of an enormous population of mosquito larvae in a control medium. Understanding this relationship will also help to explain the time lag between fish satiation and the eventual resumption of feeding in the fish that will reduce the quick escape of mosquito larvae species to other developmental stages as was observed at the feeding densities where fish satiation was attained. However, further research is needed in this area to ascertain how frequently guppies are satiated in the field so as to assure its proper functioning as bio-control agent when applied alone or as a component of integrated control of mosquito larvae in natural aquatic ecosystems. Earlier researchers have demonstrated that other factors such as drain pollution, sex of the fish and the presence of alternative preys were capable of affecting guppy consumption pattern (Dua *et al*., 2007; Manna *et al*., 2008; Seng *et al*., 2008; Saliu *et al*., 2011)

5.2. PREY PREFERENCE OF *POECILIA RETICULATA*

The selection pattern of *P. reticulata* showed that the fish prey of choice was *Chironomus* larvae however, the predator-prey interactions clearly revealed a simultaneous consumption of *Chironomids* and mosquito larvae species. Considering this fact then, the prey choice of *P. reticulata* may be unconnected to its ability to capture the more

vulnerable prey species. It is therefore unlikely that the choice for *Chironomid sp*. was associated with taste or mere preference for the larvae species as reported in a study by Manna *et al*., (2007). In a heavily mosquito infested drainage, which is often the situation in the field, the capture success of *P. reticulata* may be enhanced due to overcrowded larvae, however a right guppy-mosquito density must be introduced so as not to overwhelm the fish. The great abundance of the prey of choice, chironomid larvae compared to mosquito larvae in the analyzed water sample is an indicator that supports these assumptions.

The presence of alternative preys is advantageous in that it allows the sustenance of guppy species in the absence of the target mosquito larvae but in the case when the preference for alternative prey forms is high compared to co-occurring target mosquito larvae, the regulation of the mosquito species by guppy fish alone may fail however, in a support control such as inherent in this study, mosquito larvae population are reduced by the simultaneous actions of a biological control agent and larvicides. The indirect effects of guppies exposed to alternative preys have also been reported by other authors (Bence, 1988; Blaustein and Karban., 1990; Blaustein, 1992; Dua *et al*., 2007; Manna *et al*., 2008). Lundkvist *et al*., (2003) and Kumar and Rao, (2003) reported that predators like some dysticid beetles and copepods showed positive selectivity of mosquito larvae against other prey forms unlike what was obtained with guppies in this study. Unfortunately, the use of these organisms as bio-control agents on a large scale remains controversial (USAID, 2006).

5.3. MEAN LETHAL CONCENTRATION OF LARVICIDES ON MOSQUITO LARVAE AND *POECILIA RETICULATA*

The report from this study undermines the choice of chlorpyrifos as a candidate larvicide for integrated larval control since the compound remained the most toxic insecticide on the non-target guppy fish. The LC_{50} value of 4.23 μgL^{-1} obtained for chlorpyrifos in this study was lower than those by earlier workers. Selvi *et al*., (2005) reported a 96 h acute LC_{50} value of 1.79mg/L of technical chlorpyrifos methyl against male guppies. In another study, Rice *et al*., (1997) reported a value of 0.30mg/L and 0.25mg/L under 24 h and 46 h exposure periods respectively of chlorpyrifos against 30-day-old Japanese medaka, *Oryzias latipes*. A range of 100 - 678μgL^{-1} have also been documented for other standard test species (CERC, 1986).

Poecilia reticulata was not susceptibility to spinosad compound at acute toxicity bioassay when compared to the organophosphates indicating the higher level of safety of this larvicide on the non-target species. Previous reports have equally shown that spinosad was safe to some non-target organisms (Thompson *et al*., 2000; Perez *et al*., 2002; Stebbins, 2002; Williams *et al*., 2003; WHO, 2005). Pest Management Regulatory Agency in (2001), reported the toxicity of spinosad to aquatic invertebrates including *Daphnia sp*, *Chironomid sp*, shrimps and mollusk, albeit, in comparism to an organophosphate, spinosad was 5 times less toxic to non- target species during continuous exposure studies (Stark & Vargas, 2003). Toxicity to fish by spinosad is classified as low or moderate with 96 h LC_{50} values between 5 and 30 p.p.m depending on the species (Thompson *et al*., 2000). The impact of spinosad on non-target aquatic organisms is still poorly understood (Bond *et al*., 2004) hence, future research on the com-

pound will necessitate an in-depth sub-chronic and chronic toxicity test on different fish species before a final recommendation is made on spinosad use for replacement of organophosphates in field mosquito larval control.

The values of the 50% mean lethal concentration of 73.06 μgL^{-1} and 59.34 μgL^{-1} obtained for *C. quinquefasciatus* and *Anopheles gambiae* respectively using spinosad larvicide compared favorably with those from other workers. Anthonio *et al*., (2008) estimated a 24 hLC_{50} value for spinosad against *Aedes aegypti* as 0.060mgAI/L (range of 95% confidence limits 0.045 – 0.079). The 24 h toxicity of two formulations of spinosad under different water resources against 3rd larval instar of *Culex pipens* was obtained by Baghat *et al.*, (2007) as 0.002 ppm and 0.007 ppm for liquid and dust formulations respectively and they concluded that the dust formulation had better initial kill on the *Culex* mosquitoes than the liquid form. Temarek (2003) reported that spinosad was not affected by the existing resistance mechanism common to conventional insecticides and that the naturally derived larvicide showed effectiveness against a range of mosquito species. Laboratory and field trials have also been carried out to further demonstrate the effectiveness of spinosad against various types of mosquitoes as well as, its non-repellency to mosquito oviposition presumably due to its distinctive aroma of damp earth, characteristic of the presence of *Actinomycetes*, that may prove attractive to gravid females (Bond *et al*. 2004; Perez *et al*., 2007).

Culex quinquefasciatus was found to show more tolerance to the test larvicides than *Anopheles gambiae* which did not agree with the works of Otitoloju & Don-Pedro (1997), who reported that *Anopheles sp.* was more tolerant to dieldrin and cypermethrin

in a laboratory bioassay. Nevertheless, the result from the present study was expected since immunity may have been conferred on the *Culex sp*. based on the nature of their natural habitat (gutters, sewages and open drains) which are usually prone to pollution. The higher tolerance by *C. quinquefasciatus* larvae is suggestive of the concentration of insecticide to be applied for mosquito control practices. The concentration capable of killing the more tolerant larvae species, in this case, the culex mosquitoes should be applied to ensure adequate control of other less tolerant species that may co-habit with it.

Despite the high mortality by chlorpyrifos on *C. quinquefasciatus* at acute toxicity study, the larvicide quickly lost its biological activity on the target mosquito species compared to actellic and spinosad. The implication of this is that though chlorpyrifos was the most effective larvicide against the mosquito larvae species, it is unlikely to sustain their control with time compared to actellic and spinosad. Spinosad was the least persistent on the non-target fish species indicating that this larvicide was compatible with the fish and is not likely to deter the functionality of the fish as a component of integrated mosquito larval control. On the other hand, the toxic effects by the organo-phosphates persisted on the guppy fish implying that these larvicides were not compati-ble to the fish and are not the best choices for integrated mosquito larviciding at the test concentrations in which they were applied.

The biological activity of the test larvicides on the mosquito species declined with time indicating the need for a more frequent site retreatment which may eventually lead to the accumulation of chemicals in water bodies affecting the non-target guppy fish.

However, with the determination of a compatible concentration and larvicide for use, marked damage on the biological control agent may be reduced or averted considering the fact that these larvicides easily lost their biological activity on the non-target fish species. Similar trends of decreased in toxicity level of test insecticides following increase in time of exposure as obtained in this study have been reported (WHO, 1986; WHO, 2005; Selvi *et al*., 2005; Perez *et al*., 2007).

The suitability of larvicidal compounds for use in insect vector control programmes depends on a variety of properties and characteristics including cuticular disposition to penetration by insecticide and enzymatic breakdown (Don-Pedro, 1989), the behavioral responses of insect vectors that come in contact with the compound and the persistence of the larvicide in the environment (Perez *et al*., 2007).

5.4. SUB-LETHAL TOXICITY ON *POECILIA RETICULATA*

5.4.1. Induction of Micronuclei and Nuclear abnormal cells with Giemsa Assay

Spinosad induced more MN in the fish gill cells than actellic which implies that spinosad inhibited cell division and thereby affected growth in the exposed guppy fish than actellic. The incidence of micronucleus (MN) in guppy exposed to actellic and spinosad increased with increasing concentration of each larvicide which corroborated with the findings by various researchers that used end points other than MN such as chromosome breakages on other organisms (Cohen and Shaw, 1964; Adler, 1973; Adler, 1974). Matter and Grunwiler, (1974); Kliesch *et al*., (1981); Das and Nanda, (1986) reported that MN increased with increasing concentration of the drug Mitomycin C in different laboratory bioassays. In a more recent study, Archipchuk and Garanko

(2005) reported similar elevated response to concentration increase on in vivo fish cells treated with copper and cadmium ions.

The total absence of MN in the control (untreated dechlorinated tap water) clearly indicated that the MN had certainly been induced by the test larvicides. This result was unexpected since *P. reticulata* are known to inhabit polluted drains and thus, are usually prone to contamination. It is possible that several mitotic divisions of the initially induced MN from drain pollutants were eventually lost as a result of the long period of laboratory rearing of the fish prior to bioassay.

Some variations existed in the time of induction of MN and NA cells in the fish. The elevated responses in the frequencies of MN that were found at the highest concentrations of the larvicides were most pronounced at days 3 and 14 for spinosad and actellic respectively. At their lowest concentrations however, damages from each larvicide were peaked on day 14. The explanation for this could be that actellic exerted a more lasting inhibitory effect on cell division than spinosad subsequently, hindering the passage of the affected cells into the tissue erythrocyte at that concentration. At the lowest concentration however, both larvicides expressed similar inhibitory effects on mitotic division in the affected cells.

Earlier researchers have demonstrated various time dependent MN responses. Campana *et al*., (1999), reported that at 15 days of exposure of *Cheiredon interruptus interrruptus* to a pyrethroid, the maximum response in MN frequency was found with the lowest dose. Cavas and Ergene-Gozukara (2005a) showed a significant increase in MN only at the highest concentrations and longest exposure duration on treating *Oreochromis*

niloticus with petroleum and chromium processing effluents. A gradual decrease in the frequency of MN with an increase in exposure periods as well as, at higher concentrations was also obtained by Das and Nanda (1986), who worked with paper mill effluents on a fish species.

The elevated response in the NA cells with the highest concentration of spinosad and actellic were most pronounced at days 1 and 14 respectively as against days 3 and 14 earlier obtained for MN induction. At their lowest concentrations however, peak damage were obtained on days 14 and 3 as against day 14 that was previously obtained with MN induction. The rapid response of NA cells as compared to those obtained from MN induction is indicative that NA cells were very prompt to detect negative influences emanating from these larvicides making it a better cytological biomarker than MN. Carlin and Dragonir, (1980); Archipchuk and Garanko, (2005) have also reported similar result upon the exposure of fish and rat cells to benzo(a)pyrene, N-acetylaminofluorene, cadmium and copper compounds.

Several studies have indicated that NAs were induced in response to exposure to genotoxic agents (Tolbert *et al*., 1992; Serrano-Garcia and Montero-Montoyo, 2001). The pattern of induction of nuclear abnormality (NAs) in organisms is controversial depending on the genetic system or assay used (Campana *et al*., 1999). Cavas and Ergene-Gozukara (2005a) reported an insignificant increase in NAs in *Oreochromis niloticus* exposed to chromium effluent. In another study, they observed the repression of all the analyzed Nucleolar parameters in fin cells of a fish species exposed to lambda-cyhalothrin (Cavas and Ergene-Gozukara, 2003a). In this study, the cumulative analysis

of the NAs (PCE & BN) induced by the larvicides showed an insignificant increase from the control experiment indicating that these compounds were not cytotoxic to the fish.

The mechanism underlying the formation of NAs is not fully understood although; they may result from problems segregating tangled and attached chromosomes (Cavas and Ergene-Gozukara 2005a). It has also been suggested that gene amplification via the breakage-fusion-bridge cycle could cause NAs like lobed nuclei and blebbed nuclei during the elimination of amplified DNA from the nucleus (Shimizu *et al*., 1998).

Polychromatic cells (PCE) were quicker to respond to the effects from the larvicides compared to binucleated cells (BN) particularly with spinosad compound. Treatment with actellic showed an insignificant increase in PCE except at its lowest concentration. The higher concentrations of actellic probably caused cell mortality. Cavas and Ergene-Gozukara (2005a) have reported similar response with the frequency of binucleated cells (BN) at the lowest and highest concentrations of chromium effluents. Consequently, the induction of PCE was faster at the lowest concentration of actellic compared to MN. This is an indication that PCE was indeed very sensitive even at its lowest concentration thus, agreed with earlier findings that NAs were quicker end points to the impacts from chemicals than MN (Cavas and Ergene-Gozukara, 2003a; Cavas and Ergene-Gozukara 2005a; Arkhipchuk and Garanko, 2005).

The repression of normal cells was observed in both control and treatment group, though the result was insignificantly different from each other. However, the decrease in response was more in the treatment group than in the control indicating that the larvi-

cides were definitely responsible for the repressive activity found in the treated cells. This was further supported by the observed peak periods of NCE repression that coincided with the periods of change of bioassay. The repressive response observed in the control may be associated with external stress factors including fish handling and the laboratory conditions in which the fish were subjected to.

As a possible parameter of mutagen-induced cytotoxicity, the ratio of PCE to NCE in the fish gill cells was assessed and showed a significant increase with relation to concentration and time. This infers that at the test concentrations, the larvicides were not mutagenic to *P. reticulata.* PCE/NCE ratio is a key component of cytotoxicity assessment routinely included in micronucleus tests with mammalian test organisms (Criswell *et al*., 1998; Celik *et al*., 2003). However, there is a dearth of information on the combination of PCE/NCE ratio with micronucleus in fish toxicity tests (Cavas and Ergene-Gozukara, 2005b). Pacheco and Santos, (2002) reported that PCE frequencies in peripheral blood of *Anguilla anguilla* decreased while erythrocyte micronucleus frequencies increased as a result of Benzo[a]pyrene, dehydroabietic acid and bleached kraft paper mill effluent treatments. This study has demonstrated the combination of PCE/NCE ratio with micronucleus and nuclear abnormal cells in *P. reticulata* gill cells exposed to spinosad, actellic and chlorpyrifos larvicides contributing immensely to the dearth of information in this area of research.

5.4.2. Induction of Micronuclei cells with Acridine Orange Assay

The three larvicides failed to significantly induce micronuclei in the fish cell at lower concentration than those used under the Giemsa protocol hence, did not overtly reduce

growth activity in the fish as was obtained with the Giemsa stain. This is implicative that concentration is a very important parameter in the toxicity of chemical to aquatic non-target organisms and must be highly considered during larvicidal practices.

The application of AO protocol is one improvement over Giemsa assay in detecting micronuclei in organisms (Hayash *et al*., 1990; Hayashi *et al*., 1998; Polard *et al*., 2011; Nersesyan *et al*., 2006; Oliveira-Martins and Grisolia., 2007). Acridine Orange is a nucleic-acid-selective fluorescent cationic dye that emits green light (525nm) when excited and only if bound to DNA. Thus, no ambiguous artefacts may confuse the observer in AO stained slides unlike in the use of Giemsa (Polard *et al*., 2011). As a consequence, the scoring is faster and more reliable (Hayashi *et al.,* 1983). The higher frequency of MN observed under the Giemsa protocol may be as a result of ambiguous artifacts stained along with the nuclei since Giemsa is a non-specific staining agent. This finding corroborated with that of Polard *et al*., (2011) and goes to support previous reports that fluorescent AO staining is better adapted to piscine erythrocytes MN assay than classical Giemsa staining (Hayashi *et al.,* 1983; Cavas, 2008; Polard *et al*., 2011). The baseline MN measured under the acridine orange protocol are consistent with results from Cavas and Ergene-Gozukara, (2005b); Cavas, (2008); Polard *et al*., (2011).

At this stage, the present study had confirmed that the three larvicides were not cytotoxic but genotoxic to *P. reticulata* at sublethal concentrations however, at further reduction in concentration, the compounds failed to inhibit growth in the fish evidenced by their inability to significantly induce MN in the fish gill cells using the more specific Acridine orange bioassay. It was then necessary to further investigate the impacts of

these larvicides at these reduced concentrations on the non-target biological component of the integrated control using additional biomarkers including behavioural and ultra-structural responses that will finally assist the choice of a compatible larvicide to guppy for integrated larval control.

5.4.3. Behavioral Responses in *Poecilia reticulata*

Spinosad compound has again proven to be the safest of the three types of larvicides used in this study following the results obtained from the behavioural analysis. The behavioural responses observed with spinosad was not different from that of the control implying that this larvicide was compatible with the fish unlike in the organophospho-rus treatments where marked responses different from the control were recorded mostly as haemorrhage of the operculum, reduced feeding and hypoactivity particularly with chlorpyrifos. The initial hypoactivity caused by spinosad treatment on guppy was short lived as against that observed in the organophosphates. This was evidenced by the initial slow movement in the fish which soon after, rebounced back to life resuming its feeding activity just as in the control thus, indicating the negligible effect of the compound on guppy making it the best choice of larvicides for use in integrated larval control compared to actellic and chlorpyrifos. From the aforementioned then, it be-comes more logical to first expose the fishes to the larvicides few moments before their introduction into the test media so as to reduce the larvicidal impact on the fish and al-low the quick resumption of feeding in the fish. This will help to ensure adequate con-trol of the mosquito larvae especially those that escaped the initial toxic effect of the larvicides.

Reduction in feeding was a response that was common to the three larvicides unlike haemorrhage of the operculum which was specific to chlorpyrifos alone. The implication of this is that haemorrhage of the operculum can probably serve as a diagnostic tool for assessing the behavioral response of fish species exposed to chlorpyrifos compound as this response was peculiar to the compound alone however, more research is needed to validate this assumption. Distinct behavioural responses in fish species such as heamorrhage and scoliosis in the caudal region as well as, forward movement of pectoral fin have also been associated with chlorpyrifos treatment (Rice *et. al*, 1997).

The reduced feeding in the fish was probably associated with the damages observed in the gastrointestinal cells of *P. reticulata* exposed to the various larvicides during the ultrastructural analysis. The different responses that were recorded in this study were consistent with the findings of the following researchers: Drummond *et al*., (1986); Jarvinen *et al*., (1988); Halcombe *et al*., (1982); Coats, (1990) and Rice *et al*., (1997).

The three larvicides did not inhibit hatching at their highest concentration but seemed to possess the ability to induce premature birth in the fish. This was true because hatching occurred earlier in the treated fish unlike in the control where hatching occurred at a time close to the normal 28days gestation period for guppy. The non-inhibition of hatching by these larvicides at the tested concentrations indicated their inability to affect the reproductive potentials of the fish which is important for the fish as agent of biological control. However, there is a strong indication that these larvicides may cause premature birth in the fish. Premature offspring are likely to possess unfit traits that can

eventually impair the predatory ability of *P. reticulata* thereby resulting in mosquito control failure.

Behavioural monitoring is a promising diagnostic tool for screening and differentiating chemicals according to their mode of action (Drummond *et al*., 1986). From this study, there appears to be a strong correlation between concentration and intoxication in *P. reticulata* and this supports the common adage that "Nothing in itself is poisonous only dosage determines poison".

5.4.4. Ultrastructural Responses in *Poecilia reticulata*

The ultrastructural analysis in this study showed that exposure of guppy to the test larvicides has potential side effects that are concentration dependent. Apart from the gills, the intestinal mucosa is the major port of entry for xenobiotic and many pathogenic agents. Because of this challenge, it serves to prevent damage to the gastrointestinal tract from foreign chemicals or organisms by the desquamation of its intestinal epithelium mucus (Sorensen, 1991). But in the case of continuous or high exposures of the gut to foreign chemicals, there may be severe impairment of the fish immunity, maturation and basic functionality particularly as predatory agent. Usually, this damage begins as molecular malfunction within specific organelles (Wayne *et al*., 2009) and may gradually progress into a disease or chronic disorder.

The marked damages observed in this study especially at higher concentrations of the larvicides were obviously signs of stress from the bio-larvicide and corroborated with earlier ultrastructural toxicity reports by various pesticides (Ba-Omar *et al*., 2011; Al-Ghanbousi *et al*., 2011).

Some of the marked damage observed particularly lysosomonal defects may have impaired digestion and recycling of cellular components that are no longer needed in the fish and this probably was responsible for the reduced feeding observed in the fish. There are over 40 heritable lysosomonal storage diseases known, each characterized by the harmful accumulation of a specific substance or class of substances commonly poly-saccharides or lipids that would normally be catabolized by the hydrolytic enzymes present within the lysosome or transported out of the lysosome (Wayne *et al*., 2009).

Spinosad at 49 μgL^{-1} showed no marked difference from the control but for the presence of large secretory vesicles and mucus cells that were not present in the control. These were likely to be initial protective responses by the fish to the impacts from the bio-larvicides. Al-Ghanbousi *et al*., (2011) showed the hyper production of mucus in *Aphanius dispar* following exposure to low concentration of deltamethrin. Another but similar report, demonstrated the increase of secretory vesicles in the gill of *A. dispar* upon exposure to temephos (Ba-Omar *et al*., 2011). Researches have suggested that mucus secretion by gills and intestines play a major role in the protection of these tissues from environmental impacts of xenobiotics (Sorensen, 1991; Pawert *et al*., 1998; Matey *et al*., 2008) however, it is likely that under high concentration or continuous exposures to the larvicides this protective ability in the fish may become compromised hence the need to apply low concentrations of larvicides with integrated approach for effective larval control.

Additionally, the disappearance of the nucleolus and the elongation of the nucleus under the lowest concentration of spinosad probably suggested that *P. reticulata* was undergo-

ing cell division implying that spinosad at 49 μgL^{-1} did not inhibit growth in the fish which was similar to the observation made under AO assay. The use of a suite of biomarkers is indeed necessary to make final decision on the toxicity or safety of any chemical on non-target species. The lower concentration of spinosad (49 μgL^{-1}) seems to be the threshold above which severe harm occurred in the fish organelles hence, this concentration or below it are suggestive of the dosage solution for field larviciding.

The morphological damages caused by actellic and chlorpyrifos were similar and indicated the impact of these larvicides on *P. reticulata*. This was generally characterized by shriveled cytoplasm with organelles including Golgi body that is known to be responsible for the transportation of liquid in cells (Wayne *et al.*, 2009), and cell necrosis particularly in chlorpyrifos treatment. The formation of necrosis and epithelial cell rupture are signs of hypoxia and respiratory failures in organisms as reported by Richmond and Dutta, (1989).

In this study, there were few cristae and rupture of mitochondria matrixes particularly in the chlorpyrifos treatment. The cristae are sites of oxidative phosphorylation and electron transport while the matrixes are sites of Kreb cycle enzymes (Taylor *et al.*, 1997). The damage to these components may explain the presence of the necrosis and cell rupture that was observed in the fish which strongly implies that guppy respiratory system was impaired at these concentrations of larvicides. Though damages were found in the gastrointestinal tract of guppy, it had not reached a threshold that can cause fish mortality. It is however assumed that with continuous exposure to the larvicides, fish mortality may begin to occur.

Spinosad is a nicotinic poison and this was reflected in the various damages in the fish which manifested mainly as cell swelling and rupture without due consideration on the respiratory system of the fish. In this study, the adverse damage by spinosad was found within a range of concentration that was previously recommended for field mosquito larviciding by Hertlein *et al*., (2010). It is therefore important to re-evaluate this concentration range before making a final suggestion on spinosad dosage solution for field mosquito larviciding.

The damages found in the fine structure of *P. reticulata* may have been responsible for the initial behavioral responses earlier observed in the fish. The general activity of the organism and its respiratory system were impaired mostly by the organophosphates evidenced by the severe damage found in the enterocyte of the exposed fish's nuclei and mitochondria cells respectively. In contrast, spinosad treatment showed little or no impact on the fish enterocytes except at its higher concentration of 110 μgL^{-1}. Rupture in the lysosome at the higher concentration of spinosad may have been responsible for the reduced feeding in the fish that was observed during the behavioral study. Overall, the results from each biomarker were similar and generally demonstrated the strength of the test larvicides as toxicants to the bio-control agent.

5.5 INTEGRATING *POECILIA RETICULATA* AND LARVICIDES AGAINST *CULEX* MOSQUITOES

The feeding activity of *P. reticulata* was generally inhibited in the presence of actellic and chlorpyrifos as opposed to spinosad treatment indicating that spinosad was the most compatible larvicide to the non-target biological component of the integrated control.

The lowest concentration of spinosad produced the greatest control at the optimal predator prey density when compared to the organophosphates. This concentration of spinosad was capable of killing 30% of the more resistant *C. quinquefasciatus* larvae at acute toxicity study. This percentage mortality was too low and may not likely result in an effective larvae control programme especially in a highly mosquito infested area and under single approach. However, the involvement of a biological control fish agent in a compatible manner and at the right feeding density will act as a support control. This integration will eventually lead to the reduction of the remaining 70% mosquito larvae species that may have earlier escaped intoxication by the larvicides.

Spinosad had previously been used to effectively control mosquito larvae species under various single approaches (Bond *et al*. 2004; Perez *et al*.2007; Hertlein *et al*.,2010) but as far as literature is concerned, this study is the first to attempt the use of an integrated approach of larvicides and *P. reticulata* in a compatible manner to control mosquito larvae species.

The insignificant decrease and consistent increase in feeding observed at the lowest concentration of actellic is indicative that this concentration may be a threshold below which the feeding potential of the fish will not be impaired therefore, actellic at concentrations below 18 μgL^{-1} will probably be compatible for integrated mosquito larval control just as spinosad at 49 μgL^{-1}.

The lowest concentration of actellic failed to inhibit the feeding activity of guppy at higher predator densities of 5 and 10 but reverse was the case at fish density of 1. It is assumed that the toxic effect by the larvicides were evenly distributed amongst the two

sets of fish population thereby, it quickly lost its activity on the fishes unlike when the effect from the larvicide was concentrated on a single fish in the test chamber. It is most likely that this scenario caused an increased stress on the single fish hence, making it unable to effectively carry out its larvivorus potential. It is therefore very essential to first have a right estimate of predator–prey density prior to embarking on an integrated mosquito larval control. The consistent increase in the feeding potential of *P. reticulata* at higher fish feeding densities of 5 and 10 as against the use of 1 fish under the lowest concentration of actellic indicated that this compound will likely be compatible to *P. reticulata* at concentration not greater than 18 μgL^{-1}.

The reduction in feeding with organophosphate treatments compared to spinosad despite the application of integrated approach is implicative that this approach of mosquito larval control can only be effective when the right concentration that is compatible to the biological control component is determined and applied at the optimal predator-prey density. The high consumption rate recorded under the lowest concentration of spinosad treatment confirmed that this concentration of larvicide is a candidate for integrated mosquito larval control involving the use of *P. reticulata* as a biological control component. Field studies are necessary at this point to finally conclude on the sustenance of mosquito control by this approach.

CHAPTER SIX

6.1. CONCLUSION AND RECOMMENDATIONS

The purpose of this research was to control mosquito larvae through the simultaneous use of *P. reticulata* and selected larvicides that would eliminate an appreciable number of the target mosquito larvae in a body of water without adversely affecting the non-target guppy fish. Based on this, spinosad was found to be highly compatibility to *P. reticulata* at a concentration of 49 μgL^{-1}. This concentration of spinosad did not show any adverse effect on the fish's growth, behaviour and ultrastructural components as well as, was capable of killing 30% of the more resistant *C. quinquefasciatus* larvae.

The laboratory integration of *P. reticulata* and Spinosad at the optimal predator prey density of 10:350, with the lowest concentration of Spinosad (49 μgL^{-1}) produced the most effective mosquito larval control with minimal adverse effect on the non-target guppy fish. It is therefore recommended that this approach be considered beyond laboratory conditions to help make informed decision for onward addition to the existing national mosquito control programmes. It is also suggested that policy makers should reprioritize larval control through a sincere commitment and allocation of funds. These will no doubt lead to a sustainable mosquito control programme and the quick realization of the Millennium Developmental Goals (MDGs) in Nigeria.

6.2. SUMMARY OF FINDINGS

1. **Determine an optimal feeding density between *P. reticulata* and mosquito larvae**.

a. One *P. reticulata* consumed thirty mosquito larvae within 24 h in 0.5L of dechlorinated tap water;

b. Increase in predator-prey density, prey-types and time interval significantly increased the larvivorus potentials of guppy ($P<0.05$);

c. *Poecilia reticulata* at feeding density of 4 was the threshold below which the fish attained satiation evidenced by the corresponding decrease in the fish consumption rate. At higher densities than 4, there were observed exponential increase in the consumption rate of the fish.

2. **Ascertain *Poecilia reticulata* prey preference**

a. The most consumed prey type by *P. reticulata* was *Chironomid sp*. However, the feeding interaction between the organisms indicated a simultaneous selection for mosquito and *Chironomids* species;

b. *Poecilia reticulata* preferred to compete and chase after their prey prior to feeding on them and are more efficient as predators when paired.

3. **Investigate the Acute toxicity of larvicides on mosquito larvae and *Poecilia reticulata* respectively**

a. *Culex quinquefasciatus* was more tolerant to the test larvicides than *An. gambiae* larvae;

b. Chlorpyrifos was the most lethal of the test larvicides while spinosad was the least toxic especially on the non-target guppy fish;

c. *Poecilia reticulata* was highly tolerant to spinosad but very susceptible to the organophosphates evidenced by the high safety margins obtained with spinosad;

d. There was no significant loss of biological activity by the three larvicides when exposed to the fish at 24 h;

4. Assess the sub-lethal effects of the test larvicides

4.1 Induction of micronucleus and nuclear abnormal cells with Giemsa assay

a. Spinosad was significantly more genotoxic than actellic ($P<0.05$) with higher MN in the fish gill cells;

b. Actellic and spinosad demonstrated similar potentials for cytotoxicity causing an insignificant induction of nuclear abnormal cells at the tested concentrations ($P>0.05$);

c. Actellic and spinosad were not mutagenic to *P. reticulata*;

4.2. Induction of micronucleus with Acridine Orange assay

a. At the established low concentrations, micronuclei were not significantly induced in the exposed *P. reticulata* ($P>0.05$).

4.3. Behavioral responses in *Poecilia reticulata*

a. Chlorpyrifos induced the greatest behavioural changes in the fish with heamorrhage, scoliosis, loss of equilibrium, reduced feeding and eventual death at increasing concentration;

4.4. Ultrastructural Responses in *Poecilia reticulata*

a. Chlorpyrifos and Actellic caused damages in the fish mitochondria and nuclei cell components which became more severe with increasing concentration;

b. There was no marked damage on *P. reticulata* with spinosad at 49 μgL^{-1}. However, above this concentration severe damage to the fish cells occurred.

5. Investigate the impact of integrated approach on mosquito larvae population

a. The simultaneous use of spinosad and *P. reticulata* in the control of mosquito larvae failed to compromise the fish larvivorus potential at all the predator-prey densities sampled. This was evidenced by the significant increase in the consumption of mosquito larvae ($P<0.05$) by the fish under this treatment. On the contrary, the organophosphate compounds caused significant ($P<0.05$) decrease in the larvivorus potential of the fish except with the lowest concentration of actellic at higher fish densities of 5 and 10.

6.3. CONTRIBUTIONS TO KNOWLEDGE

1. The study has established a stocking density for *P. reticulata* that can be applied in routine and integrated mosquito larvae control;
2. The ultrastructural and cytogenotoxic risks of the selected larvicides on *P. reticulata* are documented;
3. Spinosad at the concentration of 49 μgL^{-1} has been identified as a candidate larvicide for integrated mosquito larval management;
4. An additional tool that is evidenced based has been established for national mosquito control programme.

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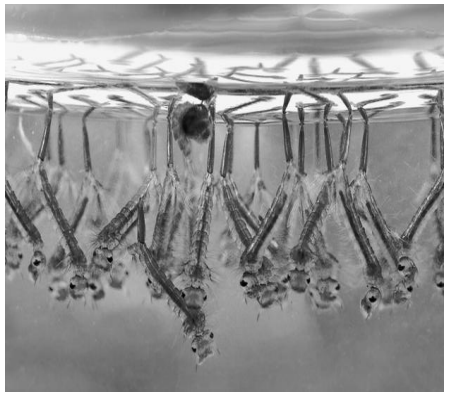
APPENDICES

Appendix 1: IDENTIFICATION OF MOSQUITO LARVAE SPECIES

A. IDENTIFICATION BY FEEDING

Culicine larvae feeding throughout the water column

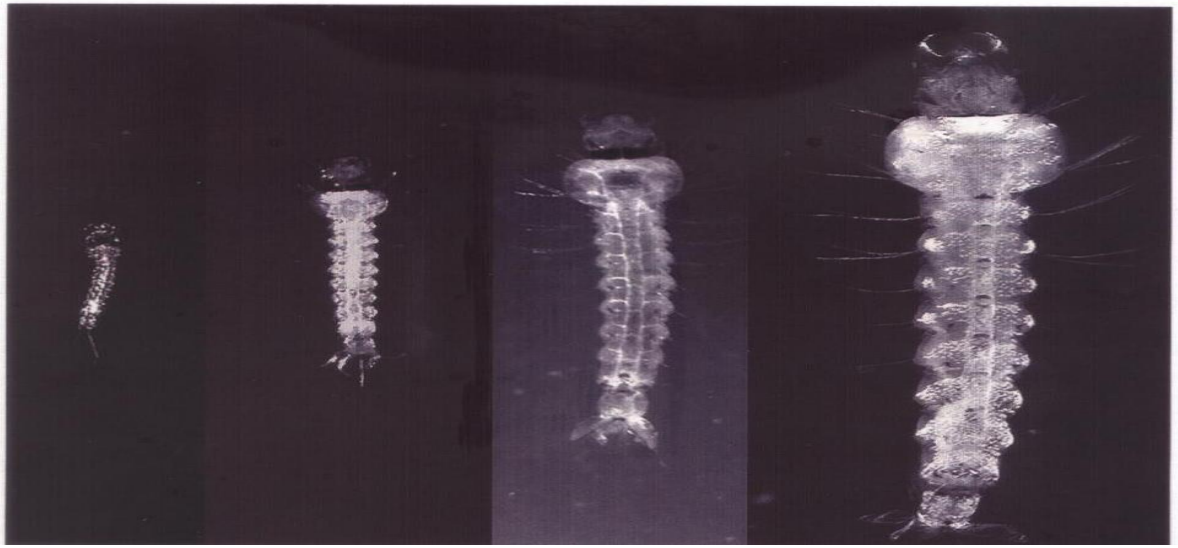
Anopheline larvae feeding at the surface and bottom of the water.



B. IDENTIFICATION BY RESTING POSITION

Resting Position of Anopheline larvae (parallel to water surface)

Resting position of Culicine larvae (perpendicular to water surface)



C. IDENTIFICATION BY LARVAL DEVELOPMENTAL STAGES

From left to right: *An gambiae* larvae 24 h post hatch (1st instar or L_1; 2 to 3 days post hatch (2nd instar or L_2; 5 days post hatch (3rd instar or L_3); 6 days to 10 days post hatch (4th instar or L_4).

Appendix 2: Two way factorial on the effect of predator-prey density and Time interval on the feeding activity of *Poecilia reticulata* (n = 10 observations)

Source of Variation	SS	Df	MS	F	P-value	F crit
1:10-100						
Predator-prey density	2569.867	9	285.5407	21.50966	5.67E-18	1.999115
Time interval	1678.333	3	559.4444	42.14271	1.94E-16	2.718785
Interaction	689.6667	27	25.54321	1.924159	0.013159	1.625513
Error	1062	80	13.275			
2:10-100						
Predator-prey density	7625.8	9	847.3111	71.00372	2.01069E-34	1.999115
Time interval	5164.3	3	1721.433	144.2542	3.53681E-32	2.718785
Interaction	1267.2	27	46.93333	3.932961	1.02246E-06	1.625513
Error	954.6667	80	11.93333			
4:10-100						
Predator-prey density	37169.34	9	4129.927	94.94085	6.21E-39	1.999115
Time interval	31227.83	3	10409.28	239.2937	7.61E-40	2.718785
Interaction	8593.425	27	318.275	7.316667	1.67E-12	1.625513
Error	3480	80	43.5			
6:10-100						
Predator-prey density	48663.63	9	5407.07	182.5171	1.7E-49	1.999115
Time interval	26083.8	3	8694.6	293.4886	4.61E-43	2.718785
Interaction	11040.03	27	408.8901	13.8022	3.08E-20	1.625513
Error	2370	80	29.625			
8: 10-100						
Predator-prey density	55842.37	9	6204.707	296.5213	1.37761E-57	1.999115
Time interval	18269.9	3	6089.967	291.0378	6.26304E-43	2.718785
Interaction	9616.1	27	356.1519	17.0204	4.14574E-23	1.625513
Error	1674	80	20.925			
10:10-100						
Predator-prey density	80559.47	9	8951.052	721.859	1.02E-72	1.999115
Time interval	12232.1	3	4077.367	328.8199	7.03E-45	2.718785
Interaction	7358.4	27	272.5333	21.97849	8.85E-27	1.625513
Error	992	80	12.4			
Total	101142	119				

P<0.05

SS = Sum of Squares; df = degree of freedom; MS = Mean of Square

Appendix 3: Concentration dependent mortality of Spinosad on *Culex quinquefasciatus*

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	culex6h	2833.333[a]	5	566.667	5.829	.006
	culex12h	2612.500[b]	5	522.500	8.957	.001
	culex24h	3144.444[c]	5	628.889	15.614	.000
Intercept	culex6h	6050.000	1	6050.000	62.229	.000
	culex12h	7812.500	1	7812.500	133.929	.000
	culex24h	12272.222	1	12272.222	304.690	.000
conc	culex6h	2833.333	5	566.667	5.829	.006
	culex12h	2612.500	5	522.500	8.957	.001
	culex24h	3144.444	5	628.889	15.614	.000
Error	culex6h	1166.667	12	97.222		
	culex12h	700.000	12	58.333		
	culex24h	483.333	12	40.278		
Total	culex6h	10050.000	18			
	culex12h	11125.000	18			
	culex24h	15900.000	18			
Corrected Total	culex6h	4000.000	17			
	culex12h	3312.500	17			
	culex24h	3627.778	17			

a. R Squared = .708 (Adjusted R Squared = .587)

b. R Squared = .789 (Adjusted R Squared = .701)

c. R Squared = .867 (Adjusted R Squared = .811)

P<0.05

Appendix 4: Concentration dependent mortality of Actellic on *Poecilia reticulata*

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6hrs	83.333[a]	5	16.667	4.918	.011
	12hrs	142.278[b]	5	28.456	25.610	.000
	24hrs	153.778[c]	5	30.756	29.137	.000
Intercept	6hrs	162.000	1	162.000	47.803	.000
	12hrs	329.389	1	329.389	296.450	.000
	24hrs	533.556	1	533.556	505.474	.000
conc	6hrs	83.333	5	16.667	4.918	.011
	12hrs	142.278	5	28.456	25.610	.000
	24hrs	153.778	5	30.756	29.137	.000
Error	6hrs	40.667	12	3.389		
	12hrs	13.333	12	1.111		
	24hrs	12.667	12	1.056		
Total	6hrs	286.000	18			
	12hrs	485.000	18			
	24hrs	700.000	18			
Corrected Total	6hrs	124.000	17			
	12hrs	155.611	17			
	24hrs	166.444	17			

a. R Squared = .672 (Adjusted R Squared = .535)

b. R Squared = .914 (Adjusted R Squared = .879)

c. R Squared = .924 (Adjusted R Squared = .892)

P<0.05

Appendix 5: Concentration dependent mortality of Actellic on *Culex quinquefasciatus*

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mortlaity 6h	3456.944[a]	5	691.389	13.100	.000
	Mortality 12h	2461.111[b]	5	492.222	4.922	.011
	Mortality 24h	2756.944[c]	5	551.389	6.108	.005
Intercept	Mortlaity 6h	10034.722	1	10034.722	190.132	.000
	Mortality 12h	18688.889	1	18688.889	186.889	.000
	Mortality 24h	20334.722	1	20334.722	225.246	.000
conc	Mortlaity 6h	3456.944	5	691.389	13.100	.000
	Mortality 12h	2461.111	5	492.222	4.922	.011
	Mortality 24h	2756.944	5	551.389	6.108	.005
Error	Mortlaity 6h	633.333	12	52.778		
	Mortality 12h	1200.000	12	100.000		
	Mortality 24h	1083.333	12	90.278		
Total	Mortlaity 6h	14125.000	18			
	Mortality 12h	22350.000	18			
	Mortality 24h	24175.000	18			
Corrected Total	Mortlaity 6h	4090.278	17			
	Mortality 12h	3661.111	17			
	Mortality 24h	3840.278	17			

a. R Squared = .845 (Adjusted R Squared = .781)

b. R Squared = .672 (Adjusted R Squared = .536)

c. R Squared = .718 (Adjusted R Squared = .600)

P<0.05

Appendix 6: Time dependent mortality of *Culex quinquefasciatus* exposed to Spinosad

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	527.942[a]	6.000	1.000	.033
	Wilks' Lambda	.000	527.942[a]	6.000	1.000	.033
	Hotelling's Trace	3167.653	527.942[a]	6.000	1.000	.033
	Roy's Largest Root	3167.653	527.942[a]	6.000	1.000	.033
time	Pillai's Trace	1.240	.544	12.000	4.000	.814
	Wilks' Lambda	.004	2.630[a]	12.000	2.000	.308
	Hotelling's Trace	211.893	.000	12.000	.000	.
	Roy's Largest Root	211.569	70.523[b]	6.000	2.000	.014

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept+time

P>0.05

Appendix 7: Time dependent mortality of *Culex quinquefasciatus* exposed Actellic

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.998	96.426[a]	6.000	1.000	.078
	Wilks' Lambda	.002	96.426[a]	6.000	1.000	.078
	Hotelling's Trace	578.558	96.426[a]	6.000	1.000	.078
	Roy's Largest Root	578.558	96.426[a]	6.000	1.000	.078
time	Pillai's Trace	.912	.280	12.000	4.000	.962
	Wilks' Lambda	.158	.253[a]	12.000	2.000	.952
	Hotelling's Trace	4.904	.000	12.000	.000	.
	Roy's Largest Root	4.812	1.604[b]	6.000	2.000	.432

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept+time

P>0.05

Appendix 8: Time dependent mortality of *Poecilia reticulata* exposed to Actellic

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.992	19.952[a]	6.000	1.000	.170
	Wilks' Lambda	.008	19.952[a]	6.000	1.000	.170
	Hotelling's Trace	119.712	19.952[a]	6.000	1.000	.170
	Roy's Largest Root	119.712	19.952[a]	6.000	1.000	.170
time	Pillai's Trace	1.445	.869	12.000	4.000	.620
	Wilks' Lambda	.026	.867[a]	12.000	2.000	.652
	Hotelling's Trace	19.343	.000	12.000	.000	.
	Roy's Largest Root	18.354	6.118[b]	6.000	2.000	.147

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept+time

P>0.05

Appendix 9: Biological Activity of Spinosad on *Culex quinquefasciatus*

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
conc	Pillai's Trace	.989	36.328[a]	5.000	2.000	.027
	Wilks' Lambda	.011	36.328[a]	5.000	2.000	.027
	Hotelling's Trace	90.821	36.328[a]	5.000	2.000	.027
	Roy's Largest Root	90.821	36.328[a]	5.000	2.000	.027
conc * time	Pillai's Trace	.985	.582	10.000	6.000	.785
	Wilks' Lambda	.194	.508[a]	10.000	4.000	.824
	Hotelling's Trace	3.229	.323	10.000	2.000	.910
	Roy's Largest Root	2.912	1.747[b]	5.000	3.000	.343

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c.
Design: Intercept+time
Within Subjects Design: conc

P>0.05

Appendix 10: Biological Activity of Actellic on *Culex quinquefasciatus*

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
time	Pillai's Trace	.784	20.010[a]	2.000	11.000	.000
	Wilks' Lambda	.216	20.010[a]	2.000	11.000	.000
	Hotelling's Trace	3.638	20.010[a]	2.000	11.000	.000
	Roy's Largest Root	3.638	20.010[a]	2.000	11.000	.000
time * conc	Pillai's Trace	.891	1.930	10.000	24.000	.091
	Wilks' Lambda	.282	1.945[a]	10.000	22.000	.093
	Hotelling's Trace	1.935	1.935	10.000	20.000	.100
	Roy's Largest Root	1.534	3.682[b]	5.000	12.000	.030

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c.
Design: Intercept+conc
Within Subjects Design: time

P>0.05

Appendix 11: Biological Activity of Actellic on *Poecilia reticulata*

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
time	Pillai's Trace	.636	8.746[a]	2.000	10.000	.006
	Wilks' Lambda	.364	8.746[a]	2.000	10.000	.006
	Hotelling's Trace	1.749	8.746[a]	2.000	10.000	.006
	Roy's Largest Root	1.749	8.746[a]	2.000	10.000	.006
time * conc	Pillai's Trace	.638	.860	12.000	22.000	.595
	Wilks' Lambda	.444	.833[a]	12.000	20.000	.618
	Hotelling's Trace	1.063	.798	12.000	18.000	.649
	Roy's Largest Root	.842	1.543[b]	6.000	11.000	.252

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c.
Design: Intercept+conc
Within Subjects Design: time

P>0.05

Appendix 12: Repression of normochromatic cells in *Poecilia reticulata* (Mean ‰ ± SD)

	Time of Exposure (Days)					
Treatments	**1**	**3**	**7**	**14**	**21**	**28**
Control	1.5 ± 2.121	1.356 ± 1.918	1.150 ± 1.626	1.255 ± 1.775	0.557 ± 0.787	0.548 ± 0.775
Spinosad	0.718 ± 1.015	1.505 ± 2.128	0.718 ± 1.015	0.600 ± 0.846	0.580 ± 0.792	0.863 ± 1.220
Actellic	0.502 ± 0.710	0.568 ± 0.802	0.507 ± 0.716	0.639 ± 0.903	0.653 ± 0.923	0.913 ± 1.290

P>0.05

Appendix 13: Percentage ratio of PCE/NCE in *Poecilia reticulata* exposed to Spinosad

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	0 µgL-1 - 60 µgL-1	-35.000	18.963	7.742	-54.901	-15.099	-4.521	5	.006
Pair 2	0 µgL-1 - 123 µgL-1	-38.667	9.993	4.080	-49.154	-28.179	-9.478	5	.000
Pair 3	0 µgL-1 - 361 µgL-1	-42.500	9.006	3.677	-51.951	-33.049	-11.560	5	.000

P<0.05

Appendix 14: Percentage ratio of PCE/NCE in *Poecilia reticulata* exposed to Actellic

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	0 µgL-1 - 60 µgL-1	-36.429	15.820	5.980	-51.060	-21.797	-6.092	6	.001
Pair 2	0 µgL-1 - 58 µgL-1	-37.000	16.289	6.157	-52.065	-21.935	-6.010	6	.001
Pair 3	0 µgL-1 - 230 µgL-1	-52.857	10.270	3.882	-62.355	-43.359	-13.617	6	.000

P<0.05

Appendix 15: Impact of integrated control of mosquito larvae at feeding density of 1:35

Treatment	Conc (μgL^{-1})	(Mean Consumption ± SE) 0.5L	3 L	5 L
Chlorpyrifos	0.4	18.33 ± 1.31^{ns}	14.33 ± 0.33^{o}	17.17 ± 0.60^{o}
	0.6	18.33 ± 0.96^{ns}	12.67 ± 0.33^{o}	12.50 ± 0.43^{o}
	0.8	15.00 ± 0.37^{o}	13.33 ± 1.63^{o}	13.17 ± 0.83^{o}
Actellic	18	20.50 ± 0.89^{ns}	19.00 ± 0.86^{ns}	13.67 ± 0.84^{o}
	30	17.50 ± 0.89^{o}	15.33 ± 0.84^{o}	13.33 ± 0.56^{ns}
	50	16.17 ± 1.01^{o}	13.67 ± 0.88^{o}	13.83 ± 1.11^{ns}
Spinosad	49	29.83 ± 1.85^{o}	23.33 ± 0.84^{o}	26.67 ± 1.86^{o}
	73	25.33 ± 1.20^{o}	19.00 ± 0.82^{ns}	17.33 ± 1.54^{ns}
	110	20.67 ± 0.99^{ns}	19.17 ± 0.79^{ns}	20.50 ± 0.89^{ns}
Control	0	20.67 ± 0.72	19.33 ± 0.42	19.50 ± 0.43

Appendix 16: Impact of integrated control of mosquito larvae at feeding density of 5:70

Treatment	Conc (μgL^{-1})	(Mean Consumption ± SE)		
		0.5L	**3 L**	**5 L**
Chlorpyrifos	0.4	25.17 ± 0.65^{o}	23.33 ± 0.84^{o}	15.67 ± 1.20^{o}
	0.6	20.50 ± 0.89^{o}	19.00 ± 0.86^{o}	13.67 ± 0.84^{o}
	0.8	17.50 ± 0.89^{o}	15.33 ± 0.84^{o}	13.33 ± 0.56^{o}
Actellic	18	39.83 ± 0.60^{o}	38.33 ± 0.87^{o}	38.00 ± 0.68^{o}
	30	31.17 ± 0.48^{ns}	30.00 ± 0.58^{o}	29.17 ± 0.48^{o}
	50	31.67 ± 1.63^{ns}	34.00 ± 1.67^{ns}	30.67 ± 0.92^{o}
Spinosad	49	63.00 ± 2.41^{o}	59.83 ± 2.40^{o}	51.00 ± 2.58^{o}
	73	60.67 ± 1.91^{o}	49.17 ± 2.90^{o}	45.83 ± 2.77^{o}
	110	60.50 ± 2.32^{o}	49.83 ± 2.68^{o}	45.00 ± 2.61^{o}
Control	0	32.00 ± 1.71	34.17 ± 0.87	34.83 ± 0.83

Appendix 17: Impact of integrated control of mosquito larvae at feeding density of 10:350

Treatment	Conc (μgL^{-1})	(Mean Consumption ± SE)		
		0.5L	**3 L**	**5 L**
Chlorpyrifos	0.4	111.67 ± 2.23^{ns}	97.33 ± 1.26^{ns}	97.67 ± 2.64^{ns}
	0.6	105.83 ± 2.39^{ns}	94.17 ± 1.78^{ns}	92.67 ± 2.78^{o}
	0.8	94.67 ± 2.87^{o}	90.33 ± 2.40^{ns}	89.33 ± 3.47^{o}
Actellic	18	121.67 ± 1.28^{o}	137.00 ± 1.46^{o}	116.33 ± 1.20^{o}
	30	116.50 ± 1.34^{ns}	123.00 ± 1.03^{o}	113.00 ± 1.37^{o}
	50	112.33 ± 1.52^{ns}	110.33 ± 1.50^{o}	104.83 ± 2.27^{ns}
Spinosad	49	267.33 ± 9.69^{o}	234.50 ± 10.19^{o}	213.50 ± 13.86^{o}
	73	233.33 ± 14.33^{o}	217.33 ± 10.31^{o}	206.83 ± 11.26^{o}
	110	182.67 ± 13.67^{o}	150.17 ± 16.24^{o}	198.00 ± 8.89^{o}
	0	108.17 ± 3.75	89.33 ± 3.47	102.67 ± 2.64