EFFECT OF IRRIGATION ON *ANOPHELES* VECTOR LARVAL ECOLOGY AND INSECTICIDE RESISTANCE STATUS AND MECHANISMS IN MIXED CROP IRRIGATION IN HOMA BAY COUNTY, WESTERN KENYA

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OF

AGRICULTURE AND TECHNOLOGY

2023

Effect of irrigation on *Anopheles* vector larval ecology and insecticide resistance status and mechanisms in mixed crop irrigation in Homa Bay County, Western Kenya

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Molecular Biology and Bioinformatics of the Jomo Kenyatta University of Agriculture and Technology

2023

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

To Newton and Amma-Mich

ACKNOWLEDGEMENT

First, I am extremely grateful to the Lord Almighty for the strength, wisdom, providence, care, protection, patience, and grace, which have all enabled me to successfully undertake to completion this task despite the challenges that were involved.

My deepest gratitude also goes out to my supervisors, Dr. Steven Ger, for his excellent suggestions, inspiration, and help with this endeavor. I appreciate the time he invested in finishing this task. As my mentor and supervisor, I value all of his advice, support, and suggestions. I am really grateful to Dr. Andrew Githeko whose commitment of this work and and assistance in helping me realize my aspirations will never be forgotten. Your encouraging words have had a major influence in me at a personal level. Much gratitude goes to Prof. Guiyun Yan for giving me this opportunity to undertake my PhD and for all of his help and assistance in getting this task done. I am grateful for his ongoing direction and support throughout this endeavor. A special thanks goes to Dr. Harrysone Atieli, I sincerely appreciate your constant dedication, support, and direction for this work. Not forgetting all the staff at the International Centre of Excellence for Malaria Research, the community at large in Homa Bay and others who supported my work, for their brilliant ideas and moral supportthat were so timely. They have imparted in me great skills and knowledge

Last but not least, I wish to thank my family and friends for their never- ending encouragement, much needed financial assistance and all- rounded support.

You all contributed to this achievement.

God bless you all.

TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

ACKNOWLEDGEMENT iv

TABLE OF CONTENTS v

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF APPENDICES xiv

LIST OF ABREVIATIONS AND ACRONYMS xv

ABSTRACT xx

CHAPTER ONE 1

INTRODUCTION 1

1.1 Background Information 1

1.2 Statement of the Problem 2

1.3 Justification of the Study 4

1.4 Hypothesis 6

1.4.1 Null Hypothesis 6

1.5 Objectives 7

1.5.1 General objective 7

1.5.2 Specific objective 7

CHAPTER TWO 8

LITERATURE REVIEW 8

2.1 *Anopheles* mosquito 8

2.1.1 Mosquito Species Identification 9

2.1.2 Anopheline species distribution in Kenya 10

2.2 Malaria vector abundance 12

2.2.1 Larval habitats 12

2.2.2 Habitat stability 13

2.2.3 Larval habitat productivity 14

2.2.4 Larval habitat predator composition 14

2.3 Malaria Transmission 15

2.4 Malaria Vector Control Strategies 17

2.5 Insecticide Resistance 17

2.5.1 Resistance Mechanisms 22

2.5.2 Contribution of Agriculture and Public Health to Insecticide Resistance 23

2.5.3 Monitoring Insecticide Resistance 24

CHAPTER THREE **26**

MATERIALS AND METHODS **26**

3.1 Study Area 26

3.1.1 Homa Bay County 26

3.2 Larval ecology study framework 29

3.3 Sampling 29

3.3.1 Larval Collection 30

3.3.2 Water Sampling 32

3.3.3 Predator Sampling 32

3.4 Initial Processing of the Samples from the Field 32

3.5 Experimental Techniques 33

3.5.1 Larval Ecology Study 33

3.5.2 Bacterial analysis 40

3.5.3 Aquatic composition and metabolite identification 43

3.5.4 Larval susceptibility tests 47

3.5.5 Insecticide resistance study 48

3.5.6 Laboratory Analysis of mosquito and water samples 54

3.6 Data management 57

3.7 Data Analysis 57

3.7.1 Larval ecology study 57

3.7.2 Larval susceptibility tests 61

3.7.3 Insecticide resistance 61

3.7.4 Species Identification 62

3.8 Ethical considerations 62

CHAPTER FOUR 63

RESULTS 63

4.1 Larval Ecology study 63

4.1.1 Habitat availability and productivity 63

4.1.2 Monthly habitat and larval density dynamics 69

4.1.3 Habitat stability 73

4.1.4 Habitat productivity surveillance using mosquito emergence traps 75

4.1.5 Overall adult malaria vector productivity in each ecosystem 77

4.1.6 Laboratory Predator experiment 84

4.1.7 Habitat types for life table assessment 85

4.1.8 Survival rates for larval vectors 88

4.2 Bacterial culture results 92

4.3 MALDI-TOF MS results 92

4.4 Bacterial isolates identified on sequencing aquatic larval sources 94

4.5 Aquatic metabolite analysis 97

4.5.1 Fatty acids concentration and larval availability 98

4.5.2 Phytochemical concentration and larval availability 99

4.5.3 Sugar concentration and larval availability 100

4.5.4 Aquatic mineral concentration and larval availability 101

4.6 Larval chemical exposure tests 102

4.6.1 Questionnaire survey on use of chemical 102

4.6.2 Larval susceptibility tests 107

4.7 Insecticide Resistance 107

4.7.1 *An. arabiensis* insecticide phenotypic resistance bioassays 107

4.7.2 Mechanisms of insecticide resistance 109

4.8 Molecular species identification 111

CHAPTER FIVE 113

DISCUSSION 113

5.1 Discussion 113

CHAPTER SIX 123

CONCLUSION AND RECOMMENDATION 123

6.1 Conclusion 123

6.2 Recommendations 124

REFERENCES **126**

APPENDICES **169**

LIST OF TABLES

Table 4.1: Morphologically identified larval species 63

Table 4.2: Habitat availability per transmission zone during the dry and wet season. .. 64

Table 4.3: Estimated adult malaria vector productivity for larval habitats 78

Table 4.4: Summary results of the model fit of the predator experiment 84

Table 4.5: Origin of samples cultured for MALDI-TOF MS and bacterial sequencing 92

Table 4.6: Fatty acid concentrations in larval sites 99

Table 4.7: Phytochemical concentrations in larval sites 100

Table 4.8: Sugar concentrations in larval sites 101

Table 4.9: Mineral concentrations in larval sites 102

Table 4.10: Frequency of responses to chemical use 103

Table 4.11: Chemical insecticides classes commonly used 104

Table 4.12: Proportion of household that use chemicals 105

Table 4.13: Proportion of farmers using pesticides 105

Table 4.14: Summary of questionnaire survey on use of pesticides 106

Table 4.15: The status of phenotypic resistance of *Anopheles arabiensis* 108

Table 4.16: Allele frequency of *vgsc* and *ACE*-1 mutations. 110

LIST OF FIGURES

Figure 2.1: Map of Kenya showing the distribution of *Anopheles* complexes......... 11

Figure 2.2: Map of Kenya showing the varied prevalence of malaria 16

Figure 2.3: Resistance mechanisms 18

Figure 2.4: Impact of environment on mosquito response to pyrethroid insecticides19

Figure 2.5: Distribution of insecticide resistance in Kenya 21

Figure 3.1: Map of Homa Bay study site 27

Figure 3.2: An example of a mixed- crop irrigated farm in Homa Bay.................. 28

Figure 3.3: The diversity of habitat types. 31

Figure 3.4: Emergence trap sampling 36

Figure 3.5: Figurative description of calculation of effective area 38

Figure 4.1: Area of the clusters 65

Figure 4.2: Monthly weather 2018-2019 in Homa Bay, Kenya.............................. 66

Figure 4.3: Effects of seasonality.. 67

Figure 4.4: Seasonal sampling. 69

Figure 4.5: Number of habitats and habitat area during monthly dynamics............ 71

Figure 4.6: Larval density during monthly larval dynamics in 2019....................... 72

Figure 4.7: Habitat stability 73

Figure 4.8: *Anopheles* densities from September 2018 to August 2019.................. 74

Figure 4.9: Densities of female adult *Anopheles* per trap-night 76

Figure 4.10: Estimated productivity by effective area... 80

Figure 4.11: Estimated total adult vector productivity per week............................. 81

Figure 4.12: Predicted number of emerged female *Anopheles* adults....................... 83

Figure 4.13: Predation of rates *An. gambiae* larvae.. 85

Figure 4.14: Correlation between larval densities and total number of predators.... 86

Figure 4.15: Correlation between *Anopheles* larval densities and total predators.... 87

Figure 4.16: Correlation between *Culex* larval densities and total predators 88

Figure 4.17: Mosquito age specific survival rate... 89

Figure 4.18: Total number of *An. gambiae* using five- day smoothed dynamics..... 90

Figure 4.19: Total number of *An. funestus* using five- day smoothed dynamics...... 91

Figure 4.20: Distribution of bacterial genera detected by MALDI-TOF MS........... 93

Figure 4.21: Distribution of bacterial species detected by MALDI-TOF MS.......... 94

Figure 4.22: Frequency of isolated genera from larval habitats 95

Figure 4.23: Phylogenetic relationship between isolated bacteria............................ 97

Figure 4.24: Frequency of responses to chemical use. 103

Figure 4.25: Percentage mortality of phenotypic resistance.................................. 108

Figure 4.26: Metabolic enzyme activity for *An. gambiae* 111

Figure 4.27: Gel photos... 112

LIST OF APPENDICES

Appendix I: Ethical approval letter....169

Appendix II: Ethical clearance from Ministry of Health....170

Appendix III: Questionnaire for households....171

Appendix IV: Questionnaire for agro- vet shops....173

Appendix V: Questionnaire for crop farmers....176

Appendix VI: Questionnaire for livestock farmers....179

Appendix VII: Questionnaire for veterinary officers/ agricultural extension workers 183

Appendix VIII: Emergent traps field form....186

Appendix IX: Predation rates laboratory form....188

Appendix X: Vertical life table field form....189

Appendix XI: Larval and water sampling field form....191

Appendix XII: Phenotypic resistance laboratory data form....194

Appendix XIII: Microplate enzyme assay laboratory data form....195

Appendix XIV: Species identification laboratory data form....196

Appendix XV: Molecular assays (*kdr* and *ACE-1*) laboratory data form....198

Appendix XVI: Larval bioassay laboratory data form....199

Appendix XVII: Identity matrix table....200

LIST OF ABREVIATIONS AND ACRONYMS

µg	micrograms
µl	microliters
ACE	Angiotensin Converting Enzyme
AChE	Acetyle Cholinesterase
An.	*Anopheles*
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical chemists
AR	*arabiensis*
BLAST	Basic Local Alignment Search Tool
bp	base pair
CDC	Centre for Disease control
cDNB	1-chloro-2,4'-dinitrobenzene
CI	Confidence interval
$CuSO_4$	Copper Sulphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double distilled water
DDT	Dichlorodiphenyltrichloroethane
dGTP	Deoxyguanosine triphosphate
DHA	Docosahexaenoic
DNA	Deoxyribonucleic acid

dNTP	dinucleotides triphosphates
DPPH	2-Diphenyl-1-picryl hydrazyl
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid
EIR	Entomological inoculation rate
EMB	Eosine methylene blue
EPA	Eicosapentaenoic
ExoSAP	Exonuclease 1 and shrimp alkaline phosphatase
F	Forward
FUN	*funestus*
G	grams
G119S	guanine to serine substitution at position 119
GA	*gambiae*
GC	Gas chromatography
GLMM	Generalized linear mixed model
GST	Glutathione S- transferase
H_20_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
ICEMR	International Center of Excellence for Malaria Research
ID	Identification
IRAC	Insecticide Resistnace Action Committee

IRS	Indoor residual spraying
ITN	Insecticide treated nets
ITS	internal transcribed spacer
IVM	Integrated Vector Management
JKUAT	Jomo Kenyatta University of Agriculture and Technology
K_2SO_4	Potassium Sulphate
Kdr	knockdown resistance
kdr- E	knockdown resistance east
kdr-W	knockdown resistance west
KDT	Knockdown time
Km	kilometers
KMIS	Kenya Malaria Indicator Survey
KNBS	Kenya National Bureau of Statistics
KOSFIP	Kimira- Oluch Smallholder Farm Improvement Project
KPO_4	Potasium phosphate
L	Length
L1014F	leucine to phenylalanine substitution at position 1014
L1014S	leucine to serine substitution at position 1014
LLITNs	Long lasting insecticide treated nets
LSM	larval source management
M	metres
M	mutant

MALDI-TOF MS Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

ml milliliters

MUERC Maseno University Ethical Review committee

Na^+ Sodium ions

NaOAc Sodium Acetate

NCBI National Centre for Biotechnology Information

O Original Area

ODK Open data kit

PAU Pan- African University

PBO Piperonylbutoxide

PBS Phosphate buffered saline

PCR polymerase chain reaction

qPCR Real- time polymerase chain reaction

R Reverse

rDNA ribosomal Deoxyribonucleic acid

RI Refractive index

rpm revolutions per minute

s.l sensu lato

s.s sensu strict

SNP Single nucleotide polymorphism

TBE Tris-borate- Ethylenediamine tetraacetic acid

TFA Trifloon Acetic Acid

TMBZ	Tetramethyl-Benzidine Dihydrochloride
UK	United Kingdom
UN	Universal
USA	United States of America
UV	Ultra Violet
VGSC	Voltage- gated sodium channel
W	Width
W	Wildtype
WHO	World Health Organization

ABSTRACT

Malaria is a debilitating parasitic disease that causes high economic and health burden especially in sub- Saharan Africa. The control and elimination of malaria is greatly jeopardized by drug resistance and increasing insecticide resistance witnessed in different regions with high vector population. The main aim of this study was to determine the effect of irrigation on malaria vector larval ecology, larval survivorship and insecticide resistance in irrigated and non-irrigated areas of Homa Bay County, Western Kenya. Larval sampling was conducted throughout the dry and wet seasons of 2018 and 2019 to assess the availability and productivity of vector aquatic stages in temporary, semi-permanent, and permanent habitats. The productivity of female adult vectors from various habitat types per week was determined using emergent traps. In 2019, monthly larval abundance measurements and predator experiments were also undertaken to assess the densities of mosquito aquatic stages throughout the year and the survivorship of *Anopheles arabiensis* larvae and habitat productivity in four permanent habitat types respectively. Furthermore, the duration of habitat stability was compared between cohorts of selected habitats followed for a year in irrigated and non-irrigated eco-systems. Water samples were collected from malaria vector larval positive and negative aquatic habitats in the irrigated and non- irrigated sites. Bacteria were cultured from these samples, and the colonies identified using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). To validate and further identify the bacterial species present in the water samples, DNA was extracted from these bacterial cultures, polymerase chain reaction (PCR) and sequencing were performed. Finally, the metabolite composition of larval positive and negative habitats was examined (total polyphenols, free radical scavenging activity, mineral analysis, chlorophyll and carotenoid, crude protein, fatty acid and lipid/ oil, and sugar contents). In addition, the status and mechanism of insecticide resistance in malaria vectors in irrigated and non-irrigated areas and the contribution of public health interventions and agriculture to insecticide resistance was investigated. Overall, *An. gambiae* complex was the most predominant vector (95.2%), with *An. arabiensis* (a sibling species) as most common (98.3%) in both irrigated and non-irrigated areas. The irrigated areas had more habitat variety (74.8%) and *Anopheles* larvae (72.3%) than the non-irrigated areas. For *Anopheles* and *Culex*, single species infestation rate in non-irrigated areas was greater than in irrigated areas. Furthermore, larval densities decreased significantly with age in both irrigated and non-irrigated zones. During the seasonal sample period, temporary habitats were the most productive in both irrigated and non- irrigated areas. The number of semi-permanent and permanent habitats was significantly different in irrigated and non-irrigated areas during monthly dynamics sampling. In the predator experiment, fish was the most efficient predator of all examined predators. *Culex* larval density dropped as predator density increased. The mortality rate from larva to pupa was above 97% for *An. arabiensis* and 100% for *An. funestus*. The highest larval stage survival rate was between larval stages I and II, while the lowest was between larval stages IV and pupa. Life tables for each developmental stage revealed substantial mortality rates, particularly at larval stages II and III. *Bacillus* was the only genera identified from larval sources in the non-irrigated zone using MALDI-TOF MS. *Shigella* was the dominant genera in the irrigated region, while *Escherichia coli* was the most prevalent species. *Bacillus* was found in 65% of the

sequenced isolates. *Brevibacillus brevis*, *Bacillus subtilis*, and *Exiguobacterium profundum* were isolated and classified as larvicidal isolates, together with *Bacillus mojavensis*, *Bacillus tequilensis*, *Bacilus stercoris*, and *Brevibacillus agri*. Compared to non-irrigated sites, irrigated areas with larvae had lower crude fat and crude protein content. Larvae were present and absent in non-irrigated areas with comparable protein levels. The presence of larvae was seen in both irrigated and non-irrigated areas with high total chlorophyll content. Sites with larvae in both irrigated and non-irrigated locations showed higher sugar concentrations than sites without larvae; however, when compared to identical sites in irrigated areas, non-irrigated sites with larvae had higher sugar concentrations. Furthermore, significant amounts of Manganese, Calcium, and Copper were detected in larvae-infested sites in both irrigated and non-irrigated areas. Pyrethroids were the most common chemical class of pesticides used for crop and animal protection, with very high coverage of LLINs impregnated with pyrethroids and IRS impregnated with organophosphate insecticides. Larvae were susceptible to various dilutions of Diazol and Thunder, resulting in 96% - 100% mortality. Milraz, on the other hand, caused 23.2% and 11.2% larval mortality in irrigated and non-irrigated areas, respectively, at 1:10,000 dilutions, while no mortality was seen at 1:50,000 dilutions in both irrigated and non-irrigated areas. *An. arabiensis* was the sole species tested in irrigated areas and the main species in non-irrigated areas, with the rest being *An. gambiae* sensu stricto. In 2018, susceptibility to deltamethrin and malathion was reported in irrigated areas while phenotypic resistance to deltamethrin with susceptibility to malathion was observed in the non-irrigated areas. However, in 2019, phenotypic resistance was observed against deltamethrin in both areas. In 2019, suceptility to DDT and PBO-deltamnethrin was observed in both populations. There were observed low frequencies of L1014F with mutation frequencies ranging from 1% to 16%, and nearly no alteration in the *ACE-1* gene. There were higher oxidase and β-Esterase levels in mosquitoes from the irrigated and non- irrigated sites. However, the enzyme levels of oxidase, GST and esterase were not statistically different between the irrigated and non- irrigated area. Malaria breeding sites in Homa Bay have increased as a result of environmental modification. Irrigation has promoted habitat stability, potentially stabilizing malaria breeding and transmission throughout the year, making larval source management a serious problem as a supplementary strategy of malaria control. The presence of *B. brevis*, *B. subtilis*, and *E. profundum* may have altered larval availability, and the existence of related clustered isolates *B. mojavensis*, *B. tequilensis*, *B. stercoris*, and *Brevibacillus agri* allows for further study as possible larvicidal or adulticidal agents. A high concentration of fatty acids, chlorophyll, sucrose, and manganese may have an effect on larval production. The absence of insecticide reistance in immature vectors to commonly used pesticides in the area is encouraging as this might not be the origin of insecticide resistance observed in the area. However, the widespread use of pyrethroids in agriculture and public health may have contributed to the fast spread of insecticide resistance. The susceptibility of these malaria vectors to organophosphates and PBO synergists in pyrethroids suggests that IRS and ITN-based vector control strategies have a bright future.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria is a parasitic disease-causing high rates of morbidity and mortality especially in tropical areas of Central America, Asia and Africa (WHO, 2022), with over 90% of these cases reported in the Sub-Saharan African countries (WHO, 2022). This disease results in high economic burden due to high treatment costs and loses in human resource thus hampering economic growth in affected regions (Alonso *et al.,* 2019; Degarege *et al.,* 2019; El-Houderi *et al.,* 2019). Most prevention methods aim to protect pregnant women and children under the age of 5 years as these are the most affected by malaria infection (CDC, 2016). With the recent pronouncement of China as malaria free (Chen *et al.,* 2021; Zhou, 2021), more and more countries have renewed hope that a malaria-free world is still achievable (Chen *et al.,* 2021). In Kenya, malaria prevalence varies between regions with the Coast and the Lake region considered as endemic regions. The lake region has been observed to have malaria transmission throughout the year (Noor *et al.,* 2009; MoH, 2005).

Malaria vector control has been a major component in the public health sector as a tool for reducing malaria transmission in the tropics (WHO, 2006). Several control measures targeting different developmental stages of the mosquito life cycle have been adopted to curtail the growth and development, maturation and eventual dispersion of malaria vectors (Walton & Eldridge, 2009). Recent studies have reported an emerging new adaptation in mosquitoes when faced with domestic interventions including outdoor biting (Beier *et al.,* 2018; Sougoufara *et al.,* 2020). The change in mosquito vector behavior has thus resulted in reduced contact between vectors and insecticides, hence reduced effectiveness of the conventional malaria intervention programmes including the use of long-lasting insecticide nets (LLITNs) (Pates & Curtis, 2005).

It has also been observed that agricultural activities, in addition to public health have resulted to the rise of insecticide resistance (Antonio-Nkondjio *et al.,* 2011). This is due to use of chemical pesticides that might posses the same mechanism of action as those used in public health. This results in exposure of the immature mosquito stages to these chemicals resulting in resistant emerging adults (Nkya *et al.,* 2014; Reid & McKenzie, 2016).

Homa Bay County in Western Kenya is known to be an endemic region for malaria transmission (KMIS, 2020) and this trend has persisted over the years despite the wide coverage of insecticide treated nets in this area (Omondi & Kaman, 2018). Homa Bay is classified as a malaria holo-endemic region with high entomological inoculation rates (EIR) of about 300 infectious bites per person per year after the introduction of Kimira- Oluch Smallholder Irrigation Project in 2007 (Onchiri, 2014). This irrigation system has resulted in an increase of malaria vector population and mosquito dynamics (Ondeto *et al.,* 2022) thus resulting in increased malaria incidences (Omondi *et al.,* 2022a) and insecticide resistance in the vector populations (Hemming-Schroeder *et al.,* 2018; Orondo *et al.*, 2021).

As a result, the need to continuously monitor malaria vector behavior and resistance against all the classes of insecticides in Homa Bay County and understand the different mechanisms responsible for the widespread levels of resistance is essential. If a change in either behavior or susceptibility is observed, alternative measures should be employed to mitigate against the same (WHO, 2016). Another class of insecticides with a different mechanism of action could be used/ developed to the already observed resistance. This will help keep the incidences of malaria low and its probable final eradication.

1.2 Statement of the Problem

Due to effects of climate change, many countries are taking up irrigation as a source of food production for sustainability (Ding *et al.,* 2020; Higginbottom *et al.,* 2021; Karthikeyan *et al.,* 2020; Kirshanth & Sivakumar, 2018; Kukal & Irmak, 2018; Zhou *et al.,* 2022). In Kenya, many areas are quickly becoming prone to long draughts and are classified as arid and semi- arid areas. Currently, over 80% of the Kenyan land

surface area is classified as arid and semi-arid (Biamah, 2005; Maundu *et al.,* 2009). This has led to necessitating irrigation to supplement for food production so as to sustain the ever-growing population. As a result, several irrigation systems across the country have been established in Ahero, Western Kenya (Githeko *et al.,* 1993; Kamau *et al.,* 2008; Mbogo, 2015), Mwea in Central Kenya (Kamau & Vulule, 2006; Muriu *et al.,* 2008; Mutero *et al.,* 2004; Mwangangi *et al.,* 2010) and Homa Bay in Western Kenya (Omondi *et al.,* 2022a; Ondeto *et al.,* 2022; Orondo *et al.,* 2023a; Zhou *et al.,* 2022).

Homa Bay County is a semi- arid malaria endemic area with a canal- based irrigation system (Kimira Oluch Smallholder Farmer irrigation Project, KOSFIP) that was constructed to assist in food production through improved agricultural programs at household levels. As a result, the ecosystems was altered, resulting in adverse consequences to human health (Ochwedo *et al.,* 2021; Omondi *et al.,* 2022a; Omondi *et al.,* 2022b; Omondi *et al.,* 2023; Ondeto *et al.,* 2022; Orondo *et al.,* 2023a; Zhou *et al.,* 2022). With the ever- growing human population, deforestation, reclamation of marsh lands and establishment of the irrigation system in Homa Bay has resulted in increased vector densities and hence malaria transmission in the County (Ochwedo *et al.,* 2021; Omondi *et al.,* 2022a; Omondi *et al.,* 2022b; Omondi *et al.,* 2023; Ondeto *et al.,* 2022; Orondo *et al.,* 2023a; Zhou *et al.,* 2022). In addition, there has been observed a rise in the insecticide resistance frequencies thus jeopardizing the vector control strategies in place (Orondo *et al.,* 2021). This area has a high coverage of LLITNs and IRS using pirimiphos-methyl (Actellic® 300CS) was conducted during the study period (Alegana *et al.,* 2021; Omondi *et al.,* 2022a).

In Homa Bay County, agriculture is the main source of income and the establishment of a canal- based irrigation system by the government to aid in food production also let to the creation of more malaria vector breeding habitats (Orondo *et al.,* 2023a) leading to increased vector populations (Ondeto *et al.,* 2022; Zhou *et al.,* 2022) and subsequent disease transmission (Omondi *et al.,* 2022a; Omondi *et al.,* 2022b; Zhou *et al.,* 2022). The over-use or misuse of pesticides in the irrigation scheme has led to development of insecticide resistance (Orondo *et al.,* 2021) as has been observed in other schemes (Antonio-Nkondjio *et al.,* 2011; Nkya *et al.,* 2014; Reid & McKenzie,

2016). In addition, heavy metal tolerance has been observed to be a great contributor to mosquito development (Mireji *et al.,* 2008) and thus might contribute to their susceptibility to insecticides in habitats close to industries. This has also been observed in Homa Bay County where insecticide resistant mosquitoes have been reported (Orondo *et al.,* 2021).

To combat malaria transmission, the wide scale use of insecticide treated nets (ITNs) or the long lasting insecticide treated nets (LLITNs), IRS and agricultural activities in Homa Bay have impacted on *Anopheles* mosquitoes differently and reduced mosquito densities (Abong'o *et al.,* 2020; Ondeto *et al.,* 2022; Zhou *et al.,* 2022) as has been observed in other areas (Mbogo *et al.,* 1996). However, studies have shown that these vectors can reappear in regions where they were once eliminated, sometimes even with resistance to the previously used insecticides (Brooke *et al.,* 2001; Casimiro *et al.,* 2007; Hargreaves *et al.,* 2000). Resistance to recommended insecticides can jeopardize the efforts put forth towards the control of mosquito transmitted diseases such as malaria.

Therefore, Homa Bay County a semi- arid malaria endemic region with a canal-based irrigation system resulting in increased malaria vector breeding sites and thus indreased malaria transmission. The use of public health intervention tools and pesticides in farms could result in selection pressure for insecticide resistance in malaria vectors

1.3 Justification of the Study

Malaria vector control targeting the immature stages would be beneficial in Homa Bay because it is less tedious and more advantageous as compared to the control of adults as the larvae are less mobile and are found in specific locations (Floore, 2006). Insight in the characteristics of larval habitats and mosquito species composition in the habitats is thus important for an effective design of vector control programme in Homa Bay County (Ondeto *et al.,* 2022; Orondo *et al.,* 2023a; Zhou *et al.,* 2022). Understanding the factors that attract or deter vector oviposition in aquatic habitats is essential in the downstream implementation of larval source management. These factors include microbial species (that act as food or parasites) (Gimnig *et al.,* 2001;

Takken & Knols, 1999), predators and other symbiotic insects (Dambach, 2020; Carlson *et al.,* 2004) or other mosquito species (Munga *et al.,* 2014). These have been observed to determine vector population densities, development and survival.

Studies in malaria endemic regions have proven that the wide scale use of a single class and/ or related classes of mosquito insecticides has given rise to resistance (Ochomo *et al.*, 2013; Ochomo *et al.,* 2014; Orondo *et al.,* 2021) in several predominant malaria vector species, including *An. gambiae* s.s., *An. funestus* and *An. arabiensis*. Insecticide resistance has been reported in several malaria endemic countries, with pyrethroids resistance being the most common in sub-Saharan Africa (Riveron *et al.,* 2018). This is due to increase in selection pressure and mutations (Riveron *et al.,* 2018). Selection pressure has been observed to be as a result of frequent use of pyrethroids in all approved LLITNs and in most IRS programmes worldwide (WHO, 2011). In Homa Bay the emergence and steady spread of insecticide resistance has been reported in the main malaria vectors (Hemming-Schroeder *et al.,* 2018; Orondo *et al.,* 2021). The currect study assessed the status of insecticide resitance and the contributuion of agriculture to the observed resistance in malaria vectors. The resultant information is essential in downstream implementation of vector control programmes in Homa Bay.

Mosquitoes have been observed to be fast adapters to environmental changes, including establishment of irrigation systems, and as a consequence, the wide-spread use of chemical insecticides in both public health and agriculture has resulted to insecticide resistant mosquito phenotypes with better survival chances in the presence of an insecticide as compared to their susceptible counterparts (Riveron *et al.,* 2018). However, studies have observed that continuous exposure of insecticide resistant mosquito population to insecticides reduces malaria transmission (Alout *et al.,* 2014; Viana *et al.,* 2016).

Mosquito oviposition is influenced by microbial volatiles from larval environments (Mwingira *et al.,* 2020; Takken & Knols, 1999) including food from microorganisms. Algae has been observed to increase vectors' choice of oviposition environment (Otienoburu *et al.,* 2007). Reduced oviposition has been noted in

aquatic habitats with a reduction in bacterial content (Sumba *et al.,* 2004a; Benzon & Apeperson, 1988), because these bacterial volatiles function as attractants to many species (Mwingira *et al.,* 2020). Additionally, it has been noted that the volatiles released by organic materials draw gravid *Anopheles* species females for oviposition (Rodriguez, 1990).

Previous research show that, mosquitoes are drawn to aquatic habitat cues from a distance. Given that emergent vegetation commonly grows around and around malaria mosquito larval locations, aquatic vegetation may provide cues. Different vectors prefer particular habitat types over others, although this preference has been seen to shift with time. For instance, *An. funestus* primarily bred in swamps and marshes with longer-lasting vegetation (Gimnig *et al.,* 2001; Himeidan *et al.,* 2009; Kibret *et al.,* 2017c). However, this species is now known to inhabit environments devoid of plants (Debrah *et al.,* 2021). The edges of sunny, fresh, slow-moving waters had long been assumed to be preferred by *An. gambiae* s.l., but more recent research has revealed that these vectors may also infect more permanent environments with vegetation cover, such as rice fields (Orondo *et al.,* 2023a). Certain volatile molecules that vegetation produces can also serve as attractants or repulsants. Terpenoid and alcohol-based chemicals have been combined to create attractants (Torres-Estrada *et al.,* 2005). In addition, irrigation has been observed to increase bacterial abundance and diversity in larval habitats (Orondo *et al.,* 2023b). Information on the larval attractants, deterents, and possible larvicidal bacteria will provide data on possible larval control tools for further exploration.

1.4 Hypothesis

1.4.1 Null Hypothesis

Changes in the ecosystem as a result of irrigation has no impact on malaria vector larval ecology, larval survivorship and subsequently does not cause insecticide resistance in malaria vectors as a result of insecticide use in agriculture, veterinary and public affecting their malaria transmission potential.

1.5 Objectives

1.5.1 General objective

To determine the effect of irrigation on malaria vector larval ecology, metabolite and bacterial composition and insecticide resistance in mixed crop irrigation in Homa Bay County, Western Kenya.

1.5.2 Specific objective

i) To determine the ecology of malaria larval vectors, metabolite, and bacterial composition of larval habitats in Homa Bay County.
ii) To evaluate the effect of agricultural, veterinary and public health insecticide use on larval survivorship in Homa Bay County.
iii) To determine insecticide resistance biomarkers (*kdr* and *ACE1* genes and changes in metabolic enzyme levels) in malaria vectors in Homa Bay County.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Anopheles* mosquito

Mosquitoes are biting insects of public health importance and are classified in the order Diptera and family Culicidae. These insects are known to belong in two major sub-families of the *Anophelinae* and *Culicinae*. They transmit viral and parasitic illnesses to humans. In sub- Saharan Africa, the *Anophelinae* group is known to be the major vectors of malaria (Coetzee *et al.,* 2000) and O'nyong' nyong virus (Johnson, 2019; Nanfack Minkeu & Vernick, 2018). However, other genus of mosquitoes transmit other diseases of medical importance like filariasis (Dietrich *et al.,* 2019; Famakinde, 2018) and arboviruses e.g. Zika virus, yellow fever, Chikungunya, Dengue virus, West Nile virus (Whiteman *et al.,* 2020). The Anopheline mosquitoes comprise of a complex of several mosquito species including *Anopheles gambiae* and *Anopheles funestus*, both of which exists as sub-species with different vectoral capacity in different regions (Garrett-Jones & Shidrawi, 1969; Takken & Lindsay, 2003).

There are known approximately 3000 species of mosquitoes with only about 100 species being important in public health (Eldridge, 2009; Rozendaal, 1997). The Anopheline group is composed of about 400 species occurring in nature (Rozendaal, 1997) with about 60 species capable of transmitting malaria (Rozendaal, 1997). The other *Anopheles* mosquitoes are known to be vectors of filariasis and viral diseases. In most cases, malaria transmission in a locality is usually dominated and driven by two or three important vector species that are ecologically adapted to reproduce and survive in the area (Coetzee *et al.,* 2000). For instance, *An. gambiae* sensu stricto (s.s.) Giles and *An. arabiensis* Theobald usually occur in sympatry over large geographical ranges and associate strongly with the traditional rural life of many African communities (Coetzee *et al.,* 2000). In sub-Saharan Africa, the most important and predominant malaria vectors include *Anopheles gambiae*, Giles complex (*An. gambiae* s.s., *An. arabiensis, An. merus,* and *An. melas), An. funestus* Giles complex and *An. pharoensis* Theobald (Service, 1993). Nine sibling species of

the *An. gambiae* Giles complex are known to date (Coetzee *et al.,* 2013; White, 1971). Even within sub-species like *An. gambiae* s.s, further sub-divisions have been made defined by the different karyotypes and are known as chromosomal forms like Mopti, Savanna Bissau and Forest (Della Torre *et al.,* 2002). Of the several sub-species of *An. gambiae sensu lato* (*s.l.*), *An. gambiae* s.s. and *An. arabiensis*, have been incriminated as the major malaria vectors in sub-Saharan Africa. These mosquito vector species differ in their biology to enhance their survival within the population. For instance, *An. arabiensis* are known to be more exophilic, making it a lesser target for indoor residual spraying (IRS) with insecticides and other control strategies unlike the *An. gambiae* (Bayoh *et al.,* 2010; Tirados *et al.,* 2006). However, recent studies have shown both endophilic and exophilic behavior (Atia *et al.,* 2022).

Anopheles funestus Giles complex consists of at least eleven species that are morphologically identical but differ in vectoral behavior which include *An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. rivulorum* Leeson, *An. parensis* and *An. leesoni* Evans which occur in Kenya (Kamau *et al.,* 2003; Muturi *et al.,* 2009).

2.1.1 Mosquito Species Identification

Prior to implementation of any vector control strategy in a region, target species identification is paramount so as to shape necessary deployment policies. Correct identification of specific vector species is thus crucial so as to differentiate non-vector species from vector species. This helps to save time and resources that would otherwise be used to control non- vector species. Moreover, related mosquito species may show different levels of susceptibility to different mosquito insecticides (Ramphul *et al.,* 2009), thus underlining the importance of correct taxonomic classification. Currently, the widely used technique for species identification is Polymerase Chain Reaction (PCR). This technique provides markers that can be used in diagnostic assays (Collins & Paskewitz, 1996) so as to distinguish members of the *An. gambiae* complex (Bass *et al.,* 2008; Scott *et al.,* 1993).

There are known approximately over 450 *Anopheles* mosquito species have been described with only about 50 of these capable of malaria transmission (Raghavendra

et al., 2011). Malaria transmission dynamics is therefore vital in the control of this vector-borne disease. Therefore, precise identification of the Anopheline species is essential in determining the degree of contact between the vectors and the host populations thus assessing risk of human exposure and changes in *Anopheles* blood meal preference (Githeko *et al.,* 1994). This helps in identifying risk of malaria transmission and human mosquito bite exposure.

2.1.2 Anopheline species distribution in Kenya

In Kenya, the *Anopheline* species are widely distributed **(Figure 2.1)** with the main malaria vectors being complexes of *Anopheles gambiae* and *An. funestus*. Morphological identification of these species is usually the fist tool for identification, however, as these vectors exist as complexes, molecular identification is essential for identification of sibling species.

Anopheles gambiae s.s. is mostly found in Western Kenya (Machani *et al.,* 2020; Okara *et al.,* 2010; Wamae *et al.,* 2015) and Coast region (Kiuru *et al.,* 2018; Mwangangi *et al.,* 2007; Okara *et al.,* 2010). In Central Kenya, *An. arabiensis* were observed to be of higher density in the irrigated rice regions than the non-irrigated regions (Muriu *et al.,* 2008; Okara *et al.,* 2010). *An. merus,* though not a dominant species, occurs along the Kenyan coast (Kipyab *et al.,* 2013, 2015; Mbogo *et al.,* 2003; Okara *et al.,* 2010) while *An. funestus* complex is distributed at the Coast (Mwangangi *et al.,* 2007; Okara *et al.,* 2010), in Western Kenya (Machani *et al.,* 2020; Munga *et al.,* 2009) and in Central Kenya (Kamau *et al.,* 2003; Muturi *et al.,* 2009).

However, there is scarcity of information on the vectors in Homa Bay, but recent studies show that the major vector in this area is *An. arabiensis* and *An. funestus* (Abong'o *et al.,* 2018; Ondeto *et al.,* 2022; Orondo *et al.,* 2021). Previously, before the implementation of IRS, there were observed significant numbers of *An. gambiae* s.s. (Omondi *et al.,* 2015). These vector species however, was drastically depleted after IRS was conducted in 2018. This study sought to shed light on the major malaria species in Homa Bay.

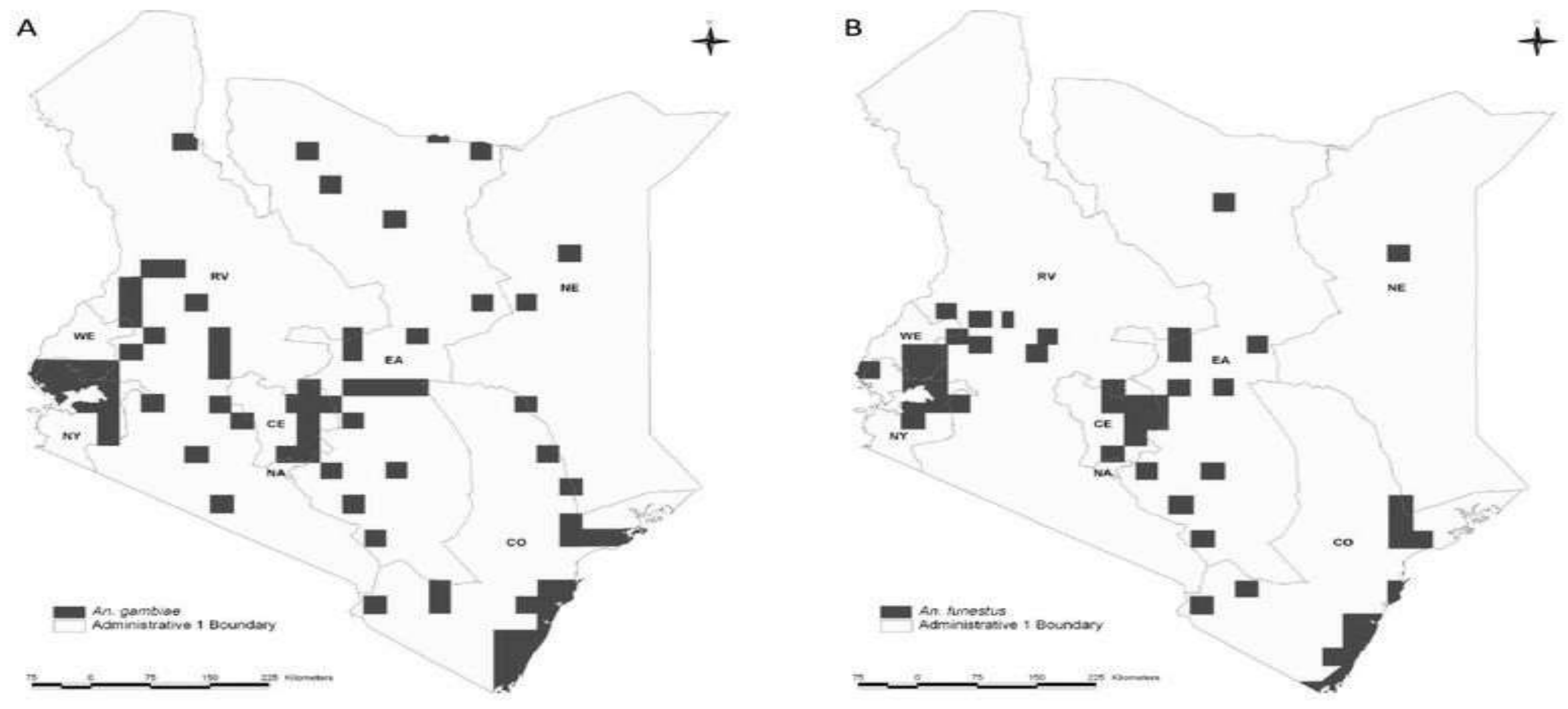


Figure 2.1: Map of Kenya showing the distribution of *Anopheles* complexes (Okara et al., 2010).

(a) *An. gambiae* sensu latu (s.l.) and (b) *An. funestus* s.l. as per previous administrative units (provinces). CE = Central Province; CO = Coast Province; EA = Eastern Province; NA = Nairobi Province; NE = North Eastern Province; NY = Nyanza Province; RV = Rift Valley Province; and WE = Western Province (Adapted from (Okara *et al.,* 2010).

2.2 Malaria vector abundance

Adult vector abundance is directly proportional to the availability and productivity of larval habitats. In addition, the proximity of these habitats to human dwellings has been observed to greatly determine malaria transmission (Awono-Ambéné & Robert, 1999; Edillo *et al.,* 2007; Shililu *et al.,* 2003). Human activities have been observed in several studies to have a major impact on mosquito abundance (Chaves *et al.,* 2021; Finda *et al.,* 2019). This is mostly impacted by land use in an effort to try and carter for the ever growing population. Studies in Kenyan highlands have shown that high *An. gambiae* larval survivorship was observed in habitats located in deforested areas and cultivated swamps as compared to those in the forested area (Minakawa *et al.,* 2005; Munga *et al.,* 2006). Generally, larval habitats and subsequently adult mosquito abundance is directly related to rainfall (Hii *et al.,* 1997) land topography and water drainage, which in most cases are driven by land use (Minakawa *et al.,* 2005; Shililu *et al.,* 2003) in addition to other factors like predators (Munga *et al.,* 2007).

Homa Bay County has in the recent past taken up irrigation and building of dams as a means of livelihood (Omondi *et al.,* 2022b). This has greatly destabilized the eco-system and thus resulted in the formation of additional habitats and thus increased mosquito population (Ondeto *et al.,* 2022).

2.2.1 Larval habitats

Adult female mosquitoes are known to lay their eggs in aquatic habitats with favorable conditions to support the survival, development and maturation of their juveniles (Day, 2016). Eggs are usually laid near the edge of the habitat as this provide for a high food supply from the algae. The following are factors that are considered before oviposition; type, size and age of the aquatic habitat (Blaustein *et al.,* 2004; Day, 2016; Torrisi & Hoback, 2013), availability of food for the immature (Blaustein & Kotler, 1993; Day, 2016), presence/ absence of predators and their population (Blaustein & Kotler, 1993; Cohen & Silberbush, 2021), presence/ absence of competitor and con- specific larvae (Day, 2016; Yoshioka *et al.,* 2012). This

results in various types of habitats being preferred by different vector species ranging from natural habitats to artificial habitats.

Studies have shown that different species prefer different habitat types with specific characteristics for oviposition. Previous studies have shown that *An. gambiae* prefer to lay their eggs in small shaded temporary, sun-lit pools with low emergent plants (Kweka *et al.,* 2012) like animal hoof-prints, small water puddles, tire tracks and ditches. *An. funestus*, however, prefer more stable habitats with emergent vegetation (Minakawa *et al.,* 2008) like marshy pools.

2.2.2 Habitat stability

In sub-Saharan Africa, aquatic habitats for malaria vectors are numerous and widely distributed, especially during the rainy seasons. Therefore, larval control using bio-control or environmental modification is non- effective and impractical when applied in all habitats. A more convenient way is to consider a targeted approach of only productive habitats (Fillinger *et al.,* 2003).

For effective larval source management (LSM), an understanding of the duration over which a habitat remains aquatic is important in addition to the productivity of the habitat (Orondo *et al.,* 2023a). This will help focus on more stable and productive habitats and not waste useful resources on unproductive habitats. This is because stable and productive habitats are a source of stable malaria transmission. Habitat stability is determined by several factors including habitat surface area size (Minakawa *et al.,* 2005), presence/ absence of canopy cover (Afrane *et al.,* 2012), weather conditions (Afrane *et al.,* 2012), habitat substrate type (Soleimani-Ahmadi *et al.,* 2013), among other physical conditions. This in turn affects the densities of pupae and subsequently adult mosquito populations (Minakawa *et al.,* 2005). *An. gambiae* larvae usually occur in small, temporary and sunlit pools (Gillies & De Meillon, 1968; Minakawa *et al.,* 1999), which run the risk of drying up quickly (Minakawa *et al.,* 2001) especially during the dry weather. On the contrary, larger habitats are more stable but are infested with larval predators (Service, 1977).Therefore, temporary habitat may be unproductive due to faster evaporation

while more permanent habitats may also remain unproductive due to high predation rates

2.2.3 Larval habitat productivity

Anopheles gambiae complex is known to use diverse small water bodies as larval habitats (Service, 1993). These habitats differ in physical as well as biological characteristics, which directly influence the distribution and abundance of larval mosquito populations (Minakawa *et al.,* 1999; Okech *et al.,* 2007) demonstrated that productive mosquito aquatic habitats as dictated by soil organic matter content and microbial activity, contributes to fitness and vector competence.

Adult female mosquitoes lay eggs on stagnant or slow moving waters for undistracted and complete development of the larvae and pupae e.g. old tires, rain barrels, streams, ponds, ditches, unused swimming pools, tree holes, flower pots and vases, roof gutters, sewage and industrial waste ponds among others (Day, 2016). Oviposition is also affected by the presence and density of predators in the habitat.

The biological and physicochemical conditions at the larval habitat also affect larval development finally affecting adult body size (Mwangangi *et al.*, 2007). In Homa Bay, it is anticipated that human activity has greatly affected larval habitat productivity and subsequently adult mosquito population. However, studies should be conducted to ascertain this hypothesis.

2.2.4 Larval habitat predator composition

Larval survivorship and maturation in the aquatic habitats are highly dependent on the status of the aquatic habitats. These aquatic habitats are a host to a variety of predatory and symbiotic insects. These predators play a major role in the regulation of malaria vectors before they emerge as adults. Studies have shown that several species have been identified to prey on mosquito immature stages including Coleoptera, Amphibians, Hemiptera, Odonata, fish, Arachnida, and Ephemeroptera (Dambach, 2020; Carlson *et al.,* 2004). These species affect larval densities, survival and maturation thus affecting adult mosquito populations and competencies. In

addition to predator populations, studies have shown that the densities of the vectors within a habitat affect the final habitat productivity of adult vectors (Lyimo *et al.,* 1992). It has been observed that the higher the density of larvae in a habitat the less productive the habitats is as compared to one that is not densely populated.

2.3 Malaria Transmission

After an infective bite from a female *Anopheles* mosquito, *Plasmodium* is transmitted to the host thus resulting in malaria. This mosquito-borne infection caused by *Plasmodium* parasites, causes a significant burden of disease, both globally and regionally (Kamau *et al.,* 2020; Varo *et al.,* 2020). In regions with high malaria transmissions, children under 5years and pregnant women are greatly affected (WHO, 2017). In Kenya, malaria prevalence varies between regions **(Figure 2.2)**. This disease has been observed to be endemic at the Coast and the Lake region with the later having malaria transmission all year round (KMIS, 2020). Homa Bay is classified as a malaria holo-endemic region with high entomological inoculation rates (EIR) of about 300 infectious bites per person per year (Onchiri, 2014) however malaria prevalence declined from 2003 to 2007 but then increased after 2007.

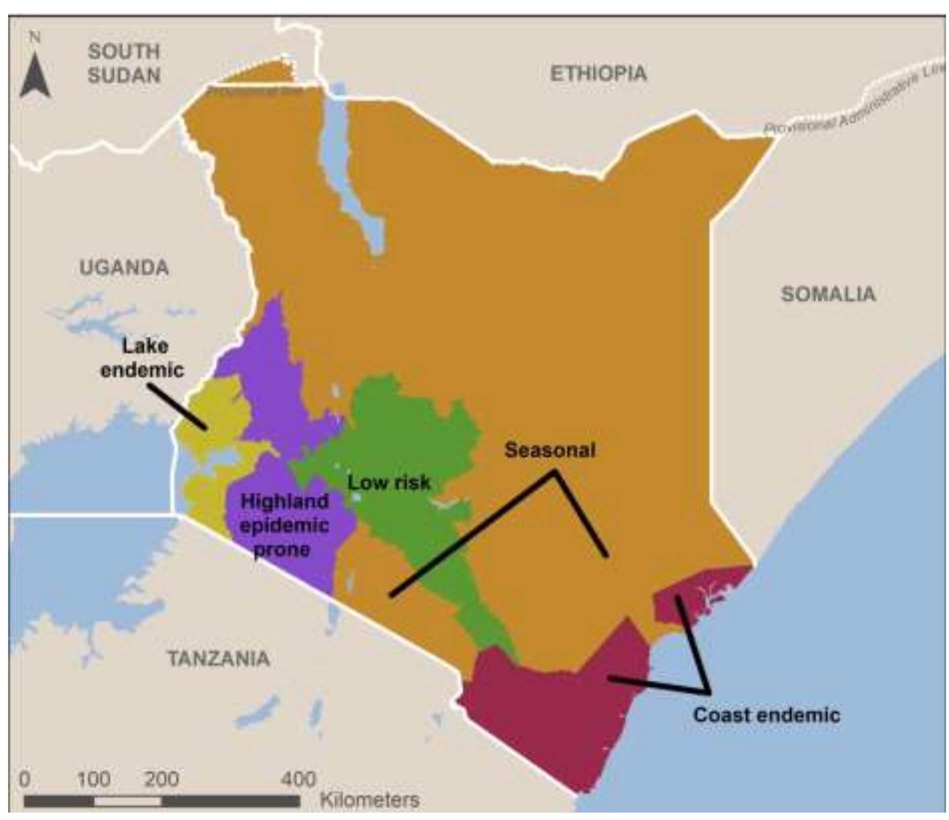


Figure 2.2: Map of Kenya showing the varied prevalence of malaria between regions (KMIS, 2020).

After the initiation of Roll Back Malaria Program by the World Health Organization (WHO), several countries outside the tropics have successfully eliminated malaria (WHO, 2008). In the tropics however, malaria has persisted and thus WHO has intensified the fight against malaria in these regions focusing on control measures (WHO, 2008).

Malaria vector control remains the main and most effective intervention strategy in malaria control programs. Kenya has conducted a massive distribution of ITNs country wide with priority given to the endemic areas of the Lake region and the Coast (KMIS, 2020). On average, 49% of all Kenyan households own at least one ITN with those in low risk areas having the lowest percentage ownership (KMIS, 2020). Orondo *et al.,* (2021) observed that bed net ownership in Homa Bay County was above 91% an indication that a greater proportion of the households owned bed nets.

2.4 Malaria Vector Control Strategies

Malaria vector control has been a major component in public health sector as a tool for reducing malaria transmission in the tropics (Beier *et al.,* 2018; Bhatt *et al.,* 2015). Several control measures that target different developmental stages of the mosquito lifecycle have been adopted to curtail the growth, development, maturation and eventual dispersion of malaria vectors (Beier *et al.,* 2018; Kamareddine, 2012). The current trend in malaria vector control is the adoption of Integrated Vector Management (IVM) strategy which involves a systemic approach to planning and implementation of vector control measures (Beier *et al.,* 2008). IVM is a rational decision-making process for the optimal use of resources in the management of vector populations, in order to reduce or interrupt transmission of vector-borne diseases (Beier *et al.,* 2008). These vector control measures include those targeting adult vectors such as use of insecticide treated nets (ITNs), indoor residual spraying (IRS), long-lasting insecticides` treated nets (LLITNs) and those targeting premature stages especially larvicides (Asale *et al.,* 2019). Several studies have observed success in vector control after implementation of IVM strategies in the field set-up (Asale *et al.,* 2019; Fillinger *et al.,* 2009; Mutero *et al.,* 2015).

In Kenya, the primary malaria vector control strategy has been use of LLITNs and IRS being the secondary method (Hawley *et al.,* 2003; Abong'o 2019). LLITNs have been widely distributed countrywide including Homa Bay. These LLITNs are impregnated with pyrethroids as the primary chemical component (Omondi *et al.,* 2022a). IRS was also done in Homa Bay for three consecutive years from 2018 to 2020 using Actellic 300CS which is an organophosphate (Abong'o *et al.,* 2020). Understanding the long term resulting effect of the available control strategy is vital for downstream implementation of vector control methods.

2.5 Insecticide Resistance

Resistance is defined as 'the developed ability in a strain of insects to tolerate doses of toxicants that would otherwise prove lethal to the majority of individuals in a normal population of the same species' (IRAC, 2003; Tabashnik *et al.,* 2014). This can arise due to either change in the gene sequence in the target site, increased

insecticide metabolic rates by the mosquitoes, cuticular modifications, or behavioral changes of the vector (Hemingway *et al.,* 2004) **(Figure 2.3)**.

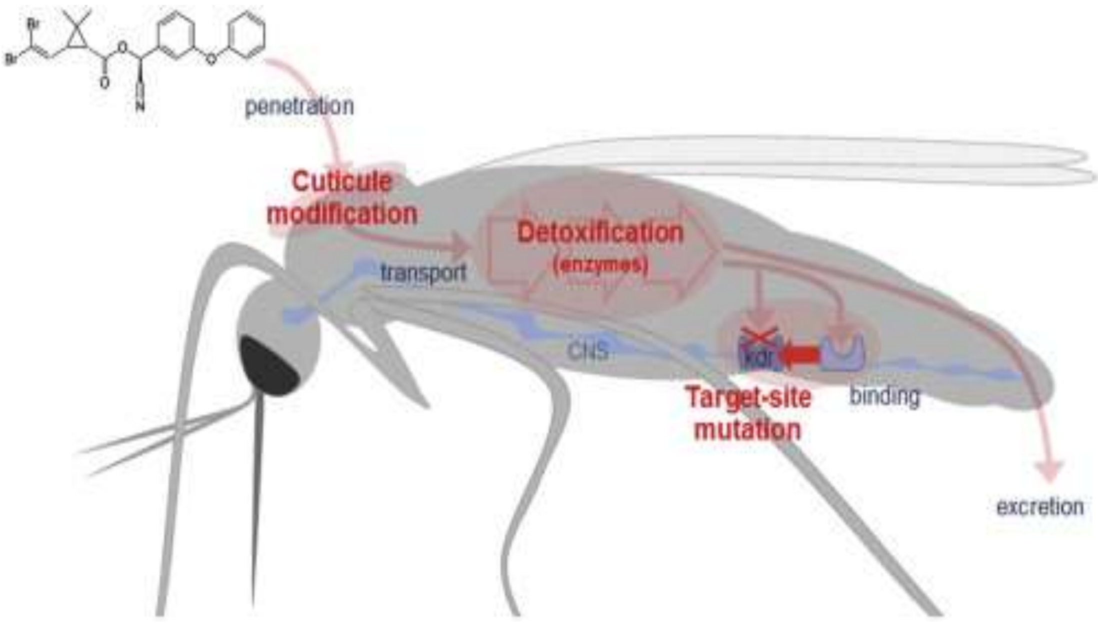


Figure 2.3: Resistance mechanisms: Mechanisms by which insects counter the effect of insecticides. Cuticular modification reduces the amount of insecticide that would otherwise penetrate into the body of the insect. The amount that is capable of passing through is transported to the CNS for detoxification by enzymes and later excreted from the body. The rest which manages to get to the target site is not able to bind to the target site leading to its elimination from the body (Nkya *et al.,* 2013)**.**

Insecticide resistance has been reported to be associated by environmental factors including agriculture, urbanization, xenobiotics, microbial community in the environment and vector control tools (Nkya *et al.,* 2013) (**Figure 2.4**). Most of these vector control strategies involve the use of chemicals which are xenobiotics (Hemingway *et al.,* 2002). The use of IRS and LLITNs are widely implemented methods of vector control (WHO, 2018; WHO, 2006). Insecticide resistance, especially against pyrethroids which are the major class of chemical insecticide used on all approved LLITNs and in most IRS programme worldwide has been confirmed in some parts of sub- Saharan Africa in the mosquitoes (https://anopheles.irmapper.com/). Should this be allowed to spread to other parts where resistance has not been reported, it would threaten the sustainability and

operational impact of IVM programmes. However, World Health Organization (WHO) is encouraging the use of another insecticide class in areas where LLITNs have been deployed as a measure against the rapidly growing insecticide resistance (WHO, 2014).

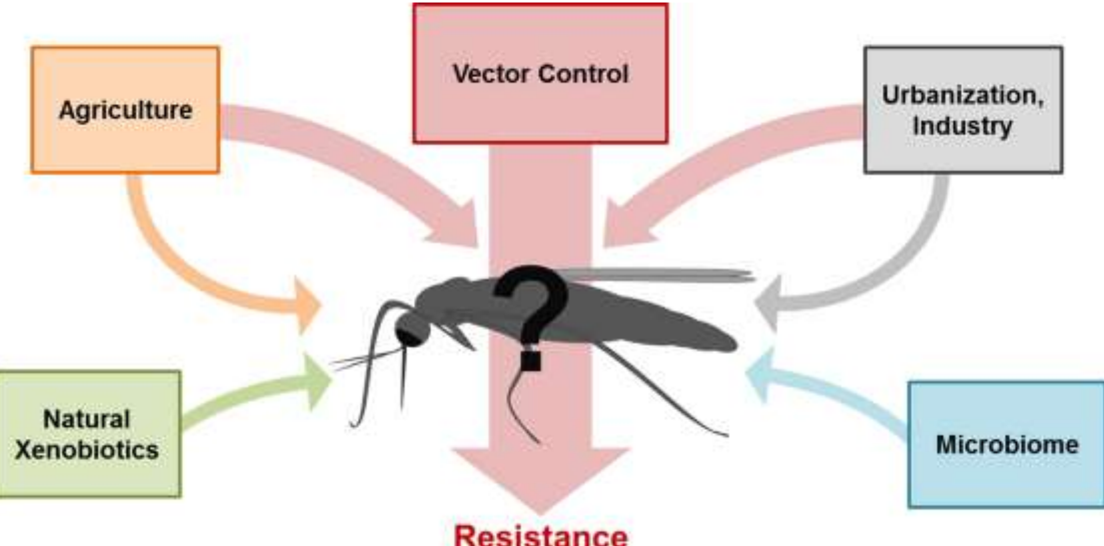


Figure 2.4: Impact of environment on mosquito response to pyrethroid insecticides (Nkya *et al.,* 2013).

Insecticide resistance has been reported in malaria vector species, including *Anopheles gambiae* s.s., *An. funestus* and *An. arabiensis* as a result of wide scale use of mosquito insecticides (https://anopheles.irmapper.com/). WHO has reported that 61 countries had detected resistance against one class of insecticide while 18 countries have reported resistance against all four classes (pyrethroids, organophosphates, organochlorides, and carbamates) between 2010 and 2016, 12 of which are in the sub- Saharan Africa (WHO, 2018). It has also been suggested that the development of resistance could also be associated with cross resistance due to larval intervention by treatment of larval habitats using different chemicals (Barbosa *et al.,* 2018).

There has been observed four types of resistance mechanisms in malaria vectors (WHO, 2018). These include metabolic resistance whereby enzyme metabolism is enhanced and detoxification is sped up to reduce the chemical's toxicity (Vontas *et*

al., 2020). Target-site resistance occur when a genetic mutation modifies the chemical receptor protein thus blocking or reducing the toxic effect of the insecticide (Hancock *et al.,* 2022; Hemingway *et al.,* 2004). Cuticular resistance (reduced penetration) occurs when the absorption of insecticide is reduced due to changes in the mosquito's cuticle (Balabanidou *et al.,* 2018; Balabanidou *et al.,* 2016; Xu *et al.,* 2020). Studies have reported an emerging new adaptation in mosquitoes when faced with domestic interventions, the development of behavioral avoidance (Gatton *et al.,* 2013; Kreppel *et al.,* 2020; Sanou *et al.,* 2021) whereby the insect avoids spaces with insecticides (WHO, 2018). The change in mosquito vector behavior has thus resulted in reduced contact between vectors and insecticides and hence reduced effectiveness of the malaria intervention programmes (WHO, 2018). Resistance can thus arise due to either change in the gene sequence in the target site resulting in either East or West Africa knockdown (*kdr*) mutation or *ACE-1* gene mutation in mosquitoes, increased insecticide metabolic rates by the mosquitoes, behavioral changes, or cuticular changes of the vector (WHO, 2018).

Currently, there are four classes of insecticides authorized by the WHO to be used in malaria vectors control which include pyrethroids, Dichlorodiphenyltrichloroethane (DDT) which is the only organochlorine being used, organophosphates and carbamates (WHO, 2011). Pyrethroids and DDT have been seen to share a common target site which is the voltage –gated sodium channel (VGSC) and this can result in cross resistance in case of mutations at this target site (Ranson *et al.,* 2000). Carbamates and organophosphate however inhibit acetyl cholinesterase (AChE) enzyme encoded by *ACE-1* gene resulting in the blockage of synaptic neurotransmission in *An. gambiae* (Weill *et al.,* 2004).

In Africa, insecticide resistance has been reported to be widespread in West Africa (Djègbè *et al.,* 2011; Hunt *et al.,* 2011; Namountougou *et al.,* 2019). There has also been evidence of resistance to some insecticides in various regions that seem to be localized to specific regions. In Mozambique, for instance, *An. funestus* s.s. remained fully susceptible to DDT and Malathion despite a high level of pyrethroid (lambda-cyhalothrin) resistance in *An. funestus* s.s. populations in Southern Mozambique (Casimiro *et al.,* 2014). In South Africa, *An. funestus* was found to be resistant to

pyrethroids (Amenya *et al.,* 2008; Hargreaves *et al.,* 2000) while in Tanzania (Kulkarni *et al.,* 2006; Matowo *et al.,* 2010; Pinda *et al.,* 2020) and Sudan (Ismail *et al.,* 2018; Korti *et al.,* 2021) *An. arabiensis* was shown to have developed resistance to pyrethroids. In Kenya, studies indicate a reduced susceptibility to pyrethroid insecticides in *An. gambiae* s.l. in Western Kenya (Githinji *et al.,* 2020; Orondo *et al.,* 2021; Owuor *et al.,* 2021). Insecticide resistance is currently spreading across the country as observed by Ondeto *et al*., (2017) **(Figure 2.5)**.

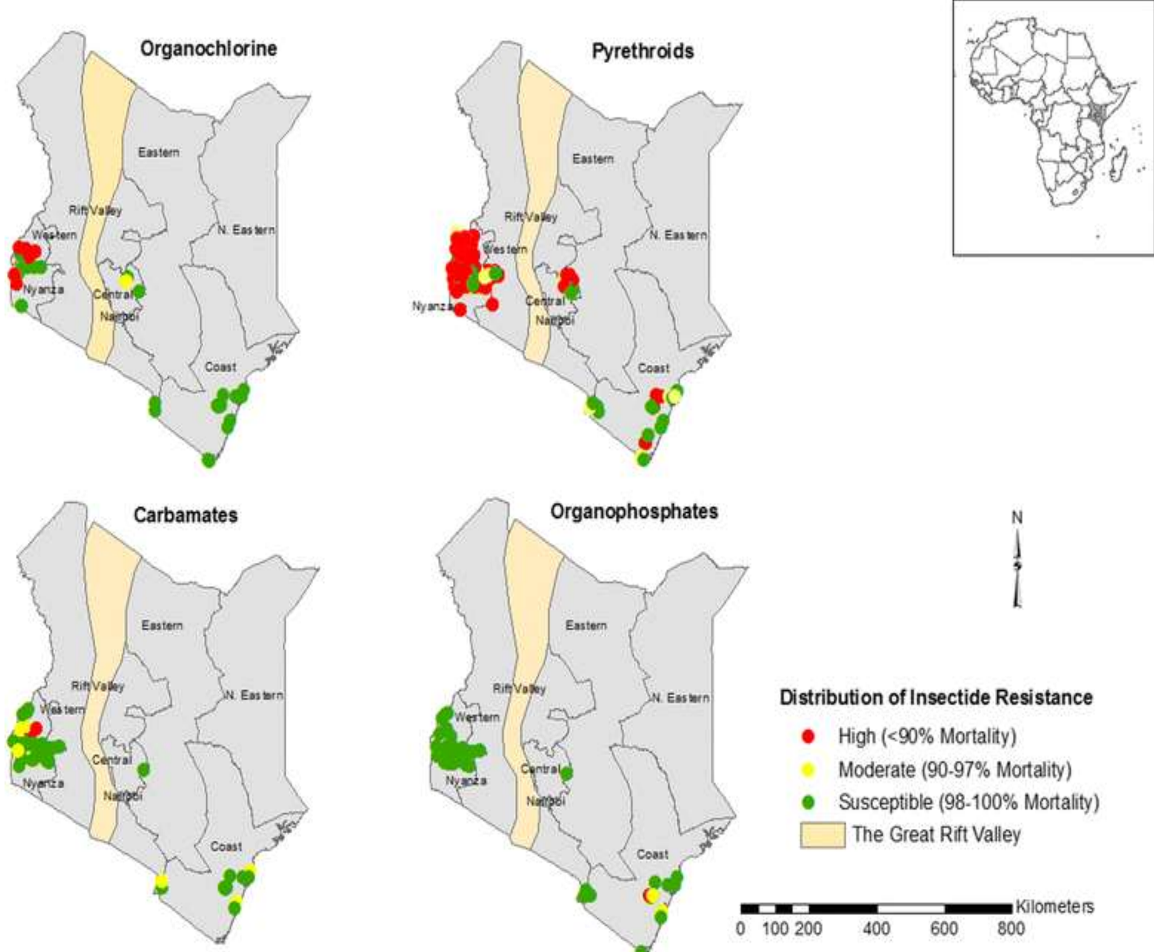


Figure 2.5: Distribution of insecticide resistance in Kenya (Adapted from (Ondeto *et al.,* 2017).

As a result, there is need to continuously monitor resistance against all the classes of insecticides and understand the different mechanisms responsible for the widespread levels of resistance. This is essential for downstream implementation of vector

control strategies. This will keep malaria incidences in check and not jeopardize the efforts so far put forth towards the reduction of malaria incidences.

2.5.1 Resistance Mechanisms

Four main resistance mechanisms have been proposed however, only two have been extensively studied and reported (Corbel *et al.,* 2007; Hemingway & Ranson, 2000). The first is propelled by changes at the insecticide target site resulting in mutations (knock down rate, *kdr* mutations) (Corbel *et al.,* 2007). Pyrethroids and DDT insecticides act against the sodium ions (Na^+) channels, disrupting their operation (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000). Acetyl cholinesterases on the other hand are the target for organophosphates and carbamates action (Hemingway *et al.,* 1986). The other mechanism is through increased rate of insecticide metabolism (Hemingway & Ranson, 2000). The rate of insecticide metabolism can be increased due to changes in enzyme composition due to overproduction of the enzyme or alteration in the catalytic activity of the enzyme (WHO, 2018). The main enzyme groups involved in insecticide resistance are the esterases, monooxygenases (P450s) and glutathione-S-transferases (GST) (Hemingway & Ranson, 2000; WHO, 2018).

Monooxygenases of the CYP6 family has been observed to be over expressed in pyrethroid resistant strains (Amenya *et al.,* 2008). Over-expression of this enzyme family has also been observed in pyrethroid resistant *An. funestus* which has been associated with thickened cuticle. Cuticle thickening results in slow insecticide penetration thus increased detoxification of the insecticide (WHO, 2018; Wood *et al.,* 2010).

With the impact of insecticide resistance on ITNs not being clear yet, the different mechanisms of resistance have been studied in different regions where resistance have been seen (N'Guessan *et al.,* 2007). In Kenya, metabolic resistance (Ochomo *et al.,* 2013) and voltage-gated Na^+ channel knock down resistance (*kdr*) (Ochomo *et al.,* 2015; Orondo *et al.,* 2021; Owuor *et al.,* 2021) to pyrethroid has been found in *An. gambiae*.

Pyrethroid resistance associated with target site insensitivity, *kdr*, has arisen independently at least twice in this species (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000). Widespread pyrethroid resistance in West Africa is due to a leucine-phenylalanine substitution at position 1014 of the sodium channel gene (L1014F *kdr* allele), in the S6 hydrophobic segment of domain II (Martinez-Torres *et al.,* 1998). A different mutation at the same amino acid position, causing a leucine- serine substitution (L1014S *kdr* allele), is associated with pyrethroid resistance in *An. gambiae* from Kenya (Ranson *et al.,* 2000).

To counter the wide-spread insecticide resistance, use of a different class of insecticide is encouraged for use in IRS and carbamates are gaining importance in IRS programmes (WHO, 2012). Sequential use of different classes can be applied. This involves the use of a single insecticide until its effect is reduced then switching to another insecticide with a different mode of action. A combination of several insecticides with different modes of action can also be applied or using different classes in neighboring geographical regions (WHO, 2018). Several studies have also shown a single nucleotide substitution at position 119 in the *ACE-1* gene encoding for AChE from glycine to serine (G119S) (Binyang *et al.,* 2022; Diallo *et al.,* 2022; Elanga-Ndille *et al.,* 2019; Ngangue-Siewe *et al.,* 2022; Weill *et al.,* 2004).

Development of resistance has been shown to occur in some species but not in others (Ramphul *et al.,* 2009; Wang *et al.,* 2020). With resistance being a constantly evolving process that needs to be constantly monitored for better management and control, the current study will determine the distribution and mechanisms of insecticide resistance in malaria vectors in Homa Bay due to continuous use of agricultural pesticides and use of indoor malaria vector interventions including ITNs/ LLITNs and IRS. The results of this study will provide an insight and a better understanding of the effectiveness of the insecticides used in malaria vector control towards the reduction of malaria incidences in Kenya.

2.5.2 Contribution of Agriculture and Public Health to Insecticide Resistance

Use of chemicals in public health and agriculture has resulted in emergence and intensified insecticide resistance with agriculture being incriminated as the major

resistance driver (Antonio-Nkondjio *et al.,* 2011; Chabi *et al.,* 2018; Mouhamadou *et al.,* 2019). Emergence of insecticide resistant vectors has been observed with the continuous use of pyrethroids as a vector control tool (Lindsay *et al.,* 2021; Machani *et al.,* 2020; Pwalia *et al.,* 2019). Assessment of the susceptibility of malaria vectors to other classes of insecticides have been observed to vary in different regions. High susceptibility against organophosphates and carbamates have been reported in some regions (Antonio-Nkondjio *et al.,* 2011) however resistance to carbamates reported in other areas especially in cultivated regions due to metabolic resistance (Antonio-Nkondjio *et al.,* 2011). DDT and pyrethroid resistance has been observed in agricultural areas due to selection pressure as a result of agricultural pesticides (Antonio-Nkondjio *et al.,* 2011).

In addition the use of the pesticides in farms lead to early exposure of the vectors still in breeding habitats. Studies have shown that juveniles of malaria vectors can develop insecticide resistance even before they emerge as adults (Li *et al.,* 2021; Wang *et al.,* 2020). It is therefore essential that both the agricultural sector and the public health sector collaborate for the effective management of insecticide resistance in the malaria vectors (Matowo *et al.,* 2020) and other pests.

2.5.3 Monitoring Insecticide Resistance

Over the years, WHO has produced and published several guidelines and instructions for investigating for the presence of insecticide resistance. This includes the use of a standardized bioassay technique in adult mosquitoes (WHO, 1998, 2016). Generally, WHO recommends that insecticide resistance is characterized as follows: Susceptibility is thus seen when the mortality is recorded to be between 98% - 100%. Mortalities less than 98% are a representation of a possibility of resistance development and further tests should be done to confirm. Mortality less than 90% is evidence of resistance in the test species (WHO, 2016). To undterstand the mechanisms of insecticide resistance, further tests should be conducted including genetic mutations and changes in enzymatic levels.

Susceptibility to all the four insecticide classes (organophosphates, organochlorine, carbamates, and pyrethroids) ought to be monitored frequently. Insecticide resistance

management strategies must be implemented before the resistant gene becomes common and stable in the population; otherwise, the gene will not recede. If nothing is done and insecticide resistance eventually leads to widespread failure of the insecticides, the progress achieved so far in reducing the burden of malaria would be lost. This would result in IVM failure and reduced effectiveness of malaria control resulting in increased malaria incidences of malaria morbidity and mortalities.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

3.1.1 Homa Bay County

This study was part of the bigger study to evaluate the effect of environmental modification in sub- Saharan Africa with regard to the changing epidemiology, transmission and pathogenesis of malaria vectors. The study was conducted in randomly selected cluster villages in irrigated and non- irrigated areas of Homa Bay County, Western Kenya where mosquito larval samples were collected. All experimental works were conducted at the sub- Saharan Africa, International Center of Excellence for Malaria Research (ICEMR), Homa Bay, located within Tom Mboya University and at the Centre for Disease Control (CDC), Kisumu.

Homa Bay County (0.6221° S, 34.3310° E) is located in the Southern part of Nyanza, along the shores of Lake Victoria's Winam Gulf which is the largest fresh water lake in Africa (**Figure 3.1**). This area experiences a semi-arid climate and is known to be malaria endemic with an altitude of 1,330m above sea level and humidity of 71%. Homa Bay County covers an approximate area of 3,183.3 km^2 with a population of about 1.13m million people as indicated by the 2019 Kenya Population and Housing Census (KNBS, 2019).

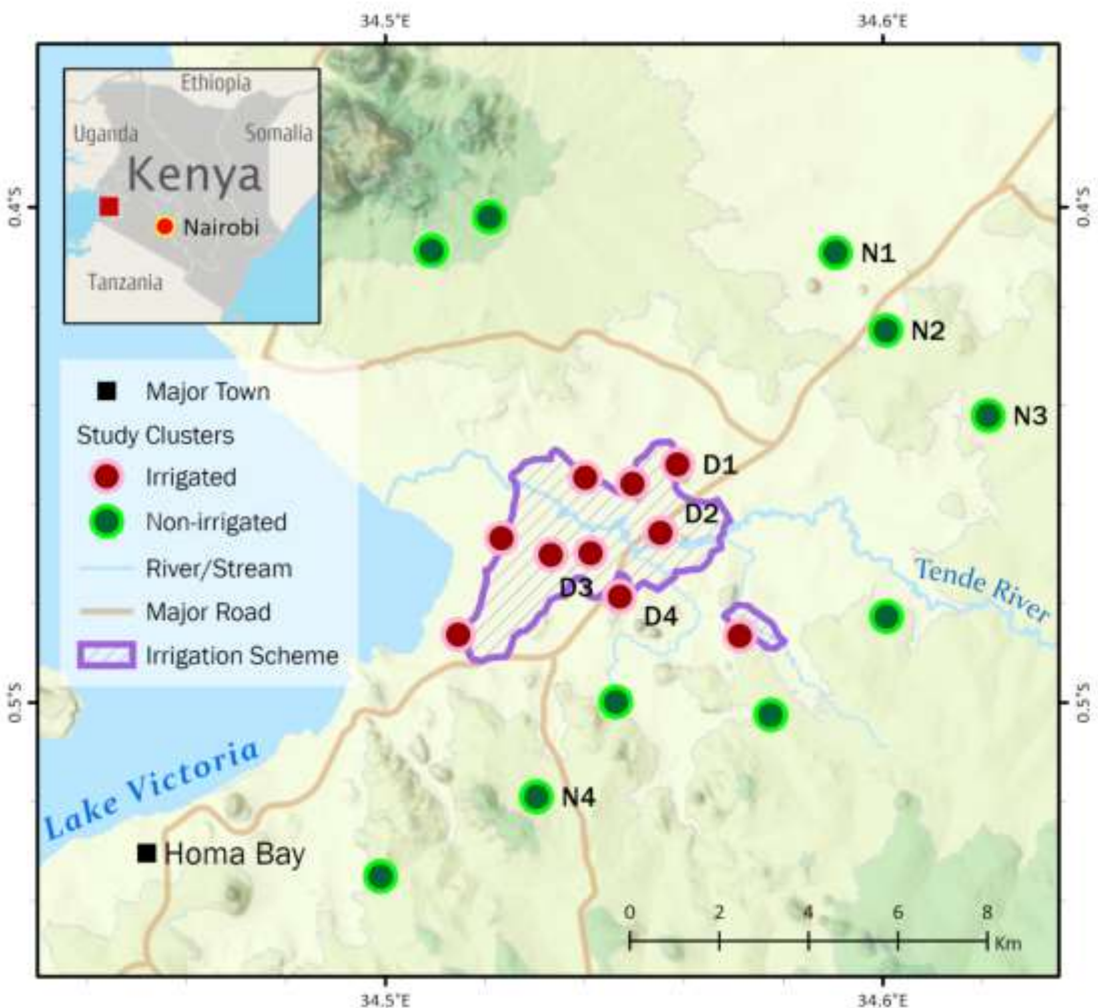


Figure 3.1: Map of Homa Bay study site showing the sampling clusters (coloured) in the irrigated and the non-irrigated ecosystems. D1-D4 and N1-N4 indicate clusters where monthly dynamics were conducted in irrigated and non-irrigated ecosystems, respectively (Orondo *et al.,* 2023a).

This region experiences an average annual temperatures and rainfall of 30°C and 1,100mm respectively with two rainy seasons; the long rains between March – May and the short rains between September – November. A concrete channel irrigation system was constructed by the Ministry of Environment, Natural Resources and Regional Development Authorities of Kenya and was named Kimira- Oluch Smallholder Farm Improvement Project (KOSFIP). This was done to aid in crop production for subsistence and cash crop farming. The local community practice crop and animal farming in addition to fish farming. The irrigation waters is used to assist

in food production in individual farms which mainly practice mixed crop farming as opposed to single crop farming (**Figure 3.2**) where several crops are grown in a single piece of land.

Figure 3.2: An example of a mixed- crop irrigated farm in Homa Bay.

The main crops grown in this area includes maize, millet, beans, groundnuts, fruits, rice and cotton while the major livestock kept in this area include cattle, goats, sheep and donkeys. Fish farming is mainly practiced along the lake shores and in the irrigated areas of the County. The communities in this area live in houses made of iron sheet roofs with mud or stone walls.

The main malaria vectors are *An. gambiae* s.l. and *An. funestus* s.l. (Abong'o *et al.,* 2018; Abong'o *et al.,* 2020; Ondeto *et al.,* 2022). Over time, malaria control in this area relied on distribution of insecticide treated nets. However, in 2018-2020 indoor residual spraying (IRS) using Actellic ® 300SC (organophosphate) was conducted resulting in significant reduction of *An. funestus* s.l. (Abong'o *et al.,* 2020) population.

This study was done in the irrigated and non- irrigated areas of Homa Bay County. The non- irrigated areas are located over 2km from the irrigated area. Samples were

collected from randomly selected village clusters. A total of 20 survey clusters were selected for this study; 10 each from irrigated and non-irrigated areas (**Figure 3.1**).

3.2 Larval ecology study framework

The larval ecology study was undertaken using the framework as described by (Githeko *et al.,* 2012). This framework allows for larval habitat profiling in terms of availability, stability, and productivity. These features can be explored to allow for targeted applications of larval source management, larval control. This framework was used in the irrigated and non-irrigated areas of Homa Bay to determine the impact of environmental modification on larval ecology. The irrigated area is defined as an area with either concrete- or earth-lined irrigation canals which is a source of water supply to the farmlands and originates from the main water supply. The non-irrigated area on the other hand, is an area with no irrigation canals to assist in agriculture yet this area also practices agriculture. These two ecosystems were selected with a boundary of at least 2 km between them.

The two ecosystems were further sub-divided into 10 clusters, drawn from the local villages. Larval sampling was done in 2018 and 2019 from the irrigated and non-irrigated areas as shown in **Figure 3.1**. Every encountered habitat was classified as either temporary, semi-permanent, or permanent. Temporary habitats were those habitats that could continuously hold water for approximately 2 weeks, while semi-permanent habitats were those that remained aquatic for a season (2-3 months). Permanent habitats were habitats that remained aquatic for up to 12 months (Mereta *et al.,* 2013).

3.3 Sampling

Before the commencement of the study, ethical approval was obtained and consent sort from relevant authorities including the County Government of Homa Bay, chiefs of the respective villages and villagers themselves (**Appendix I** and **Appendix II**). Village meetings were conducted to explain to the villagers what the study entailed and what samples will be followed and the protocols to be conducted.

Sampling was done in Homa Bay in 2018 (January - February, June - July, September- November), 2019 (January- December), 2021 (November – December) and 2022 (January) in the village clusters. This study followed both a repeated cross-sectional design and a longitudinal study design depending on the experiments to be conducted. The repeated cross-sectional study was conducted seasonally; during the dry season (January – February) and the long rains (June – July) in 2018 and 2019. All encountered habitats within a cluster and 200m outside the boundary of the cluster were sampled. A maximum of 20 dips at different spots were done for large extensive habitats while for smaller habitats, sampling was done as much as was possible.

During sample collection, all field-collected specimens were morphologically identified as *An. gambiae* s.l. using Gillies and De Meillon taxonomic keys (Gillies & De Meillon, 1968). These samples were then transported to International Center of Malaria Research (ICEMR) laboratory and Biochemistry and Pan African University (PAU) laboratories in Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Juja for laboratory processes.

3.3.1 Larval Collection

Larval sampling was done following the standard dipping technique with the help of a standard dipper of 350ml (WHO, 1975). Larvae were sort from all aquatic habitats, categorized as permanent, semi-permanent or temporary habitats. Permanent habitats included man-made ponds, swamps, concrete irrigation canals, fish ponds, and river edges. The semi-permanent habitats included drainage ditches, irrigated earth canals, natural ponds, and rice paddies. The temporary habitats included animal hoof/human foot-prints, tire tracks, road paddles, rock pools, rain pools, and water containers (**Figure 3.3**).

Figure 3.3: The diversity of habitat types surveyed in the study area in Homa Bay, County.

During the survey period, all the 20 clusters were visited and sourced for potential mosquito aquatic habitats during the seasonal surveys in 2018 and 2019 (January – February and June – July). Larval population dynamics study was conducted monthly in 2019 (January – December). All aquatic habitats were sampled, geo-coded, and documented. Several other important metadata associated with the habitats were also recorded. These included habitat types, habitat dimensions, and the characteristics of the surrounding environments. Details of the sampled larvae were also recorded which include the species as observed morphologically, larval stages, number of larvae sampled from each habitat, presence, type and densities of possible larval predators.

During sampling, *Anopheline* larvae were morphologically identified using (Coetzee *et al.,* 2000) protocol. These were then sorted to separate them from other mosquito larval species and predators using dropper pipettes. Other larval species were also identified and their larval stages recorded per habitat. The *Anopheline* species were transported to the laboratory indicating the place and date of collection in separate sealable and labeled bottles or 1.5ml eppendorf tubes for rearing or species identification respectively. Each collection site was geo-referenced and details entered in the Open Data Kit (ODK) on tablets. These samples were then taken to ICEMR laboratory for rearing to be used in the bioassay tests as per WHO recommendations and further laboratory processing.

3.3.2 Water Sampling

Water samples were collected in 2021 (November, December) and 2022 (January) in irrigated and non- irrigated areas. During each sampling season, approximately 15ml of habitat water was collected into plastic water bottles from larval infested habitats and non-infested habitats into plastic water bottles.

3.3.3 Predator Sampling

Aquatic habitats were surveyed for potential mosquito larvae predators which were carefully collected during the period of the predator experiment in 2019 (January, February). These predators were transported to the laboratory in plastic bottles containing water from the habitats and placed in cooler boxes. These bottles were not completely filled and the cap loosely fitted.

3.4 Initial Processing of the Samples from the Field

In the laboratory, some larvae were preserved in absolute ethanol for molecular analysis. These larvae were further observed under a microscope to confirm their morphological identification using morphological keys (Coetzee *et al.,* 2000). The water samples were stored at -20^{0}c awaiting bacterial, mineral and metabolite analysis.

The larvae sampled for rearing were brought into the insectary for sorting and removal of unwanted organisms (including other mosquito larval species and predators) that might have been sampled mistakenly. The *Anopheline* larvae were carefully put in clean larval trays and the water from the source habitat sieved using a cloth to eliminate undesired species that might be present in the water. After 24 hours, the larvae were further separated according to their larval stages, with the first and the second larval instar stages separated from the third and forth instar stages to reduce the risk of predation. Rain water (to avoid introduction of chemicals) was used to top- up the larval basins to provide for a conducive environment for larval development. During rearing, larvae from different habitats but belonging to the same ecological zone (irrigated or non- irrigated) were pulled together. These larval

basins were then placed in the larval rearing room in the insectary and the larvae were fed daily on Whiskas® cat food (Trademarks© Mars, Incorporated, McLean, Virginia, U.S.A.).

3.5 Experimental Techniques

3.5.1 Larval Ecology Study

3.5.1.1 Larval habitat availability and productivity

Larval habitat availability is defined as the presence of an aquatic habitat that is capable of harboring immature larval species (Githeko *et al.,* 2012). Larval habitat productivity, in terms of immature vectors, refers to the densities of immature vector species in each habitat type (temporary, semi-permanent, and permanent). A cross-sectional study was conducted, sampling larval habitats in the irrigated and non-irrigated areas in 2018 and 2019 (January, February, June and July). Larval sampling was done during dry season, in February to March and after the rainy season in June to July. All survey clusters (20) in the study area were visited to source for potential mosquito aquatic habitats. All encountered aquatic habitat was sampled, geo- coded, and documented with several important metadata such as habitat identifications, types, dimensions, and the surrounding environments recorded. In addition, sampling was also done 200m from the edge of each cluster. The encountered habitats were classified as either temporary, semi-permanent and as previously described. The temporary habitats included animal/human foot-prints, tire tracks/road paddles, rock pools, rain pools, and water containers, while semi- permanent habitats included drainage ditches, irrigated earthen canals, natural ponds, and rice paddies. Permanent habitats included man-made ponds, swamps, concrete-lined irrigation canals, fish ponds, and river edges. Larval sampling was done using standard larval 350ml dippers. The sampled mosquito larvae were identified using taxonomic keys by (Coetzee *et al.,* 2000) in the field and the sampled larvae counted. A subset of the sampled larvae was preserved in absolute ethanol, labeled, and transported to the laboratory for species identification by polymerase chain reaction (PCR). The rest of the sampled larvae in the field were returned to their original habitat from where they

were sampled. Larval densities of *Anopheles* and *Culex* larvae were recorded. The instar stages of the different *Anopheles* larvae species were also indicated.

3.5.1.2 Monthly habitat and larval population dynamics

Four survey clusters in each area (irrigated and non-irrigated) were randomly selected and visited monthly in 2019 (**Figure 3.1**). Larval sampling was done in all the aquatic habitats that were encountered within the cluster and 200 m from the edge of each cluster. All sampled habitats were geo- coded and documented with several important metadata as described previously, and habitat area size recorded at each survey. Larval sampling and morphological identification were also done through the same methods as previously described. Larval densities of *Anopheles* and *Culex* and the instar stages of different *Anopheles* larvae species were recorded. Predator presence, types and numbers were also recorded. Sampling was not done in July and December 2019 due to logistical challenges and heavy flooding in the study site respectively.

3.5.1.3 Habitat stability

Habitat stability is defined as the duration over which a habitat remains aquatic (Githeko *et al.,* 2012). A cohort of 100 semi-permanent and permanent aquatic habitats were selected in September 2018 and marked in the irrigated (50) and the non-irrigated (50) areas. The irrigated area habitats consisted of man-made ponds, swamps, river edges, concrete irrigation lining, drainage ditches, and fish ponds while the non-irrigated area was composed of swamps, river edges, natural ponds, drainage ditches, and man-made ponds. These habitats were followed every 2 weeks between September and November 2018 and then monthly in December 2018 to August 2019. During each visit, the aquatic status and the habitat characteristics of each habitat were recorded. Larval sampling and morphological identification were carried out. A sub-set of the sampled larvae were taken to the laboratory for PCR molecular speciation.

3.5.1.4 Habitat adult vector productivity using emergence traps

Habitat adult vector productivity is defined as a habitat that is capable of supporting immature stages of larval development until emergence into adults (Githeko *et al.,* 2012). The productivity of *Anopheles gambiae* vectors in their natural aquatic habitats was determined using modified emergence traps (Service, 1993). These traps were used to capture emerging adults from habitats. The surface area of the traps was 2.5 m^2 and these traps were used in habitats with a surface area that was greater than the surface area of the traps. The design of the emergence traps could not allow for oviposition by other gravid mosquitoes within the trapped area. Before the placement of the traps, habitats were sampled to ensure that the habitats contained *Anopheles* larvae. These traps were relocated every 2 days within the same habitats (**Figure 3.4**). Traps were placed in randomly selected spots within specific habitats. The selected habitats included 6 replicates of rice paddies, swamps, man-made ponds, drainage ditches, and fish ponds. This experiment was done for 10 consecutive days and replicated three times.

Every morning, larval sampling was done in the habitats with the traps and the larval densities were recorded. The emerging adults were also collected daily for 10 consecutive days for 3 months (September–November 2019). Emerged mosquitoes were collected daily using mouth aspirators into pre-labeled paper cups for morphological identification using taxonomic keys (Gillies & Coetzee, 1987) (Gillies & De Meillon, 1968). The productivity of the habitat was determined by the number of emerging female adult mosquitoes per habitat type per day.

A **B**

C

Figure 3.4: Emergence trap sampling with position of the emergence trap in the habitat (A) and sampling adult mosquitoes from the habitat (B) into the previously prepared paper cups for transportation and downstream analysis (C)

3.5.1.5 Overall adult vector productivity in each ecosystem

During sampling, dimensions of all habitats were taken by measuring its length (L) and width (W) in meters. This is termed the 'Original Area' (O). Previous field observations have reported the clustering of vector larvae within 2m from the edge of the habitat. This area is termed the 'Effective area' (R2/ R3) (**Figure 3.5**). Assuming 2α is 2m, this implies that 95.44% of larvae will be found within R2 ring area. However, if either L/2 or W/2 is less or equal to 2m, there is no R2 since the larvae will emerge within the whole area. As you move further towards the centre of the habitat, the fewer larvae are sampled. The area with larvae and much closer to the centre of the habitat and within 3m is known as R3.

The larval dipper cup used in this study (**Figure 3.5B**) is 13cm/ 5inches in diameter with capacity of 350ml. Standard dipping recommends a maximum of 20 dips. Therefore, the coverage will be:

$$\pi * 0.065^2 * 20 = 0.27m$$

This assumes that during a larval survey, larvae are collected from a total habitats area of 0.27m. However, larval distribution is not uniform from the shoreline to the centre especially in large permanent habitats thus it is assumed that larval distribution in large permanent habitats follows some sort of distribution, i.e. Gaussian distribution; half normal distribution. It is further assumed that in small temporary habitats (<0.27m), all larvae are sampled within the 20 dips during a survey while in large semi-permanent and permanent habitats, larval distribution follows the half normal distribution and 1α = 1m and 2α = 2m which implies that 95.44% of the larvae will be found within 3m from the shoreline.

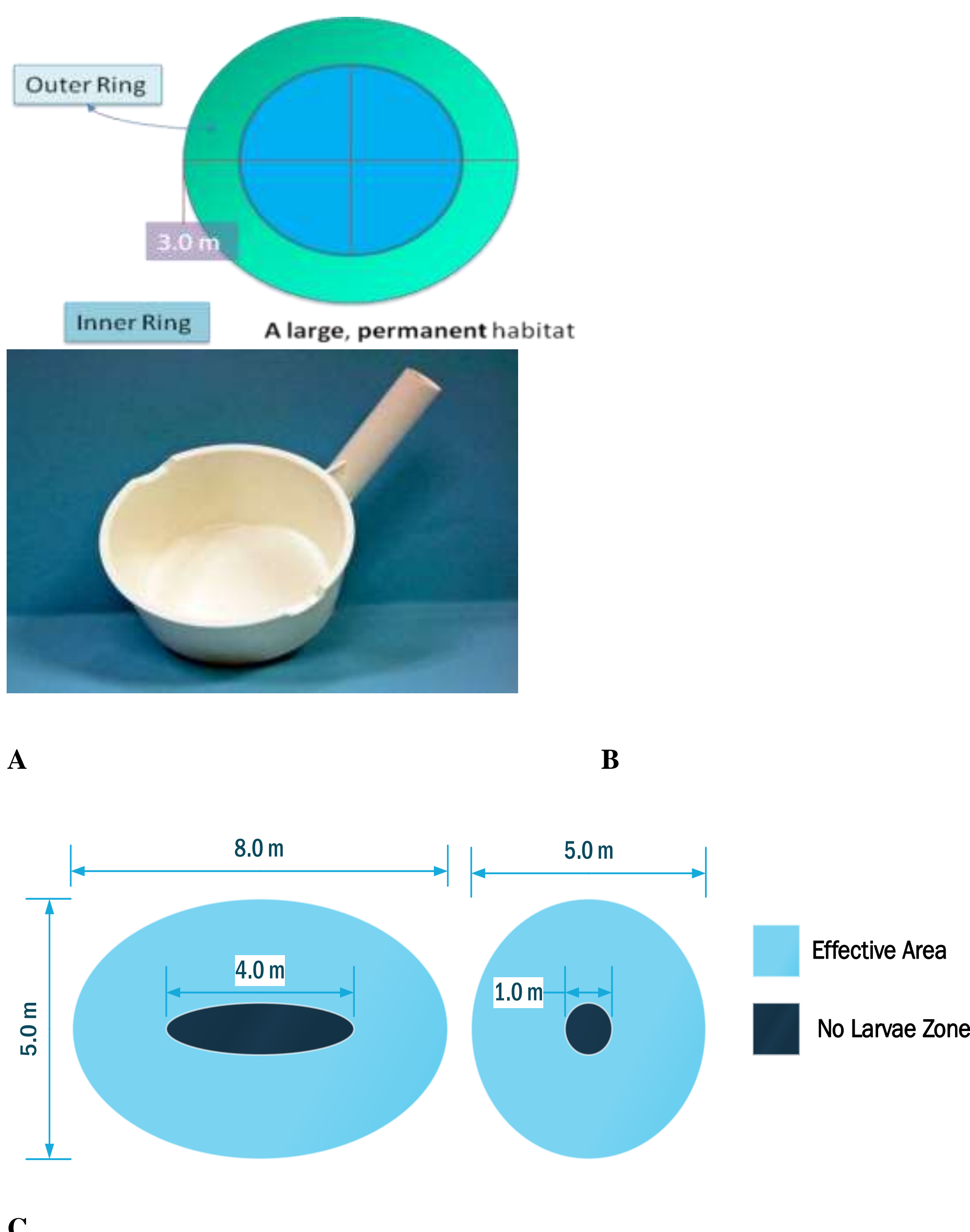


Figure 3.5: Figurative description of calculation of effective area with a description of how habitats generally appear in nature (A), a diagram of a dipper (B), and where larvae are mostly found (C) in a habitat (Orondo *et al.*, 2023a).

Based on larval habitat stability and availability data from the field survey, and habitat productivity data from the emergence traps, the overall weekly productivity of adult malaria vectors in the irrigated and non-irrigated ecosystems in our study site was modeled. In each ecosystem, the total area size of productive aquatic habitats was calculated for each cluster and its surrounding 200m buffer area in a week based on the field survey from September 2018 to August 2019 for permanent, semi-permanent and temporary habitats. Adult mosquito productivity was the product of productive aquatic habitats area size and habitat productivity estimation based on emergence traps. For large permanent habitats, field observations indicated that mosquito larvae tended to concentrate only within several meters from the edge of large habitats, and very few Anopheline larvae could be found in the area farther away from the edge. Based on field experience, areas within two-meter distance from the habitat edge were considered the only effective productive area that permitted mosquito larvae to develop into pupae and adults. For aquatic habitats with <4m diameter, all areas were considered productive. Productivity of temporary habitats was based on an earlier publication in the area *(Himeidan et al.,* 2009). Due to cluster size difference between irrigated and non-irrigated ecosystems, the total number of adults produced from permanent, semi-permanent and temporary larval habitats for the irrigated and non-irrigated zones on weekly basis were calculated and standardized based on study cluster area size.

3.5.1.6 Laboratory predator experiment

The following classes of predators were used in the study: Coleoptera (Beetles), Amphibians (tadpoles), Hemiptera (boatman, backswimmers, and water scorpions), Odonata (damsel fly nymph and dragonfly nymph), fish (Gambusia), Arachnida (Spiders), and Ephemeroptera (Mayfly). A negative control (no predator) basin was also included in the experiment. The experiments were carried out daily, with the predators and mosquitoes being replaced every 24 hours. Larval instars 2 and 3 were used in this experiment. The predator: larvae ration was at 1:20, with a maximum of 5 predators and 100 larvae per basin, as directed by previous studies (Dalal *et al.,* 2020). After 24 hours, larval counts were performed.

3.5.1.7 Vertical life tables field experiment

The survivorship and mortality of *An. gambiae* were determined using vertical life tables in field surveyed natural habitats. A vertical life table was constructed using natural habitats, allowing for continuous oviposition resulting in overlapping generations within the habitat. This is as opposed to a horizontal life table where a cohort of habitats is followed over time until a single generation is exhausted (Edillo *et al.,* 2004; Munga *et al.,* 2007). Four permanent habitat types were selected and followed for 29 days. These included six replicates of rice paddies, drainage ditches, fish ponds, and five replicates of man-made ponds. Larval sampling was conducted daily for 29 days in September and October of 2019. Larval species and densities of various instar stages, as well as predator types and densities, were recorded.

3.5.2 Bacterial analysis

3.5.2.1 Bacterial identification via culture

Water samples were collected from November 2021 to January 2022 from the irrigated and non- irrigated areas from larval infested and non- infested aquatic habitats. The water samples were pooled into 12 pools and used for analysis. Each month consisted of four pooled samples. The samples were further grouped based on their irrigation status (irrigated versus non-irrigated) and larvae status (larvae present versus larvae absent). The samples were added to peptone water for enrichment at a ratio of 1:10 and incubated at 36°C ± 2°C for 16-20 hours before being cultured in nutrient agar at 37ºC for 24 hours. Single colonies were then isolated and plated on nutrient agar/ broth (for the growth of various types bacteria), Eosin Methylene Blue (EMB) agar (a differential medium for gram-negative bacteria and provides a color indicator distinguishing between organisms that ferment the lactose (e.g., *E. coli*) and those that do not (e.g., *Salmonella, Shigella*) while allowing for cultivation of *bacilli*) and MacConkey agar (a selective medium for the growth of gram-negative bacteria alone and a differential media for lactose fermentation while inhibiting growth of most gram-positive bacteria) at 37ºC for 24 hours (**Figure 3.8**). Individual colonies were then grown in nutrient broth for 24 hours and were finally preserved in 50% glycerol for Matrix Assisted Laser Desorption Ionization-Time of Flight Mass

Spectrometry (MALDI-TOF MS) and sequencing. DNA was extracted from the colonies for sequencing (Lay Jr, 2001).

3.5.2.2 MALDI-TOF MS analysis

MALDI-TOF MS was used for microbial identification using the 16S ribosomal protein. The matrix (powder form) was diluted in 125 µl diluent (330 µl of acetonitrile, 330 µl of double distilled water, 330 µl of ethanol, 330 µl of trifloon acetic acid (TFA)), vortexed for 2 minutes then centrifuged at 1200 rpm for 30 seconds. This solution was used to dilute the sample to dissociate clustered macro-molecules. The direct smear plus formic acid sample preparation method was utilized. A colony was transferred from the agar surface to the target plate and was overlayed with 0.5 µl of 25% formic acid solution. After drying 0.5 µl of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (10mg/ml) diluted in 250 ul solutions comprising of acetonitrile, high performance liquid chromatography (HPLC) grade water, and ethanol in a ratio of 3:3:3 and containing a final concentration of 3% of trifluoroacetic acid was added. The target plate was then analyzed in MALDI-TOF spectrometry for bacterial identification. The plate was read using Shimadzu software in Shimadzu machine (Axima Confidence, Shimadzu, Japan) against the SARAMIS database. Spectra range was recorded in linear mode within a mass range between 2000 to 20000 Daltons (Singhal *et al.,* 2015).

3.5.2.3 Microbial sequencing for microbial species identification

From the bacterial colonies, bacterial genomic DNA was extracted from 31 randomly selected individual colonies. PCR was done using Go Taq Master Mix (Promega, wiscosin, USA). 12.5µl of Go Taq master mix, 9.5µl nuclease free water, 0.3µl each of each forward and reverse primers and 2µl of the DNA template was then added to make up 25µl of a final PCR reaction volume. PCR was run under the following conditions: initiation at 95°C for 5 minutes, 30 seconds of denaturing at 94°C, 45 seconds of annealing at 57°C, 45 seconds of extension at 72°C and the thermocycler (AB Applied Biosystem) was set at 35 cycles. This was followed by final extension at 72°C for 7 minutes. The amplicon integrity was assessed using agarose gel electrophoresis before purification and sequencing. The amplicons were cleaned

using Exonuclease 1 and Shrimp Alkaline Phosphatase (ExoSAP) -IT. 1µl of ExoSAP was loaded onto the 96 -well plate followed by addition of 8µl of the DNA amplicons. Conditions for purification were set as follows in the thermocycler: 37^0C for 15 seconds for 1 cycle followed by 80^0C for 15 seconds for one cycle.

Sequencing was done using standard kit on ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Internal 16S bacterial primers and the 3700/3730 BigDye ® Terminator v3.1. Briefly, 4µl of the cleaned PCR product was mixed with 0.3µl BigDye Terminator, 1.75µl of 5X sequencing buffer, 0.32µl of each primer and 3.63µl of PCR water. The thermocycler conditions were set as follows: initiation at 96°C for 60 seconds, 30 seconds of denaturing at 96°C, 15 seconds of annealing at 50°C, 4 minutes of extension at 60°C and the thermocycler (AB Applied Biosystems) was set at 25 cycles.

This was followed by ethanol and sodium acetate precipitation of the 10µl sequence reactions in the PCR tubes to remove unincorporated dye labelled terminator to produce clear signals. 90µl of the ethanol and sodium acetate premix was added to each well, sealed and incubated at -20^0C for 30 minutes. These were then spun at 3000rpm for 30 minutes. Seals were then removed and the plates inverted over paper towels to drain the tubes. The plates were then span at 50rpm for 1 minute and 150µl of ice- cold 70% ethanol was added to each well. The plates were again sealed and span at 3000rpm for 10 minutes. The plates were inverted again on clean paper towels to drain the excess fluids before being inverted on clean paper towels and left to air-dry on the bench. The samples were resuspended in Hi-Di for denaturation at 96^0C for 1 minute then 10µl of Hi-Di™ Formamide was added to each well and electrophoresis was conducted using ABI 3730 x1 capillary Sequencer (Applied Biosystems).

3.5.2.4 Sequence editing, assembling and alignment

Sequences were assembled, edited and aligned using Geneious Prime Ver 2022.2.2. The cleaned bacterial sequences were aligned and Mega 11.0 was used to analyze the sequences and compute the nucleotide diversities. The generated consensus sequences were blasted using the nucleotide Basic Local Alignment Search Tool (BLASTn) on National Center for Biotechnology Information (NCBI) GenBank data for identification.

The evolutionary relationship of the 54 nucleotide sequences, 31 of which were local isolates and 23 were accessions retrieved from GenBank was inferred using Maximum Likelihood method and Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 6.3041)). A Pearson's product-moment correlation was computed to assess the relationship between larval sources and bacterial species abundance in irrigated and non-irrigated areas.

3.5.3 Aquatic composition and metabolite identification

Moisture, protein, fat, crude fiber and ash were determined in the water samples according to Association of Official Analytical Chemists (AOAC) ® (2002) protocols (Feldsine *et al.,* 2002).

3.5.3.1. Determination of crude Protein

10 ml of the water sample was measured into a digestion flask together with a catalyst composed of 5g of potassium sulphate (K_2SO_4), 0.5g of copper sulphate ($CuSO_4$) and 15ml of concentrated sulphuric acid (H_2SO_4). The mixture was heated in a fume hood till the digest color turned blue signifying the end of the digestion process. The digest was cooled, transferred into a 100ml volumetric flask and topped up to the mark with distilled water. A blank digestion with the catalysts and

acid was also made. Ten (10) ml of diluted digest was transferred into a distilling flask and washed with about 2ml of distilled water. Distillation was done to a volume of about 60ml distillate. The distillate was titrated using 0.02N-hydrochloric acid (HCl) to an orange color of the mixed indicator which signified the end point.

3.5.3.2 Determination of total polyphenols

Determination of total polyphenol was carried out using the method of (Waterman & Mole, 1994) with slight modification. 10 ml of water sample was placed in amber bottle glass bottle, 50 ml of methanol was added and secure properly, then extracted for 3 hours in a shaker. The extract was kept in the dark for 72 hours for further extraction then filtered using Whatman number four, then topped up to 50ml using methanol. The extract was centrifuged for 10 minutes at 25 degrees at 150 rpm. 2 ml of the supernatant was taken and filtered using 0.45 µl micro-filter, put in a test tube then 2 ml of folinciocateu 10% was added, vortexed and 4 ml of 0.7 M sodium carbonate was added then vortexed again. The extract was incubated for two hours to develop color and absorbance was read at 765nm using gallic acid as a standard in a Ultra-violet (UV) Visualization machine

3.5.3.3 Fatty acid analysis

The fatty acid profile was determined by gas chromatography (GC). The extraction of the lipids was done by a modification of the (Bligh & Dyer, 1959) method. Water samples of 100 ml were mixed with 50ml of hexane and the mixture shaken overnight. To obtain methylated oil sample, methylation was done by obtaining 2mg of the oil sample which was placed in a flask and refluxed with 5ml of 95% methanol- HCl for 1 hour. The methyl esters were extracted with 3 portions of hexane (5ml) and then washed with distilled water (5ml). The hexane layer was dried in vacuum rotary evaporator and the residue re-dissolved in 1ml of hexane. Then 1µl was injected into the GC under split mode of 60 (Shimadzu GC-2010 equipped with auto sampler) with a capillary column, supelcowax 30mx0.53mm; injection temperature of 240^0C and detection temperature, 260^0C Cundera flame ionization detector. Identification of the fatty acid methyl esters was by comparison

of retention times with standards and expressed as percentages of total methyl esters.

3.5.3.4 Determination of the free radical scavenging activity

The radical scavenging activities of the water samplesagainst 2, 2-Diphenyl-1-picryl hydrazyl (DPPH) radical (Sigma-Aldrich) were determined by UV spectrophotometer at 517 nm (Molyneux, 2003). The following concentrations of the extracts were prepared, 0.01, 0.1, 1.0, 2.0 and 5 mg/ml in methanol (Analar grade). Vitamins C was used as the antioxidant standard at concentrations same as the extract concentrations. 1 ml of the extract was placed in a test tube, and 3 ml of methanol was added followed by 0.5 ml of 1 mM DPPH in methanol. A blank solution was prepared containing the same amount of methanol and DPPH. Methanol was used to zero the spectrophotometer and the absorbance was read at 517 nm after 5 minutes in UV-Vis spectrophotometer (Shimadzu model UV – 1601 PC, Kyoto, Japan).

3.5.3.5 Mineral analysis

100 ml of the water sample was put in a 250 ml beaker and placed on a hot plate following AOAC® 1984 protocols (Williams, 1984). The water was heated and while about to boil, 2 ml of nitric acid- water mixture (50:50) and 10 ml HCL- water mixture (50:50) were added. The samples were evaporated until the mixture reduced to approximately 25 ml. The 25 ml residues were transferred into 100 ml volumetric flasks and filled to the mark with distilled water. The lead, cadmium, mercury and arsenic contents in the samples were determined using a Shimadzu Atomic Absorption Spectrophotometer, Model AA-7000.

3.5.3.6 Lipids acid analysis

The fatty acid profile was determined by gas chromatography. The extraction of the lipids was done by a modification of the (Bligh & Dyer, 1959) protocol. Water samples of 100ml were mixed with 50ml of hexane and the mixture shaken for 30 minutes vigorously and left to stand. The hexane layer was collected and the

aqueous layer returned. The extraction was repeated one more time. The hexane fractions were combined and filtered using defatted cotton wool and anhydrous Na_2SO_4 to remove the water. The filtrate was concentrated using rotary evaporator at 40^0C or Nitrogen gas to about 0.5-1ml in pre-weighed. The rotary flask was dried in the oven at 70^0C for 30 minutes and weighed again to get the oil sample.

3.5.3.7 Determination of glucose, pectin, amylose and cellulose content

This followed AOAC 1996 protocol. Available sugars were analyzed by mixing 50 ml of the water with 50 ml Acetonitrile. 2 ml of lead acetate was added and then mixed thoroughly. The solution was filtered in 5% anhydrous oxalate and finally micro-filtered. The individual sugars were analyzed using a HPLC (Model LC-20A Series, Shimadzu Corp., Kyoto, Japan) fitted with a refractive index (RI) detector. The oven temperature was set at 30^0C, the flow rate was 0.5-1.0 ml/min, and the injection volume was 20 µl. The mobile phase used was acetonitrile and water in the volume ratio of 3:1. The sugars present were identified and their individual concentration calculated using the standards.

3.5.3.8 Extraction of chlorophyll and carotenoid using Acetone

This followed (Arnon, 1949) protocol. 25ml of water sample was added to 25ml of heated acetone. This mixture was centrifuged at 5000 –10000 rpm for 5 minutes. The supernatant was transferred to another tube and the procedure repeated till the residue became colorless. The absorbance of the solution was read at 480, 645nm and 663nm against the solvent (acetone) which was the blank.

3.5.4 Larval susceptibility tests

3.5.4.1 Questionnaire survey on use of chemical use

Open- ended questionnaires were prepared and tested before they were taken to the field. These questionnaires were then distributed to randomly selected clusters and surveys were conducted in these clusters with selected farmers, households, agro-vet shops, and veterinary officers/ agricultural extension workers to identify the chemicals used in public health, agriculture (farms/ crops) and veterinary (livestock/ animals) pest control (**Appendix III** to **Appendix VII**). The questionnaires captured various information ranging from the role of the respondent in the home, farm or shop, type of crops grown or livestock kept in the farm, classes of pesticides sold in the shops, the respondent's knowledge on pests/ diseases that affect the crops/ livestock, the pesticides used or any other method used for pest control on crops and livestock, sources of the chemicals, knowledge on the use of the chemical, how often the chemical is applied, duration of use of the chemical, how the disposal of empty containers and excess chemicals is done, and where the equipments are cleaned. Within the households, other methods used as personal protection against mosquito bites and malaria transmission were also surveyed. The surveys were conducted by trained data clerks sourced from the clusters and the survey questions were asked in English, Swahili or the local language (Dholuo), depending on the respondent's preferred language. The survey lasted for between 10 to 20 minutes.

3.5.4.2 Larval susceptibility tests

With every experimental basin, 30 wild- caught larvae belonging to instar stages 2 and 3 were used.. These larvae were introduced in basins containing 2 litres of water and serial dilutions of chemicals (pyrethroid (Thunder® OD 145), organophosphate (Diazol® 600 EC) and carbamate (Milraz® WP 76).

The chemicals were first diluted according to the manufacturer's recommendations as follows: 4ml of Thunder in 20 litres of water, 20ml of Diazol in 20 litres of water, and 40g of Milraz in 20 litres of water. Serial dilutions were then done as follows: 1:500; 1:1000; 1:2000; 1:4000; 1:10000; 1:20000; 1:50000; 1:100000; 1:200000;

1:500000; 1:1000000; 1:10000000. Positive (laboratory reared Kisumu strain larvae in chemical) and negative (wild- caught larvae in ethanol dilutions and wild- caught larvae in plain water) controls were included with each experiment. The basins were covered with a netting mesh and secured with a string to prevent oviposition by other mosquitoes and predation or other possible interference. Each experiment was done in triplicate and the results read after 24hours.

3.5.5 Insecticide resistance study

3.5.5.1 Larval rearing

In the insectary, the field sampled *Anopheles* mosquito larvae were placed in larval trays and kept in the larval chamber of the insectary. These larvae were fed Whiskas® cat food (Trademarks© Mars, Incorporated, McLean, Virginia, U.S.A.) daily. Temperature in the larval chamber was constantly monitored and maintained at 27°C-32°C while humidity was maintained between 40%-60%. The larval trays were checked daily for pupae which were collected and placed into smaller cups and in holding cages covered with mosquito mesh netting awaiting emergence into adults. The adult mosquitoes that emerged into these cages were maintained in the adult chamber of the insectary. This chamber had regulated temperature ranges of between 25°C-28°C and humidity of between 60%-75%. The adults were fed on 6% glucose solution soaked in cotton wool. The female mosquitoes that emerged in the insectary were used for experimental assays including insecticide resistance bioassays, knock-down resistance (*kdr*) and *ACE-1* enzyme molecular analyses. The adult cages were monitored daily for mortalities in the adult mosquitoes and these were removed from the cages using forceps and preserved in 1.5ml eppendorf tubes which were stored at -20°C freezer awaiting DNA extraction for downstream molecular analysis. With each sample preservation, the forceps was cleaned using 70% ethanol to avoid cross contamination. These samples were singly number coded before storage.

3.5.5.2 Phenotypic resistance tests

To determine the susceptibility of malaria vector populations to predetermined diagnostic concentrations of various chemicals, two to five day old adult female *An.*

gambiae s.l. were used in the phenotypic resistance bioassay tests. The susceptibility of the mosquito populations to pyrethroid (0.05% deltamethrin), organophosphate (5% malathion) and organochlorine (4% DDT) insecticides was done on female adult mosquitoes collected in 2018 (January, February, June and July) and 2019 (January to December). This procedure followed the standard WHO tube bioassay guidelines (WHO, 2016). Positive and negative controls were also conducted simultaneously with each set experiment. The positive control consisted of susceptible insectary reared *An. gambiae* s.s. Kisumu strain and the tube was lined with diagnostic concentrations of insecticide impregnated papers (0.05% deltamethrin, 5% malathion or 4% DDT depending on the tests being conducted). The negative control consisted of wild mosquitoes exposed to untreated papers. Each test was done in replicates using 15-25 female mosquitoes as per WHO recommendations. The exposures to the insecticide-impregnated papers were run for 60 minutes and the number of knocked down mosquitoes recorded every 10 minutes during the exposure period. A knocked down mosquito is defined as one that was not able to fly after exposure to insecticide (WHO, 2016). At the end of the exposure period (60 minutes), mosquitoes were transferred into holding tubes, fed on 6% glucose for observation. Mortality was recorded 24 hours post-exposure. In addition to the above mentioned tests, a synergist, piperonylbutoxide (PBO)–deltamethrin exposure bioassay was conducted. This exposure ran for a total of 120 minutes. 4% PBO and 0.05% deltamethrin was used. PBO exposure was done for 60 minutes followed by 60 minutes deltamethrin exposure. Positive controls (susceptible insectary reared *An. gambiae* s.s. Kisumu strain exposed to 0.05% deltamethrin insecticide impregnated papers) and negative controls (wild mosquitoes exposed to untreated papers and wild mosquitoes exposed to PBO) were used during this test. Knock-down was recorded every 10 minutes during exposure period and mortalities were recorded after 24 hours exposure. After recording the mortality 24 hours post- exposure, the live mosquitoes were aspirated into a separate holding paper cup and killed by freezing for 30 minutes at -20°C. Thereafter, the mosquitoes were preserved individually in 1.5ml eppendorf tubes, labeled and stored in -20°C freezer for species identification, detection of *kdr* mutations and *ACE-1* gene mutations. The labels captured the collection site, date of bioassay, insecticide tested and whether the mosquito was dead or alive after 24hrs.

3.5.5.3 Assessment of enzyme activities

Biochemical analyses were conducted to determine the levels of insecticide detoxifying enzymes in the *Anopheles* mosquitoes. Microplate enzyme analyses were used to determine enzyme levels and changed in the activity of oxidases, non-specific esterases and glutathione- S- transferase (GST) while TaqMan assays were used to determine the presence of mutation in the gene angiotensin-converting enzyme *ACE-1*, which is an acetyl-cholinesterase inhibiting enzyme.

3.5.5.3.1 Microplate enzyme assays

Wild female *Anopheles* mosquitoes, 3-5 days old, were knocked down in the -20^0C freezer, placed singly in 1.5ml eppendorf tubes using a forceps, and stored immediately in -80^0C to avoid enzyme degradation. During the test, mosquitoes were homogenized individually in 100µl potassium phosphate (KPO_4) buffer. KPO_4 was prepared by mixing 6.6g dibasic KPO_4 and 1.7g monobasic KPO_4 in 1000ml of distilled water. The pH was adjusted to 7.2 and the solution stored at room temperature. The mosquito homogenate was diluted with 1,200µl KPO_4 buffer. The microplate readings were done using a microplate reader with Microplate Manager® software on the BIO-RAD Imark microplate reader. All assays were done in triplicates. The colour intensity of each well was an indication of the amount of enzymes present in the mosquito.

3.5.5.3.1.1 Mosquito total protein estimation

The protein assay was used to measure the amount of total proteins in the mosquitoes. This value (optical density/ OD readings) was used to correct for size differences between mosquitoes as larger mosquitoes have more protein content as compared to smaller mosquitoes. 20µl of mosquito homogenate from a single mosquito was put in wells of the plate and analysis done in triplicate. Negative controls (KPO_4) were included in each plate. 80µl of KPO_4 buffer was then added to each well followed by 200 µl Protein dye reagent in each well. The protein dye reagent was prepared by mixing 20ml Protein dye concentrate in 80ml distilled water

and stored at 4^oC in a light proof bottle or a bottle covered with aluminium foil. The plates were read immediately with microplate reader at 620nm.

3.5.5.3.1.2 Elevated non-specific esterase assay

The estarase assay was used to measure the changes in the levels of non-specific β-esterases present in the mosquito homogenate sample. 100μl of the mosquito homogenate was pipetted in wells of the plate in triplicate. Positive and negative controls were also included in each plate in triplicate. 100μl of β-naphthyl acetate was added to each well in the plate and incubated at room temperature for 10 minutes. Thereafter, 100μl of Dianisidine was added to each well and incubated for another 2 minutes and read at 540 nm. β-naphthyl acetate was made by dissolving 56 mg β-naphthyl acetate in 20 ml acetone and 80 ml KPO_4 and stored at 4^oC. Dianisidine was prepared by dissolving in a light proof bottle 100mg of 0-dianisidine tetrazotized in 100ml of distilled water. This solution was to be prepared and used immediately. The color of Dianisidine was checked before use to ensure it was pale yellow and was discarded in the case of a color change to amber. Positive controls were prepared by making Esterase stock solution i.e. 50mg β-naphthyl in 10ml acetone and 90 ml KPO_4. Aliquots of 1ml of the stock solution were put in 1.5ml microfuge tubes, covered with aluminium foil to keep off light and frozen. A standard solution was prepared by diluting the esterase stock with KPO_4 buffer in the ratio of 1:35 respectively. The dilution solution that was used as the positive control solution was prepared by diluting the esterase stock in KPO_4 buffer in the ratio 1:70. KPO_4 buffer was added as the negative control.

3.5.5.3.1.3 Oxidase reaction

This assay was used to measure elevated heme peroxidase levels in mosquito samples. 100μl of mosquito homogenate were aliquoted in triplicates. 200μl of Tetramethyl-Benzidine Dihydrochloride (TMBZ) was added followed by 25μl of 3% hydrogen peroxide (H_2O_2). The plated were incubated for 5minutes then read at 620 nm. TMBZ was made by dissolving 50mg 3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride (TMBZ) in 25 ml Methanol. 75 ml of 0.25M sodium acetate

(NaOAc) buffer added to the TMBZ- methanol solution. This solution was stored at 4°C and was fit for use as long as the solution was colorless. The reagent was discarded on color change to light blue. NaOAc buffer was made by mixing 83 ml of 3M NaOAc (408.1g of NaOAc in 800ml of water) with 900 ml distilled water and pH adjusted to 5 with glacial acetic acid. The mixture was adjusted to a final volume of 1 liter. This solution was stored at room temp. Positive and negative controls were also included in appropriate wells. Positive controls were prepared by making oxidase stock solution (10mg Cytochrome-C from bovine heart dissolved in 100ml NaOAc). Aliquots of 1ml were put in 1.5ml microfuge tubes, covered with aluminium foil to keep off light and frozen. A standard solution was made from the stock solution by diluting the oxidase stock with KPO_4 buffer in the ratio of 1:55. The dilution solution was also made by diluting the oxidase stock with KPO_4 buffer in the ratio of 1:110. KPO_4 buffer was added in the negative control wells and used as the negative control.

3.5.5.3.1.4 Glutathione-S-Transferase assay

This assay was carried out to analyze the level of Glutathione S-Transferase in the mosquitoes. 100µl mosquito homogenate was put in each well in triplicates. Negative controls were also included in each plate. 100µl of reduced glutathione was added in each well followed by 100µl of 1-chloro-2,4'-dinitrobenzene (cDNB). The plate was read immediately (T_0) at 405 nm. The plates were then incubated at room temperature then read after 5 minutes (T_5) at 405 nm. The T_0 readings were subtracted from the T_5 readings and these values were used for analysis. Reduced glutathione was made by mixing 61mg reduced glutathione with 100ml KPO_4 buffer. cDNB was made by dissolving 20 mg 1-chloro-2,4'-dinitrobenzene (cDNB) in 10ml acetone and 90ml KPO_4 buffer stored at 4°C. Reduced glutathione and cDNB buffers could be stored at 4°C for 3-4 days

3.5.5.3.2 TaqMan Enzyme assays

TaqMan enzyme analysis was performed to determine the presence of a point mutation of the acetyl-cholinesterase inhibiting enzyme; angiotensin-converting

enzyme (*ACE-1*). DNA extracted from the mosquitoes preserved after WHO bioassay tests were used to determine the frequency of *ACE-1* mutation (*ACE-1R*) in the mosquito population. Assays were performed on both live and dead mosquitoes post bioassay tests. DNA was extracted from *An. gambiae* s.l. using Chelex method. The mosquitoes were subjected to conventional polymerase chain reaction (PCR) to determine their species. Real-time PCR (qPCR) was performed on the DNA to determine presence of *ACE-1* mutations. The mastermix was prepared using molecular water, 5x master mix (TaqMan ™ Fast Advance), primers and probes for *ACE-1* enzyme being tested. For a 10µl reaction, 2.2µl of molecular water was mixed with 5µl master mix, 0.4µl of *ACE-F* and *ACE-R* primers each, and 0.5µl of *ACE1-W* (wildtype) and *ACE1-M* (mutant) probes each. 1µl of the DNA sample was then added into the real time PCR tubes. The probe sequences were 5'-TTCGGCGGCGGCT-3' with VIC MGBNFQ dye for *ACE1-W* and 5'-TTCGGCGGCAGCT-3'with 6-FAM MGBNFQ dye for *ACE1-M*. Samples were loaded on a 96-well PCR plate. The temperature profile was set as 95°C for 2minutes for initiation followed by 40 cycles of denaturation at 95°C for 2seconds and annealing at 58°C for 1.5minute on Bio-Rad T100™ Thermo cycler.

3.5.5.4. Detection of kdr gene

DNA extracted from a subset of mosquitoes that were subjected to bioassay tests was used in this analysis. Real time PCR was done on *An. gambiae* s.l genomic DNA samples to determine the development of the *kdr* gene. These followed the Bass *et al.*, 2007 protocols to test the West African and East African forms of *kdr* mutations. The master mix was prepared by mixing molecular water, 5x master mix (TaqMan ™ Fast Advance), primers (*kdr- F* and *kdr- R*) and probes (*kdr- WT* (wild type) and *kdr- E* (east) or *kdr- W* (west)). For a 10µl reaction, 2.2 µl of molecular water was mixed with 5µl TaqMan mix, 0.4µl of forward primers and reverse primers each and 0.5 µl of the probes (*kdr- WT* and *kdr- E* or *kdr- W*). 1 µl of the DNA sample was then added into the real time PCR tubes. The probe sequences were 5'-ACGACTGAATTT-3' with 6-FAM MGBNFQ dye for *kdr- E*, 5'-ACGACAAAATTT-3' with 6-FAM MGBNFQ dye for *kdr- W*, and 5'-CTTACGACTAAATTTC-3' with VIC MGBNFQ dye for *kdr- WT*. Samples were

loaded on a 96-well PCR plate. The temperature profile was set as 95°C for 2minutes for initiation followed by 40 cycles of denaturation at 95°C for 2seconds and annealing at 58°C for 1.5minute on Bio-Rad T100^{TM} Thermo cycler.

3.5.6 Laboratory Analysis of mosquito and water samples

3.5.6.1 Larval and adult mosquito DNA Extraction

DNA was extracted from the whole body of the mosquito following a slightly modified version of (Musapa *et al.,* 2013) protocol. Single mosquitoes were put in a 1.5ml microfuge tube and ground using 200µl grinding buffer comprised of 190 µl 1X phosphate buffered saline (PBS) and 10 µl of 10% Saponin (1g of saponin in 10ml ddH_2O). The homogenate was mixed by gentle momentary vortexing then incubated at room temperature for 20 minutes. This was then centrifuged at 14,000 revolutions per minute (rpm) for 5 minutes and the supernatant discarded to wash the pellets. The remaining pellets were then washed again by re-suspension in 200 µl 1X PBS, gently vortexed and centrifuged again at 14,000 rpm for 5 minutes and the supernatant discarded. The pellets were then re-suspended in 250 µl of Chelex resin suspension (20% weight per volume Chelex-100 resin in de- ionized water) to bind DNA contaminants. This suspension was gently vortexed and then placed on a floating rack and heated in a water bath at 85^{0}C for 10 minutes to activate the resins and maximize binding of the Chelex resins to the DNA contaminants and other DNAses leaving the DNA in solution. After the heating process was completed, gentle vortexing was done and the samples were centrifuged at 14,000 rpm for 5 minutes. DNA in solution was then transferred into pre-labeled 0.5 µl microfuge vials to be used as a template in molecular analysis

3.5.6.2 Larval and adult species identification

Molecular species identification was carried out following (Scott *et al.,* 1993) and (Paskewitz & Collins, 1990) protocols. *An. gambiae* s.l. and *An. funestus* s.l. species complexes were further distinguished using ribosomal-DNA (rDNA) PCR. This discrimination of the sub species within the *Anopheles* species complex was done to distinguish between *An. gambiae* s.l. (*An. gambiae* s.s. and *An. arabiensis*) and the

An. funestus s.s. This PCR is based on species-specific single nucleotide polymorphisms (SNPs) in the internal transcribed spacer region 2 (ITS2).

DNA amplification was done using 13 µl reaction. In every reaction (sample) for *An. gambiae* s.l., there was 4 µl PCR water, 6.5 µl of 2x green reaction buffer (which contains blue and yellow dye acting as the loading dye, $MgCl_2$, dinucleotides triphosphates (dNTPs) which were a composition of adenine (dATP), guanine (dGTP), cytosine (dCTP) and thymine (dTTP) and DNA/ Taq polymerase), 31.4 nmole/µl of universal (UN) primer, 32 nmole/µl of *An. gambiae* s.s. (GA) primer and 31 nmole/µl of *An. arabiensis* (AR) primers was used. 1 µl DNA template was added to the master mix solution.

The *An. gambiae* complex rDNA primers used were GA: 5' CTG GTT TGG TCG GCA CGT TT 3', AR: 5' AAG TGT CCT TCT CCA TCC TA 3', UN: 5' GTG TGC CCC TTC CTC GAT GT 3'. The basepair (bp) length of *An. arabiensis* is 315 and 390 for *An. gambiae* s.s. These regions are set to amplify between the UN primer and the species specific primer.

For *Anopheles funestus*, a 13µl reaction was performed under the following PCR conditions: 4.5 µl PCR water, 6.5 µl of 2x green reaction buffer (which contains blue and yellow dye acting as the loading dye, $MgCl_2$, dinucleotides triphosphates (dNTPs) which were a composition of adenine (dATP), guanine (dGTP), cytosine (dCTP) and thymine (dTTP) and DNA/ Taq polymerase), 23.8 nmole/µl of *An. funestus* s.s. (FUN1) primer and 22.5 nmole/µl of ITS2A/ UN primers was used.

Anopheles funestus s.s rDNA internal transcribed spacer (ITS) 2 diagnostic primers were: FUN: 5' GCA TCG ATG GGT TAA TCA TG 3' and ITS2A: 5' TGT GAA CTG CAG GAC ACA T 3' (Koekemoer *et al*., 2002). The nucleotide basepair length of *An. funestus* is 505bp. These regions amplified between the UN primer and the species-specific primers. This region amplified between the ITS2A primer and the species-specific primer (FUN1).

All amplifications were done in a BIO-RAD T100^{TM} Thermo cycler which was set as follows: Initiation 3 minutes at 95°C, 30 seconds of denaturing at 94°C, 30 seconds

of annealing at 55°C, 45 seconds of extension at 72°C, 10 minutes of auto-extension at 72°C and the cycle went through 34 cycles. This process took about 1.5 hours after which the machine goes to 12°C until the samples are removed from the machine. The amplification was then scored using 1.5% agarose gel electrophoresis and visualized under ultra violet (UV) radiation. A DNA size marker was used to score the gel.

3.5.6.3 Bacterial DNA extraction and amplification

DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline, London, UK). Briefly, 180µl of Buffer GL was added to the bacterial culture and a micropestle was used to grind the sample. 25µl of Proteinase K solution was added to the sample and vortexed. Samples were incubated at 56^0C for 1 hour and were vortex occasionally. The sample was vortexed briefly and 200 µl of lysis buffer G3 was added. The sample was vortexed vigorously and incubated at 70^0C for 10 minutes followed by centrifugation for 5minutes at 11000 revolutions per minute (rpm) to remove insoluble material. The supernatant was transferred to a new microcentrifuge and 210 µl of absolute ethanol. The samples were vortexed vigorously and placed on an ISOLATE II Genomic DNA Kit Spin Column and collection tube. Samples were centrifuged for 1 minute at 11000rpm and the flow-through was discarded. 500µl of wash buffer GW1 was added and centrifuged at 11000rpm for 1 minute. The flow-through was discarded. 600µl of wash buffer GW2 was added to the column and centrifuged for 1 minute at 11000rpm. The flow-through was discarded and the silica membrane was dried by centrifugation at 11000rpm for 1 minute. 50 µl of preheated elution buffer G was added to the silica membrane and incubated at room temperature for 5 minutes. Elution was carried out by centrifugation at 11000rpm for 1 minute.

3.5.6.4 Agarose Gel Preparation and Electrophoresis

On completion of the PCR reaction, 1.5% w/v agarose gel was prepared by heating 1.2g agarose in 80ml 1X TBE buffer (108g Tris, 55g boric acid, 9.3g EDTA, 1000ml ddH_2O. 100ml of this solution was diluted in 900ml distilled water to make 1X TBE) in a microwave for 45 seconds. After heating the agarose - TBE buffer, the solution

was allowed to cool but not allowed to solidify, 5ml SmartGlow loading dye was added before the mixture was poured into the electrophoresis tank which had been prepared and the combs put in place. The solution was left to solidify after which the combs were removed.

The tank was flooded with electrophoresis buffer (TBE) and the sample amplicons loaded into the wells. Where the DNA size marker was used, the 100bp DNA ladder was prepared using 7µl PCR water, 2µl 6x blue loading dye and 1µl DNA ladder giving a total volume of 10µl. This was mixture was vortexed and approximately 5µl was added to designated wells in the gel. The tank was connected to the mains and allowed to run at between 90-105volts for 30-45 minutes. The fragments were visualized under ultraviolet illuminator and scoring done. The amplicon fragments scored against the DNA ladder fragments.

3.6 Data management

During field-work, all larval data was entered into Open Data Kit (ODK) in tablets and then uploaded to online database (MySQL database server). The collection site (irrigated or non- irrigated) and each habitat metadata were recorded in the field. In the insectary and laboratory, data was recorded in respective laboratory data forms and later entered in Microsoft Excel spreadsheets followed by error checks and corrections. The field forms and laboratory processing forms are shown in **Appendix VIII to Appendix XVI**.

3.7 Data Analysis

3.7.1 Larval ecology study

3.7.1.1 Larval and predator densities and habitats productivity

Larval densities per dip were calculated, and Z-tests were used to determine statistical difference between irrigated and non-irrigated ecosystems, and among different habitat types. Pearson chi-square was used to determine the differences in the occurrence of *Anopheles* larvae among the different habitat types and between the irrigated and non-irrigated ecosystems. The Kaplan-Meier survival analysis was

employed to determine the stability of larval habitats in the irrigated and non-irrigated ecosystems. *Anopheles* densities from the emergence traps were analyzed using analysis of variance (ANOVA) to determine difference in the productivity among different habitat types and between the irrigated and non-irrigated ecosystems. Finally, a generalized linear mixed model (GLMM) with Poisson error and log link function was used to analyze the statistical difference in larval densities among temporary, semi-permanent, and permanent habitats and the two ecosystems.

To calculate the effective habitat area the following formula was used:

If $\frac{L}{2}$ or $\frac{W}{2}$ < 2α (or 3α) the overall area of the habitat is:

$$\text{Original Area (O)} = \frac{L}{2} * \frac{W}{2} * \pi = \frac{LW}{4}\pi$$

Assuming 2α = 2m, implying that 95.44% of the larvae are within 2m from the shoreline

$$\text{Effective Area (R2)} = \frac{\text{LW}}{4}\pi - \left(\frac{L}{2} - 2\right) * \left(\frac{w}{2} - 2\right) * \pi = (L + W - 4)\pi$$

Assuming 3α- 3m, implying that 99.72% of the larvae are within 3m from the shoreline

$$\text{Effective Area (R3)} = \frac{\text{LW}}{4}\pi - \left(\frac{L}{2} - 3\right) * \left(\frac{w}{2} - 3\right) * \pi = (\frac{3}{2}(L + W) - 9)\pi$$

Larval reduction rates in predator experiments were calculated using a linear mixed model. Linear regression was used to determine the predator – larvae relationship in the field experiment. To determine the larval survival rates in the life table analysis, the overall average daily survival rates from one larval stage to the next was determined for both *An. gambiae* and *An. funestus* in the different habitats. 5-day

smoothed dynamics of different stage of mosquitoes by species and habitat types was also determined.

3.7.1.2 MALDI-TOF analysis

Bacterial isolates were analysed using Shimadzu software. The peaks generated were compared against ranges in the SARAMIS database. The spectra ranges were used to identify the specific bacteria present in the database with similar spectra ranges. The mass ranges used in identification were between 2000 - 20000 Daltons.

3.7.1.3 Sequence alignment and analysis

De novo assembly of the respective 31 generated raw reads was done using Geneious Prime® version 2022.2.2. The generated nucleotide consensus sequences were blasted using BLASTn for comparison and identification. Accessions from GenBank database in NCBI were retrieved and analysed and those with high similarity index were retrieved. Alignment was done using ClustalW algorithm within Mega version 11.0.13 software. Evolutionary relationship between the isolated samples and GenBank retrieved sequences was computed in Mega version 11.0.13 software.

3.7.1.4 Metabolite analysis

To assess the amount of crude protein in the water samples, calculations were done using the formulae;

$$\text{Nitrogen } \% = (V_1 - V_2) \times N \times F \times 0.014 \times 100/V \times 100/S$$

Where; V_1 = Titre for the sample (ml);

V_2 = Titre for the blank

N = Normality of standard HCL solution

F = Factor of standard HCL solution

V = Volume of diluted digest taken for distillation (10ml)

S = Weight of sample taken (g)

Protein % = Nitrogen x Protein Factor

The free radical scavenging activity was determined using the following formula and the results expressed as percentage inhibition of DPPH and mean inhibitory concentrations (IC_{50}) determined from a plot of % inhibition of DPPH versus concentration of extract:

% inhibition of DPPH = $\{(A_B - A_A)/A_B\} \times 100$

Where; A_B is the absorption of blank sample

A_A is the absorption of tested extract solution.

The amount of crude fat was calculated as follows**:**

$$\textbf{\% Crude fat} = \frac{W2 - W1\ (g)}{S\ (g)} \times 100$$

Where; W1 = Weight of empty flask (g)

W2 = Weight of flask and extracted fat (g)

S = Weight of sample

To estimate the concentration of chlorophyll, concentrations of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid were calculated using the following equation:

Total Chlorophyll = 20.2 (A645) + 8.02 (A663)

Chlorophyll a =12.7(A663)–2.69 (A645)

Chlorophyll b =22.9(A645)– 4.68
(A663)

Carotenoid = [A480 + (0.114 (A663) - (0.638-A645)] * V/1000 * W

3.7.2 Larval susceptibility tests

3.7.2.1 Questionnaires on chemical usage

Pesticide use questionnaire data was entered and analyzed in Excel. Chi-square test and t-test were used to determine the significance of the statistical, difference between pesticide use in irrigated and non-irrigated areas.

3.7.2.2 Larval susceptibility tests

Data on larval mortality was entered and analyzed in MS Excel. Mortality rates in test basins were calculated and the mortalities adjusted using the negative controls.

3.7.3 Insecticide resistance

3.7.3.1 Susceptibility Test

The WHO bioassay knock-down recorded after every 10 minutes for one hour and final mortality at 24 hours was recorded for all test runs with corresponding negative and positive controls. Abbot's formula was used to correct percentage mortality in cases where the negative control mortality was between 5% and 20%; experiments, where negative control mortality was above 20%, were discarded and 95% confidence limits of the adjusted mortalities determined. Mortalities of 98%-100% in the sample population indicated susceptibility to the tested insecticide. Mortality of between 90%-98% suggested possible resistance and less than 90% mortality indicated resistance in the tested species (WHO, 2016). Probit analysis was done using Poloplus Version 1 software to determine the knockdown time 50 (KDT_{50}).

3.7.3.2 Analysis of kdr and ACE- 1 mutation

The allele frequencies of *kdr* L1014 mutations and *ACE-1* G119 mutations were determined on Ms Excel. Genepop Hardy Weinberg exact tests were used to determine the differences between the *kdr* alleles in the irrigated and non-irrigated areas of both the dead and alive mosquitoes.

3.7.3.3 Enzyme assays

The means of the different enzymes for each site was done. Analysis of variance (ANOVA), Turkey HSD test was used to estimate the variance of enzymes in the wild mosquitoes when compared to the Kisumu laboratory raised strain.

3.7.4 Species Identification

The densities and proportions of the different species collected from the study sites were counted per study site.

3.8 Ethical considerations

Ethical approval for this study (Reference No. 00456) was obtained from Maseno University Ethical Review Committee (MUERC) and University of California, Irvine Institutional Review Board (HS#2017–3512) (**Appendix I**). Prior to sample collection, verbal consent was obtained from village chiefs, area leaders and household heads or their representatives. This study collected mosquito larvae from suitable larval sites in the study areas and information on chemical usage on farms, livestock and in public health. Human involvement was not invasive but limited to their acceptance that mosquitoes would be collected from near the houses. Field workers who assisted in the collection of mosquitoes and questionnaire data were trained before they were allowed to go to the field to ensure that they acquired good data collection techniques along with good communication skills.

CHAPTER FOUR

RESULTS

4.1 Larval Ecology study

4.1.1 Habitat availability and productivity

A total of 27,286 juvenile mosquitoes were sampled during the rain and dry seasons of the study period (2018 and 2019). These comprised of several species of mosquitoes including *An. gambiae* s.l. (11,380), *An. funestus* s.l. (272), *An. coustani* (125), *An. pharoensis* (31), other unidentified *Anopheles* species (141), *Culex* (15,269), and *Aedes* (88) species (**Table 4.1**). These were sampled from a total of 1,316 habitats (**Table 4.2**).

Table 4.1: Morphologically identified larval species sampled from all sampled habitats during the seasonal surveys in 2018 and 2019

Species		Irrigated	Non- irrigated	Total counts	%age
Anopheles species	*An. gambiae* s.l.	8,240	3,140	11,380	95.2
	An. funestus s.l.	147	125	272	2.3
	An. coustani	79	46	125	1
	An. pharoensis	28	3	31	0.3
	Other *Anopheles*	139	2	141	1.2
Culicine species	*Culex*	10,716	4,533	15,269	
	Aedes	88	0	88	
	Total	19,437	7,849	27,286	

Table 4.2: Habitat availability per transmission zone during the dry and wet season in 2018 and 2019 and morphological identification of sampled *Anopheles* species.

Parameters	Irrigated	Non- irrigated	P- value
Number of clusters	10	10	
Cluster area size	1.31km^2	1.84km^2	
Total habitats	985 (74.8%)	331 (25.2%)	
Anopheles			
Total counts (%)	8,661 (72.3%)	3,316 (27.7%)	
Habitat *Anopheles* infestation (%)	542 (55.0%)	202 (61.0%)	0.0567
Culex			
Total counts (%)	10,716 (70.3%)	4,533 (29.7%)	
Habitat *Culex* infestation (%)	510 (51.8%)	192 (58.0%)	0.0494
Overall			
Habitat infestation (%)	743 (75.4%)	269 (81.3%)	0.0293
Habitat with both mosquitoes (%)	309 (71.2%)	125 (28.8%)	0.0323
Anopheles immature counts			
1st - 2nd instar (%)	5,518 (70.3%)	2,330 (29.7%)	
3rd - 4th instar (%)	2,687 (74.0%)	945 (26.0%)	
Pupa (%)	317 (89.0%)	39 (11.0%)	< 0.0001

In comparison to clusters in the non-irrigated ecosystem, about 2.9-fold higher number of aquatic habitats were found and 2.6-fold higher number of *Anopheles* larvae were collected in the irrigated ecosystem (**Table 4.1**). The area size of clusters from the non-irrigated ecosystem was 28% larger than clusters from the irrigated ecosystem (1.84 vs. 1.31 km^2) (**Table 4.2, Figure 4.1**).

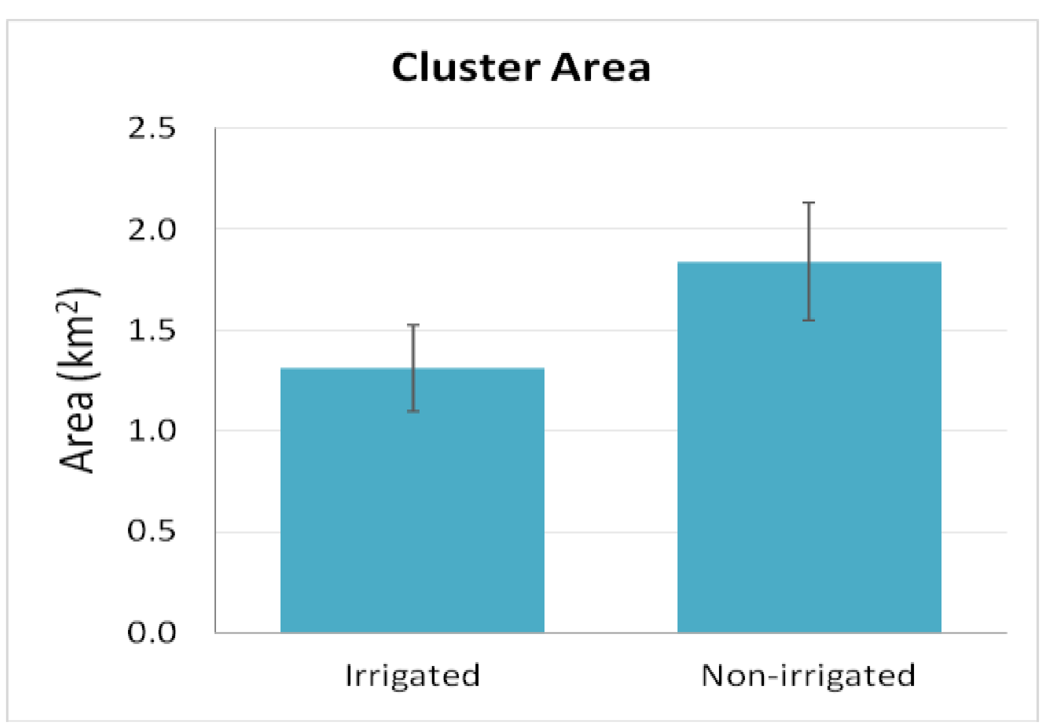


Figure 4.1: Area of the clusters in the irrigated and non- irrigated eco-system where the study was conducted

After adjusting for cluster area size, irrigated ecosystem had about 4-fold higher number of aquatic habitats. The proportion of aquatic habitats that were positive with *Anopheles* larvae was similar (**Table 4.1**). Densities of immature stages decreased significantly with age: among all immature mosquitoes collected, pupae represented a small fraction, and the pupal proportion varied significantly between the irrigated (3.7%) and non-irrigated (1.2%) ecosystems ($\chi^2 = 69.48$, df = 2, $P < 0.0001$,**Table 4.1**). In addition to *Anopheles*, similar number of *Culex* larvae and a few *Aedes* larvae were collected. Within malaria vectors, *An. gambiae* s.l. accounted for 94.7-95.4% of all *Anopheles* collected (**Table 4.1**).

The dry season occurs in January–February, and the coolest and wettest season is June–July. Precipitation and minimum/maximum temperatures were recorded monthly during the study period (**Figure 4.2**). Weather data were collected through installed sensors at ICEMR laboratory in the Tom Mboya University Collage(TMUC) Campus at Homa Bay, Kenya. Coordinate: -0.52917, 34.46431. Rainfall data were collected using the HOBO Rain Gauge (Metric) Data Logger

(RG3-M), and temperature were collected with the HOBO Temperature/RH Data Logger (MX2301A); both sensors were from Onset Computer Co., MA. USA.

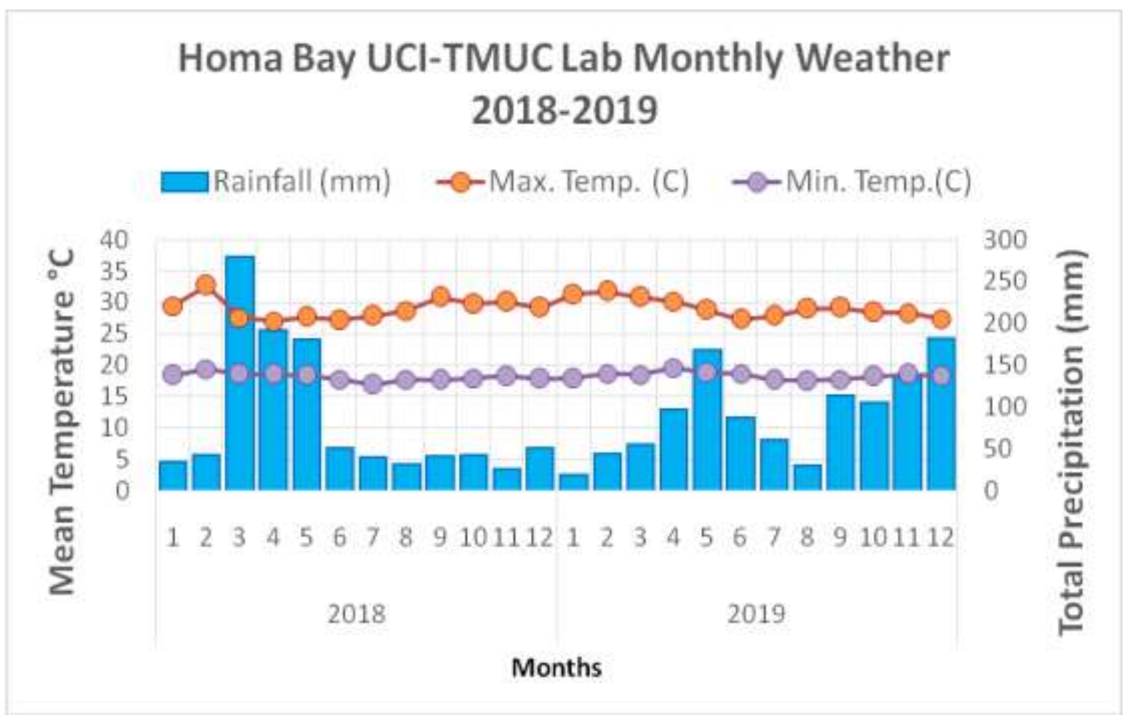


Figure 4.2: Monthly weather 2018-2019 in the study site in Homa Bay, Kenya.

Generally, effects of seasonality was experienced during the study period (**Figure 4.3**). With river beds drying up (**Figure 4.3A**) during the dry seasons and farmlands flooding during the wet season (**Figure 4.3B**). Water hydrology systems (**Figure 4.3C**) and large water bodies (**Figure 4.3E**) filled with water and remain dry (**Figure 4.3D and Figure 4.3F**) during the wet and dry seasions respectively.

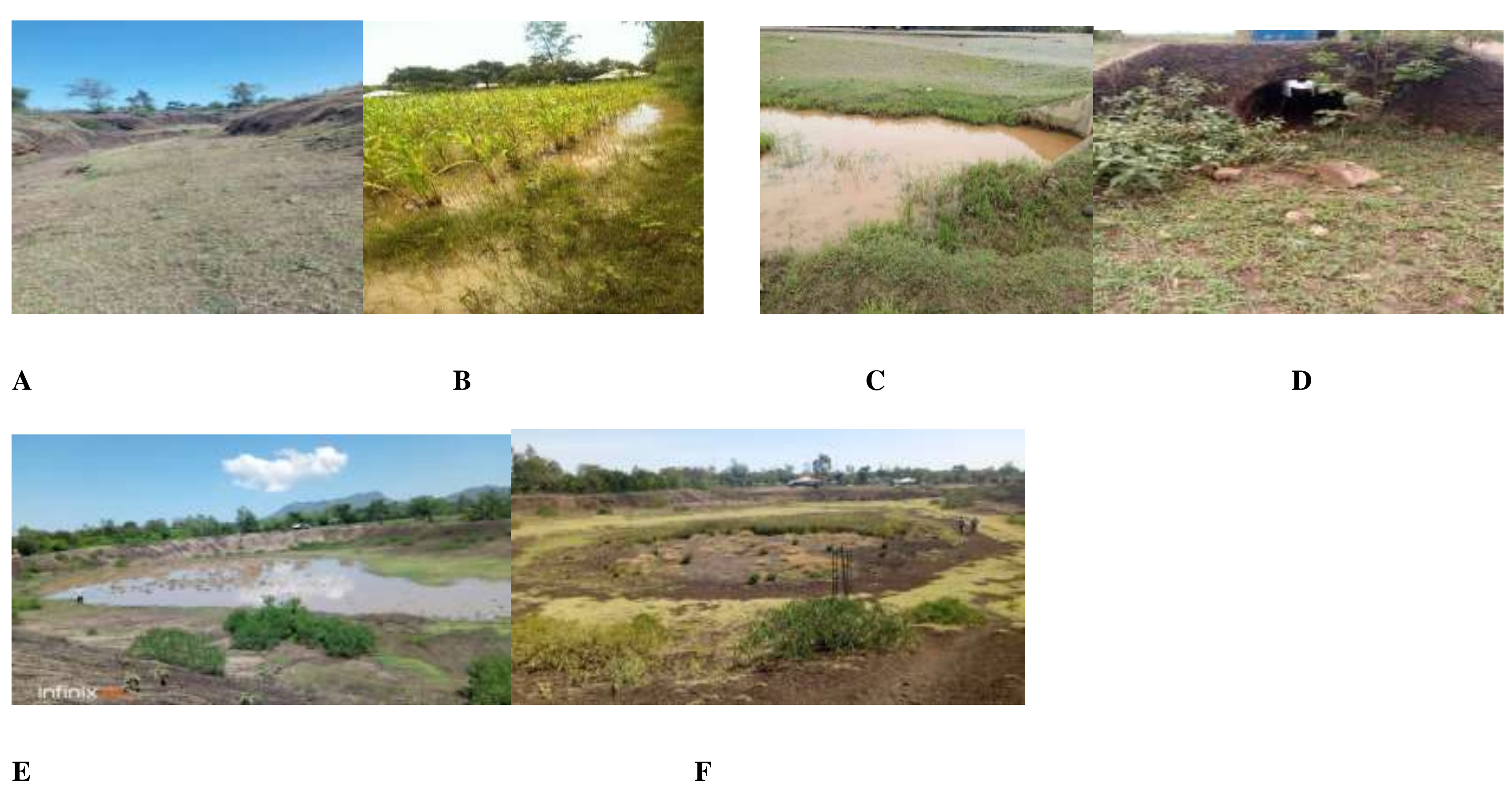


Figure 4.3: Effects of seasonality: Dry river beds (A) during the dry season to flooded farmlands (B) in the wet seasons and the changes observed in different habitat types including small temporary habitats (hydrology system) (C, D) to large permanent habitats (water ponds) (E, F).

Seasonality showed significant impact on larval habitat availability and larval densities (**Figure 4.4**). Habitat availability fluctuated more dramatically in the non-irrigated ecosystem than in the irrigated ecosystem. Significant difference in the number of available habitats was detected between the two ecosystems only during the dry season ($P<0.05$, **Figure 4.4A**). In terms of larval densities, overall the rainy season exhibited significantly higher *Anopheles* density than the dry season ($F_{(1,1311)} = 15.09$, $P<0.0001$). Differences in *Anopheles* densities between irrigated and non-irrigated ecosystems was pronounce during the dry season in the temporary (**Figure 4.4B)** and semi-permanent habitats (**Figure 4.4C**). Additionally, larval densities in permanent habitats were significantly higher ($P<0.001$) in irrigated ecosystems than in non-irrigated ecosystems during the rainy season **(Figure 4.4D**).

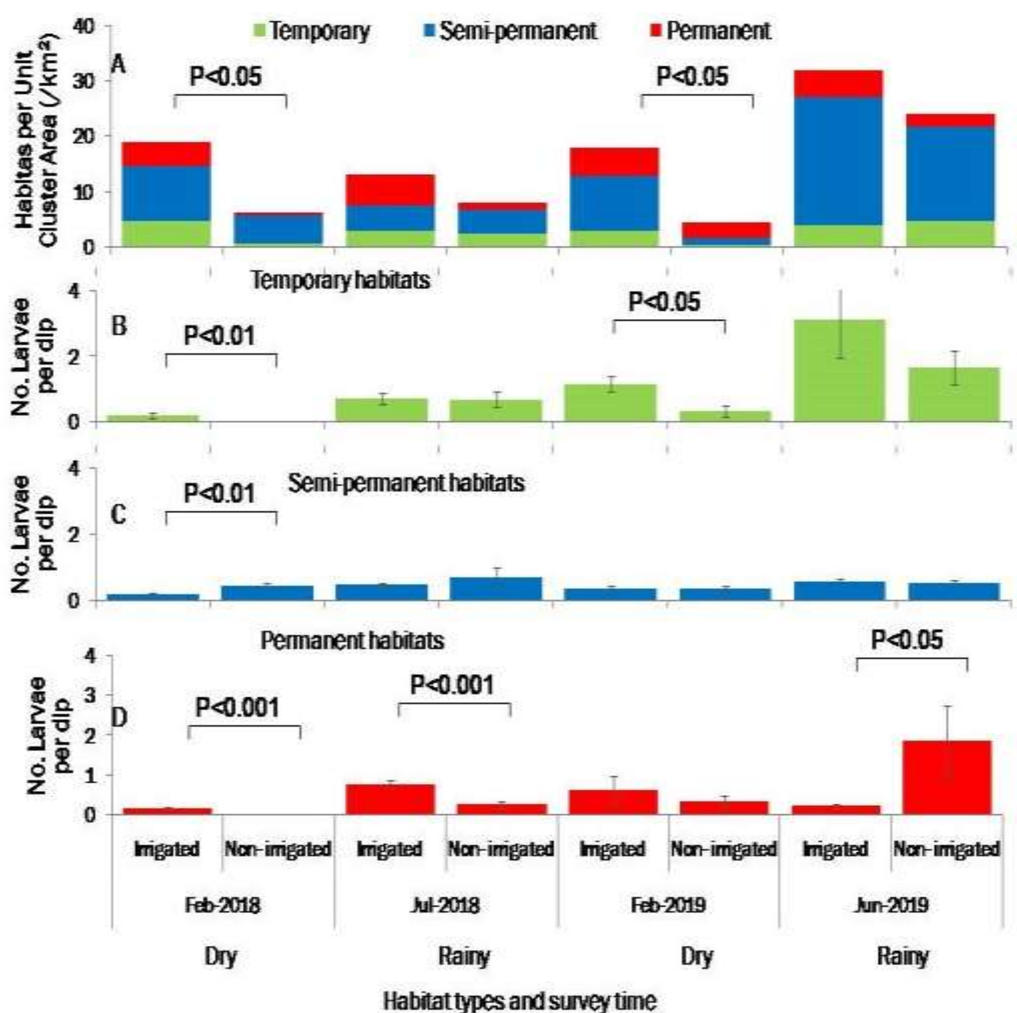


Figure 4.4: Seasonal sampling: The total number of habitats in a unit cluster area (A) and mean larval densities per dip in temporary (B), semi-permanent (C), and permanent (D) in irrigated and non-irrigated ecosystems during the seasonal sampling in 2018 and 2019.

4.1.2 Monthly habitat and larval density dynamics

To determine whether irrigated and non-irrigated ecosystems differed in aquatic habitat abundance and larval density in months between dry and rainy seasons, the availability of aquatic habitats and larval density in 8 randomly selected clusters from the irrigated and non-irrigated ecosystems were monitored. The average area size of the cluster study area in the irrigated and non-irrigated ecosystems was similar (2.63±0.06 km^2 and 2.78±0.24 km^2, respectively). There were significantly more semi-permanent (χ^2 = 56.41, df =1, P < 0.0001) and permanent (χ^2 =35.26, df

=1, $P < 0.0001$) habitats in the irrigated ecosystem than in the non- irrigated, but similar number of temporary habitats ($\chi^2 = 3.12$, df =1, $P > 0.05$) (**Figure 4.5A and 4.5B**) in both ecosystems. The irrigated ecosystem exhibited higher number of aquatic habitats and a significantly larger average area size of the aquatic habitats was than the non-irrigated ecosystem (**Figure 4.5C and 4.5D**).

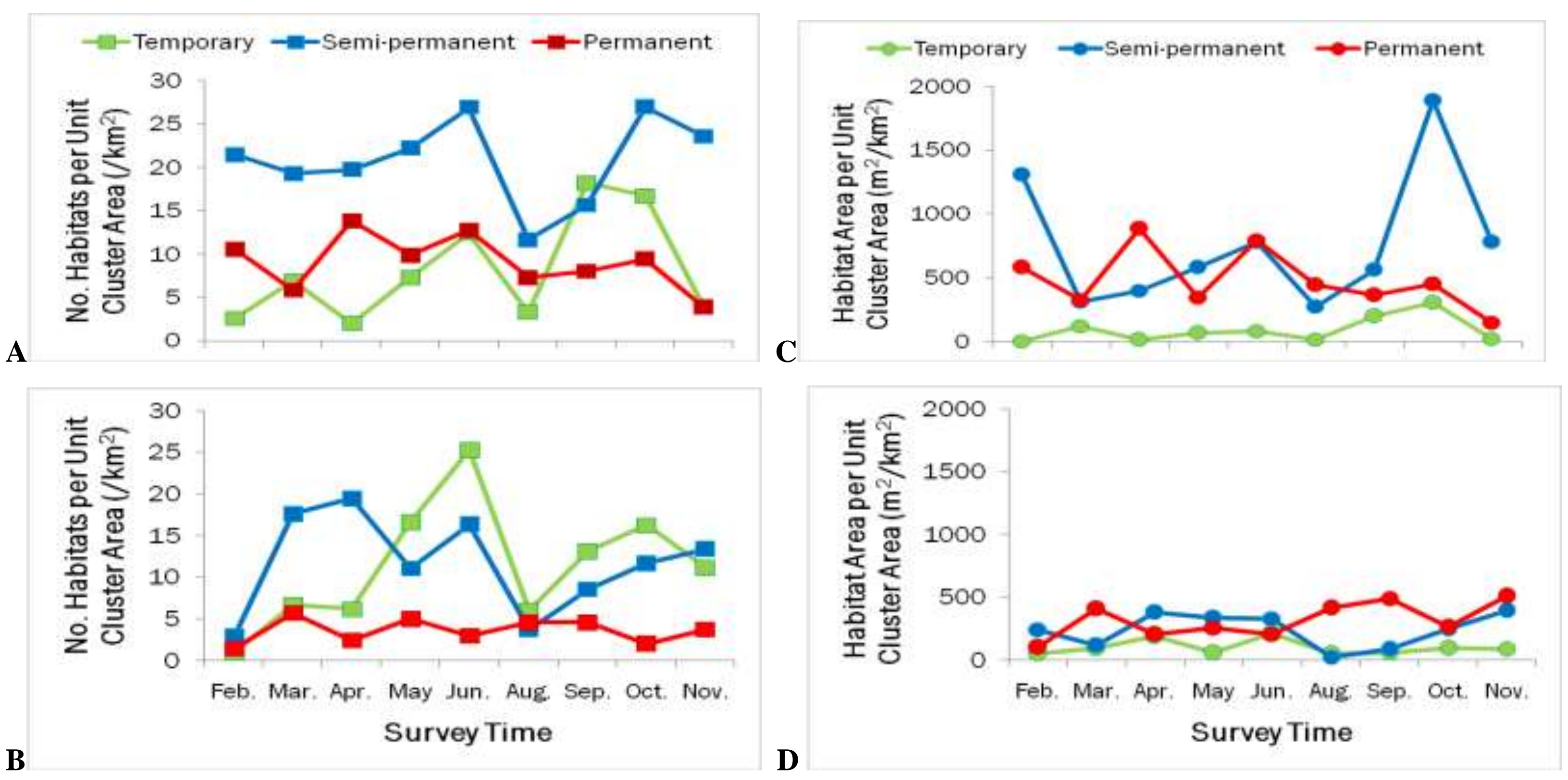


Figure 4.5: Number of habitats and habitat area during monthly dynamics in 2019: Number of habitat per unit cluster area (/km^2) in the irrigated (A) and non-irrigated (B) ecosystem; habitat area per unit cluster area (m^2/km^2) in irrigated (C) and non-irrigated (D) ecosystem.

Significantly higher *Anopheles* larval density was found in permanent habitats in non-irrigated ecosystem than in irrigated ecosystem ($F_{2,15}$= 6.25, P < 0.01), but not in temporary ($F_{2,15}$= 0.17, P > 0.05) and semi-permanent ($F_{2,15}$= 0.89, P > 0.05) habitats **(Figure 4.6A and 4.6B**). this could be due to fluctuating number of habiats in the non-irrigated area throughout the year with few available habitats during the dry season forcing gravid mosquitoes to be concentrated in the few available habitats. With the increase in breeding sites during the rain season, increase in vector densities also increase.

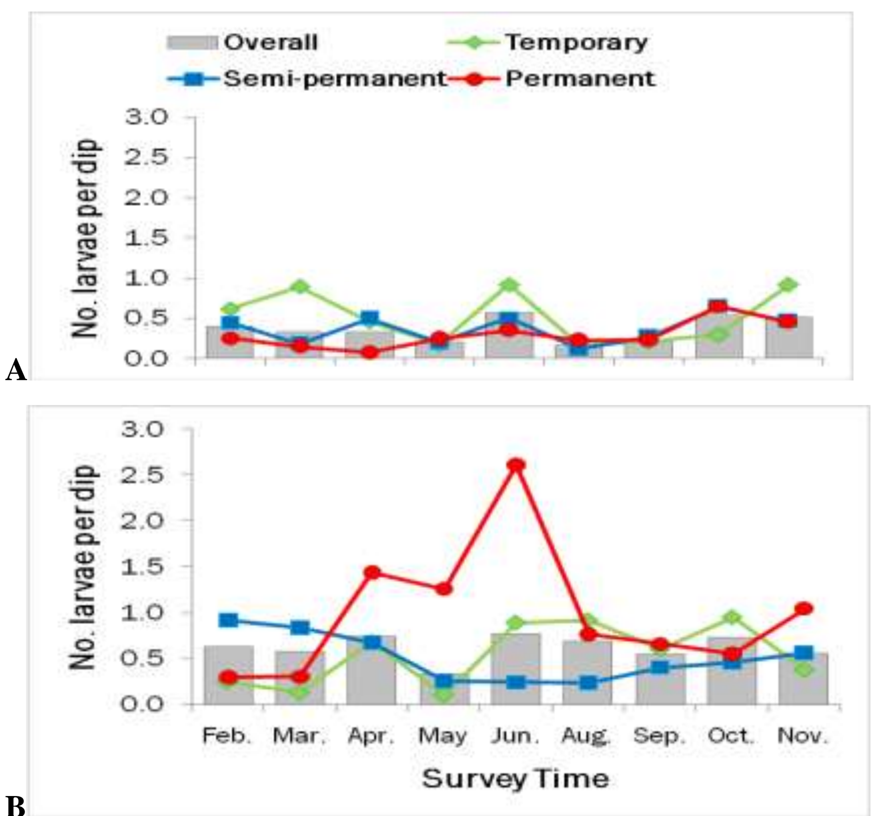


Figure 4.6: Larval density in the irrigated (A) and non-irrigated (B) ecosystem during monthly larval dynamics in 2019 in temporary, semi-permanent, and permanent habitats.

4.1.3 Habitat stability

Among a cohort of 100 aquatic habitats enrolled in September 2018, 17 habitats were classified as semi-permanent (man-made ponds, irrigation lining, and drainage ditches) and 33 were permanent habitats (swamps, river edges, and fish ponds) in the irrigated ecosystem. In the non-irrigated ecosystem, there were 28 semi-permanent (man-made ponds and drainage ditches) and 22 permanent (swamps, river edges, and natural ponds) habitats. One-year monitoring of these habitats found that habitats in the irrigated ecosystem remained aquatic longer than the habitats in the non-irrigated ecosystem. On average, 89.4% and 92.3% of the permanent and semi-permanent habitats respectively remained aquatic on the inspection days in the irrigated ecosystem (**Figure 4.7A**), significantly higher than those in the non-irrigated ecosystem (79.1% for permanent habitats, $Z = 12.23$, $P < 0.0001$; and 69.2% for semi-permanent habitats; $Z = 10.69$, $P < 0.0001$; **Figure 4.7B**). The largest difference in habitat stability between the irrigated and non-irrigated ecosystems occurred during the dry season.

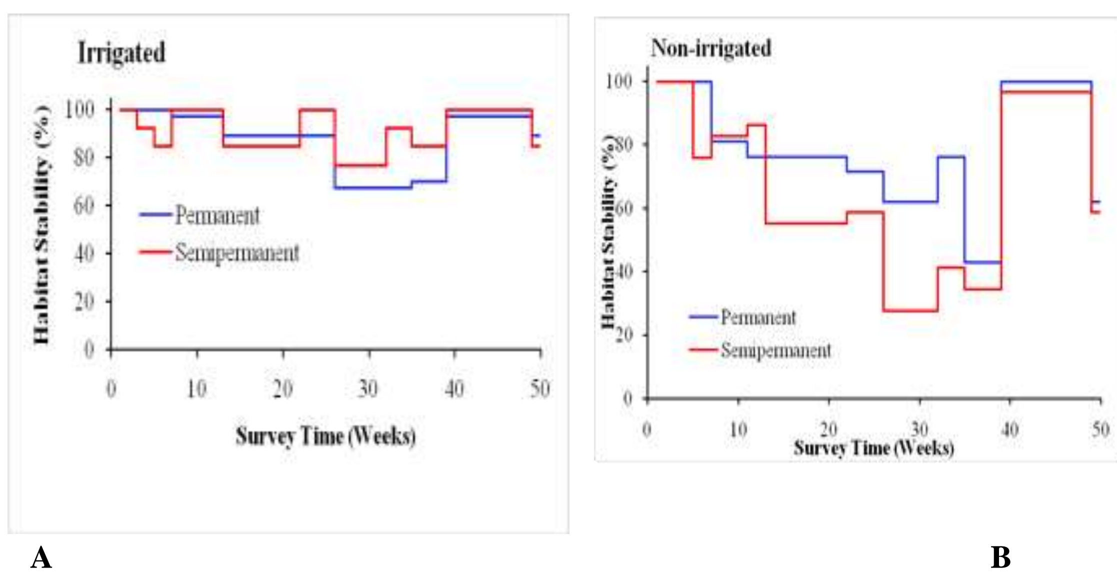
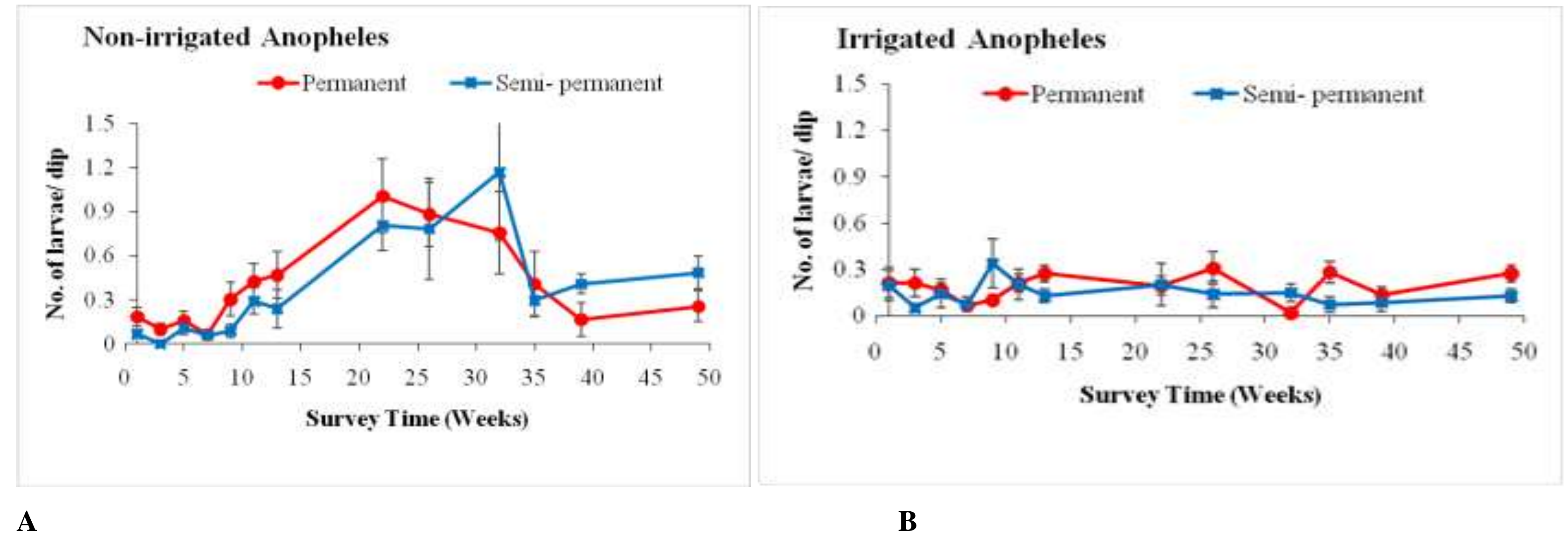


Figure 4.7: Habitat stability in the irrigated (A) and non-irrigated (B) ecosystems from September 2018 to August 2019.

In terms of larval density, permanent and semi-permanent habitats exhibited no significant difference within the irrigated ($F_{(1,23)} = 0.21$, $P > 0.05$; **Figure 4.8A**) and non-irrigated ecosystems ($F_{(1,23)} = 0.06$, $P > 0.05$; **Figure 4.8B**). However, average *Anopheles* larval densities in the non-irrigated ecosystem were significantly higher than in the irrigated ecosystem for both permanent habitats ($F_{(1,23)} = 5.62$, $P < 0.05$) and semi-permanent habitats ($F_{(1,23)} = 5.46$, $P < 0.05$). The period when the density differed the largest between the irrigated and non-irrigated ecosystems was in the dry period.



A **B**

Figure 4.8: ***Anopheles*** **densities per dip in the irrigated (A) and non-irrigated (B) ecosystems from September 2018 to August 2019.**

4.1.4 Habitat productivity surveillance using mosquito emergence traps

Overall, *An. coustani* were the predominant adults collected from the five habitat types, followed by *An. pharoensis* and *An. gambiae s.l.*, while *An. funestus* were collected only from the rice paddies (**Figure 4.9A**). The number of emerged *Anopheles* female mosquitoes varied significantly from 0.08 to 0.23 per trap-night among the 5 habitat types ($F_{(4,85)} = 2.54$, $P < 0.05$; **Figure 4.9A**), with drainage ditches having the highest productivity and the fish ponds the lowest productivity. The larval habitat types used for adult mosquito productivity surveillance did not differ significantly in larval densities (**Figure 4.9B**). Habitat productivity of adult mosquitoes varied significantly from1.1% to 4.4% ($P < 0.01$), with the highest in swamps and the lowest in man-made ponds and fish ponds (**Figure 4.9C**).

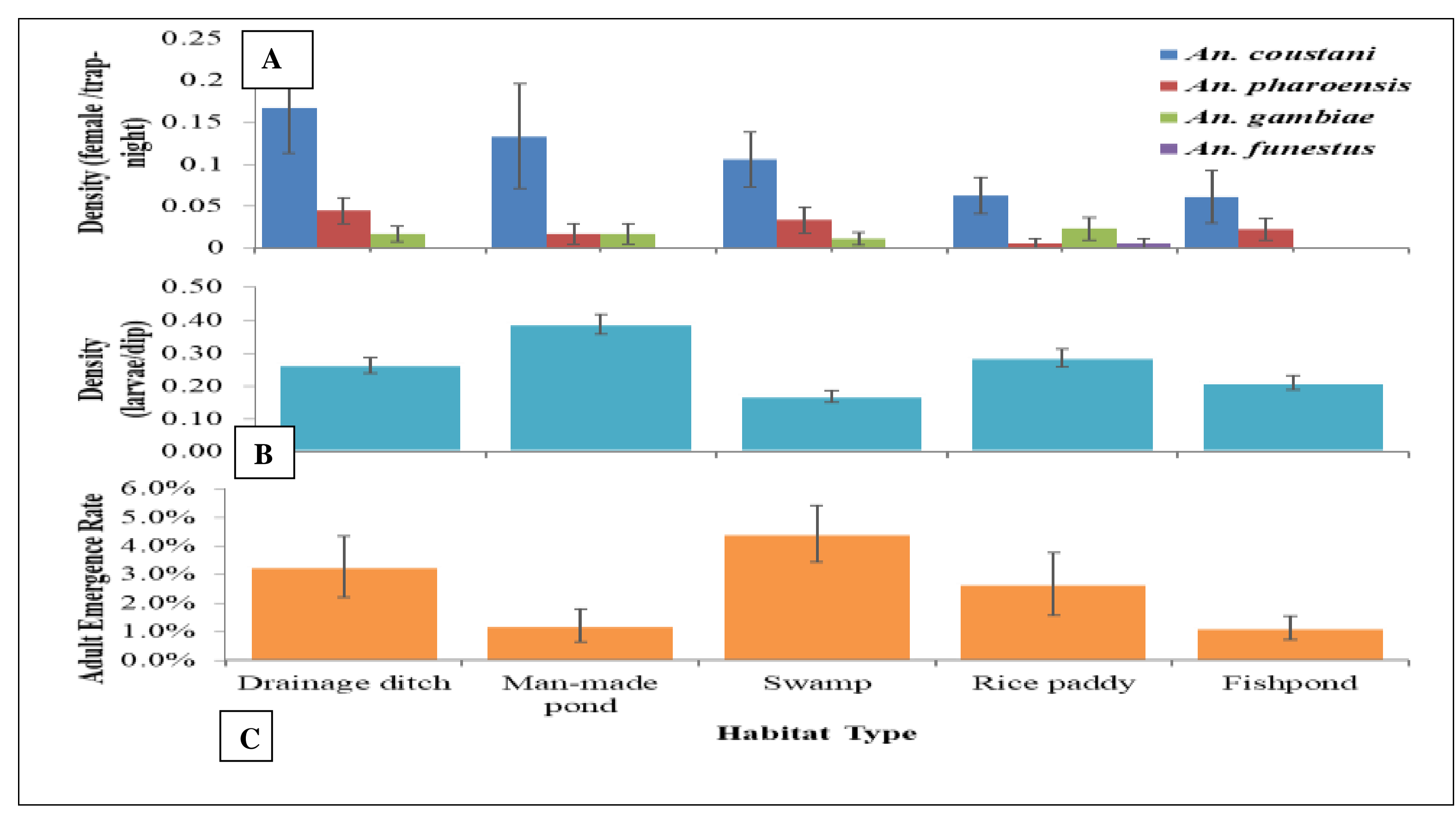


Figure 4.9: Densities of female adult *Anopheles* per trap-night collected from the emergence traps (A), densities of larval *Anopheles* per dip collected from the habitats (B), and adult emergence rate (C) from the different types of habitats where emergence traps were conducted.

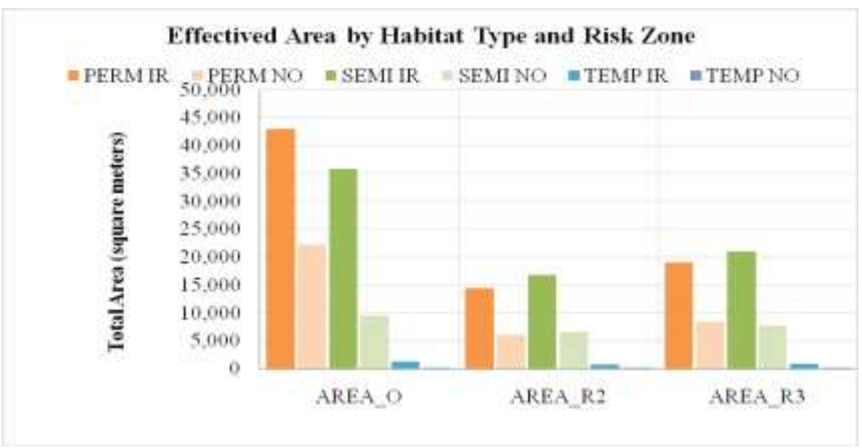
4.1.5 Overall adult malaria vector productivity in each ecosystem

After assessing for habitat availability, dynamics, and stability, the overall malaria vector productivity for all the habitats in irrigated and non-irrigated ecosystems in the study clusters was estimated. For both irrigated and non-irrigated ecosystems, semi-permanent habitats produced the largest number of adult vectors as they were most abundant and most productive possibly due to less predators as compared to the permanent habitats. The daily productivity of adult vectors from permanent and semi-permanent habitats in the irrigated ecosystem was 1.4-3.2 fold higher than those in the non-irrigated ecosystem, and similar for the temporary habitats (**Table 4.3**). The overall adult malaria vector productivity of clusters from the irrigated ecosystem was estimated to be 137.0 female malaria mosquitoes per km^2 cluster area per day,1.9 fold higher than clusters from the non-irrigated ecosystem (72.1 female malaria mosquitoes per km^2 cluster area per day) (**Table 4.3**).

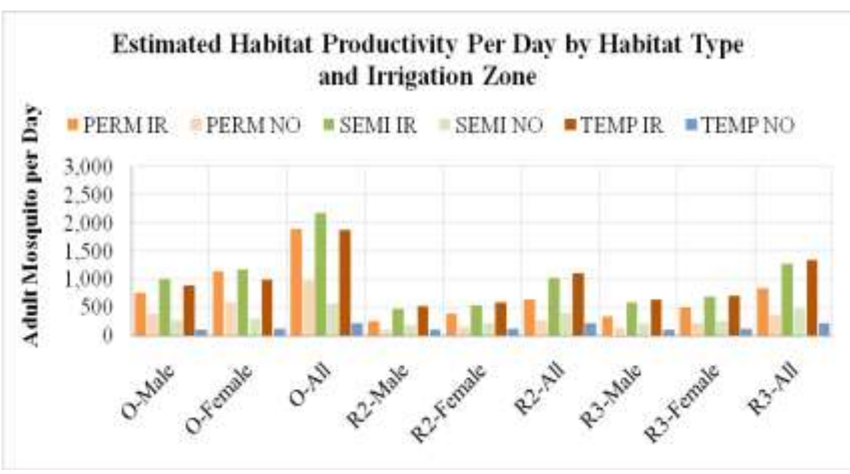
Table 4.3: Estimated adult malaria vector productivity for larval habitats in the study clusters in the irrigated and non-irrigated ecosystems.

Habitat type	Irrigated				Non-irrigated			
	Permanent	**Semi-permanent**	**Temporary**	**Total**	**Permanent**	**Semi-permanent**	**Temporary**	**Total**
Cluster Area (km^2)(95% CI)		**2.63** (2.49, 2.77)				**2.78** (2.23, 3.34)		
Habitat counts (/km^2-cluster area)	9	20.8	8.1	37.9	3.6	11.6	11.3	26.5
(95% CI)	(6.6, 11.5)	(17, 24.7)	(3.4, 12.9)		(2.5, 4.7)	(7.1, 16.1)	(5.7, 17.0)	
Habitat area (m^2/km^2-cluster area)	951	2,029	175	3,155	1,551	309	116	1,976
(95% CI)	(579, 1316)	(236, 3996)	(0, 359)		(486, 2252)	(155, 493)	(69, 177)	
Habitat effective area (m^2/km^2-cluster area)	485	748	91	1,324	339	230	97	666
(95% CI)	(302, 662)	(361, 1168)	(15, 170)		(208, 429)	(136, 343)	(54, 147)	
Female vector emerge rate (/m^2, from emergence trap)	0.085	0.104	0.2	-	0.085	0.104	0.2	-
Daily female vector productivity (number of female adults per km^2 cluster area)	41.2	77.6	18.2	137	28.8	23.9	19.4	72.1

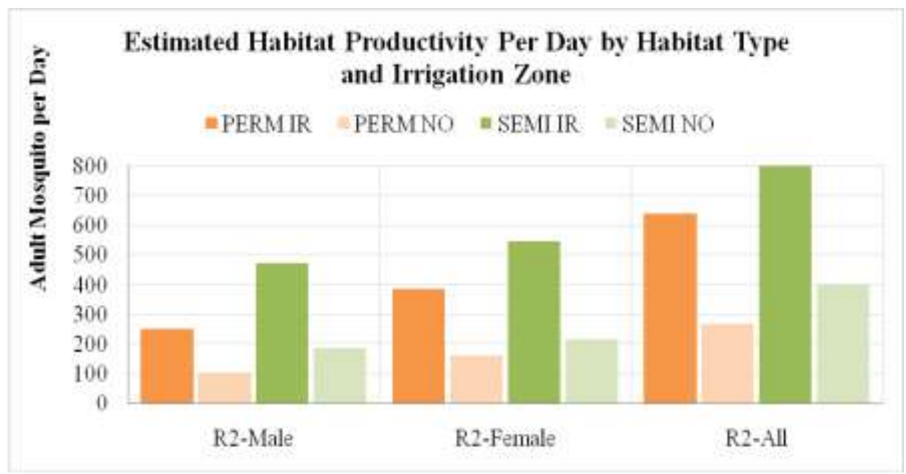
The original area (O) was observed to contain the most larvae when all habitat types were sampled. The permanent and the semi- permanent habitats in the irrigated area were the most productive. Temporary habitats were not productive in both ecosystems and in all places within the habitat (O, R2, R3) and when the R3 region was included, more larvae were observed thus implying more productivity as compared to only when the R2 region was sampled (**Figure 4.10A**). The original area (O) produced relatively more females than male adult mosquitoes but this difference was not significant (**Figure 4.10B**). Generally, the semi-permanent habitats in the irrigated area were the most productive habitats for both male and female adult mosquitoes (**Figure 4.10C**).



A



B



C

Figure 4.10: Estimated productivity by effective area (O, R2, R3) as measured by habitat type and irrigation zone (A), estimated habitat productivity per day by habitat type and irrigation zone in the O, R2 and R3 region (B), and estimated habitat productivity per day by habitat type and irrigation zone in the R2 region (C)

Key:

PERM IR – Permanent habitats in the irrigated area

PERM NO - Permanent habitats in the non- irrigated area

SEMI IR – Semi- permanent habitats in the irrigated area

SEMI NO - Semi- permanent habitats in the non- irrigated area

TEMP IR - Temporary habitats in the irrigated area

TEMP NO - Temporary habitats in the non- irrigated area

O - Original area

R2 – Effective area

R3 – Less effective area

When the data from the stability experiment was used to predict the productivity of temporary, semi- permanent, permanent and all habitats combined it was observed that the habitats in the irrigated ecosystem were the most productive (**Figure 4.11** and **Figure 4.12A-D**).

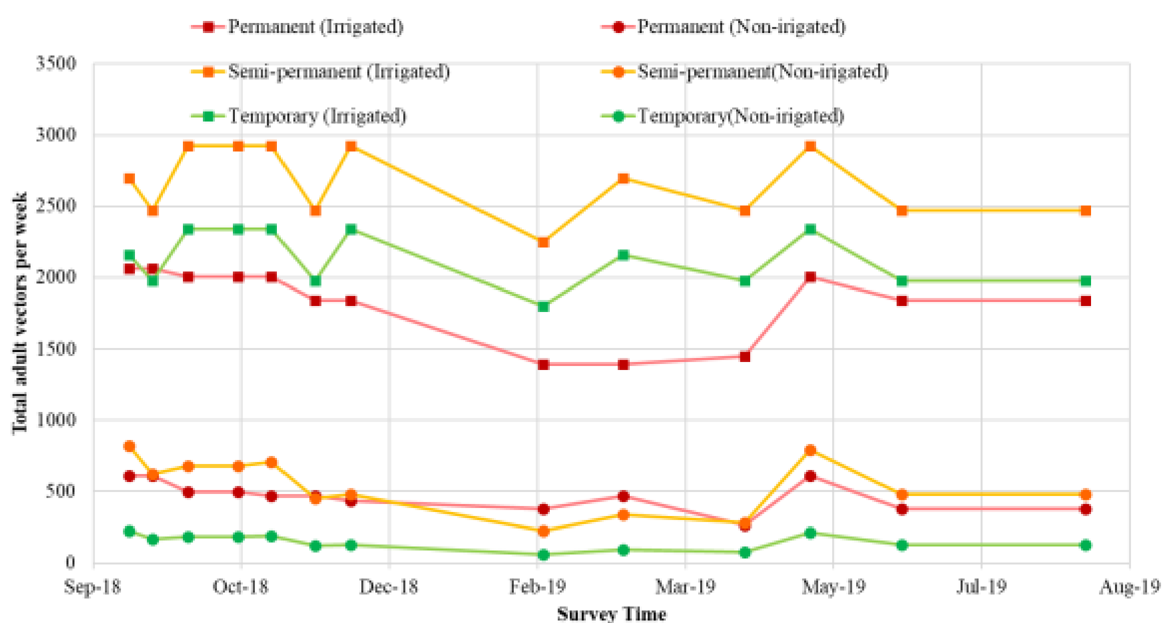
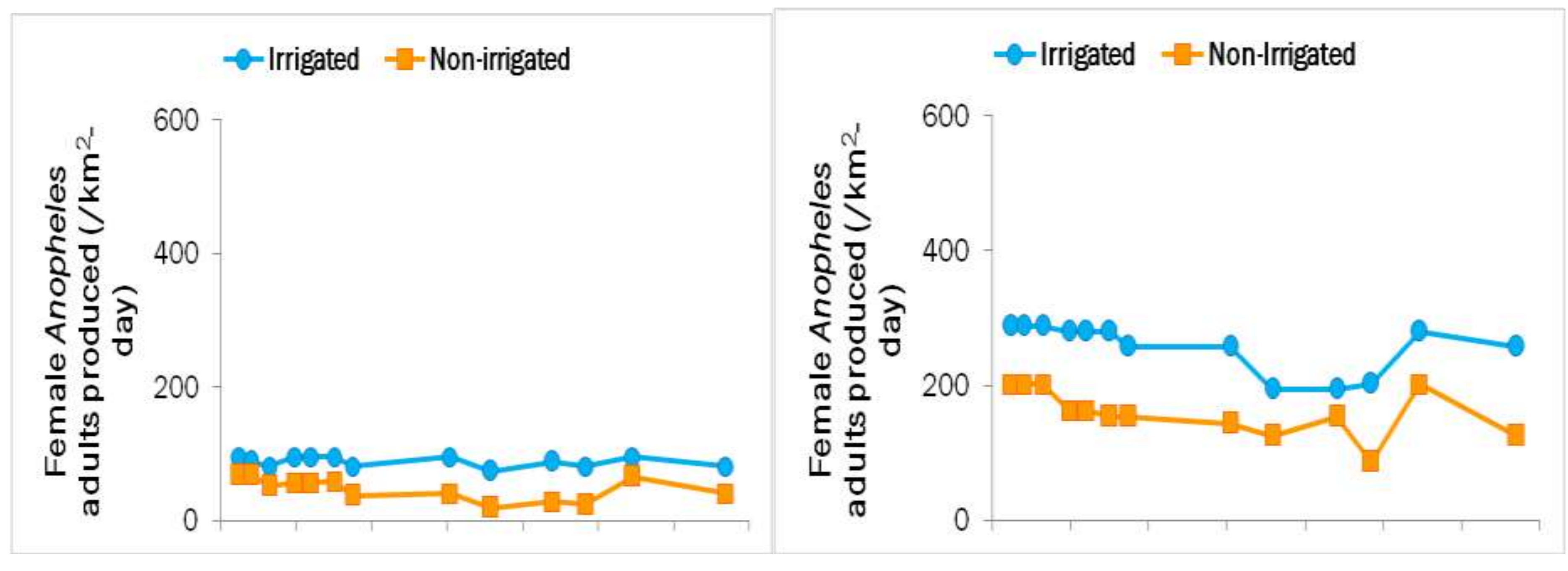
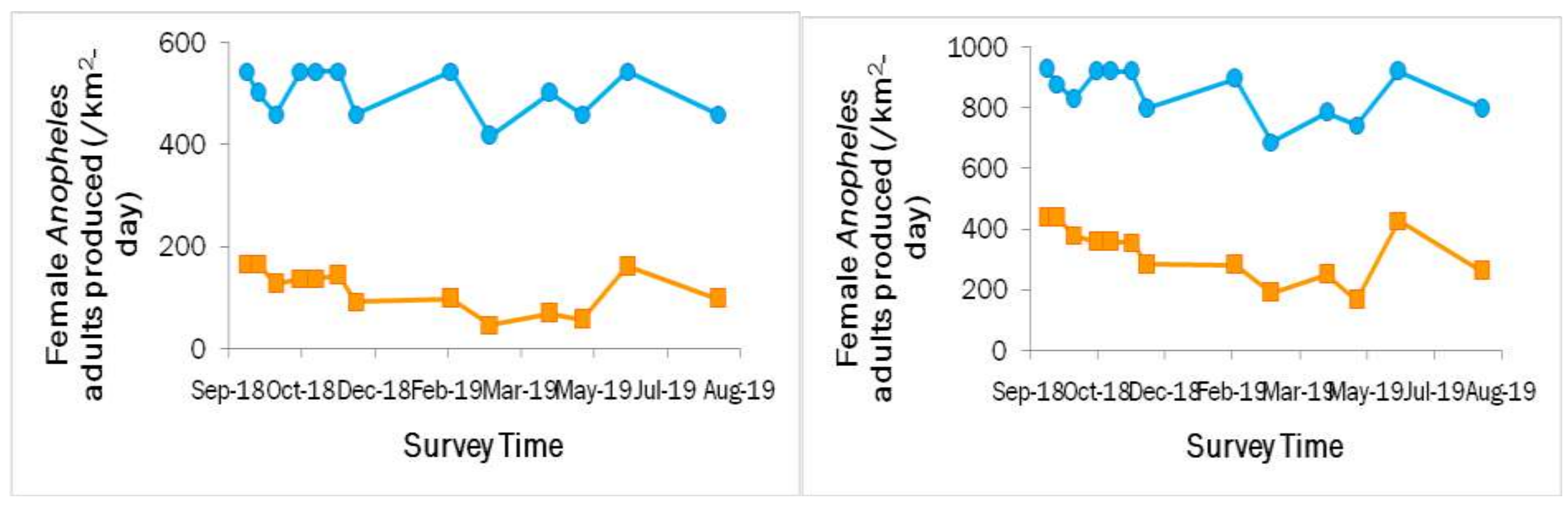


Figure 4.11: Estimated total adult vector productivity per week between September 2018 to August 2019.



A

B



C D

Figure 4.12: Predicted number of emerged female *Anopheles* adults produced in unit cluster area per day (/km2-day) in Temporary (A), Semi-permanent (B), Permanent (C)habitats, and all/ combined (D) habitat types.

4.1.6 Laboratory Predator experiment

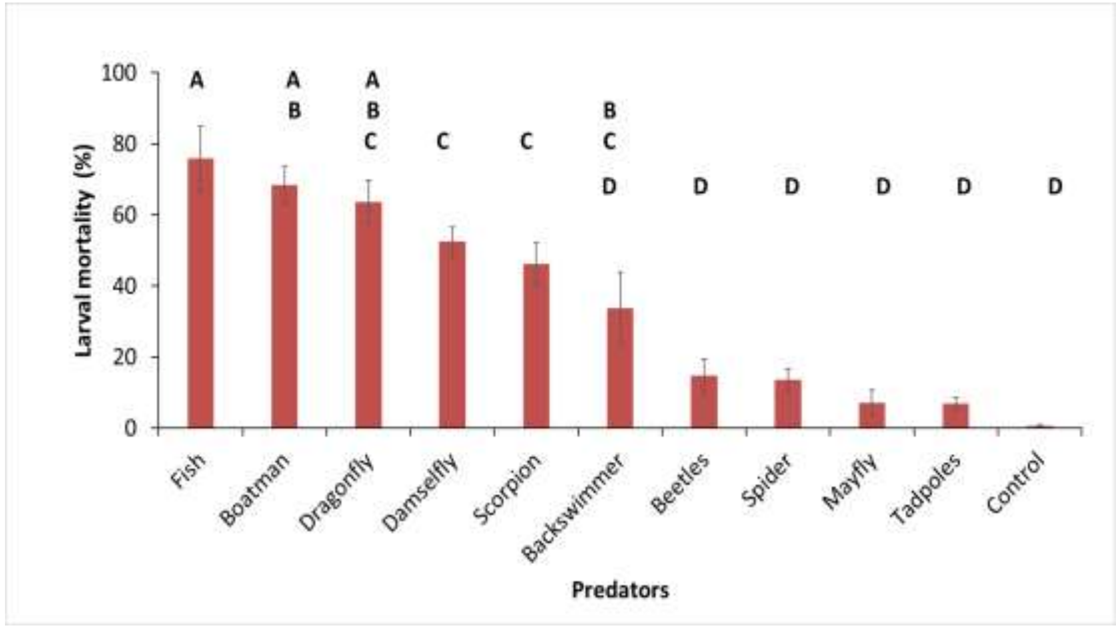
The predators were morphologically identified in the field and under a microscope in the laboratory. The following predators were used: a total of 71 water beetles (Family Hydrophilidae and Dytiscidae, Order Coleoptera), 108 tadpoles (Class Amphibians), 111 water boatman (Family Corixidae, Order Hemiptera), 68 water scorpion (Family Nepidae, order Hemiptera), 99 damselfly nymph (Family Lestidae, Order Odonata), 65 *Gambusia affinis* fish (Family Poeciliidae, order Cyprinodontiformes), 10 backswimmer (Family Notonectidae, Order Hemiptera), 30 water spiders (Family Dictynidae, Order Araneae) 26 mayfly (Family: Baetidae Order: Ephemeroptera), and 22 dragonfly nymph (Family Corduliidae, Order Odonata). In general, predators had a significant effect ($F=35.5$, $P< 0.0001$) on larval numbers (**Table 4.4**) per 24 hours.Fish was observed as the most efficident predator while the tadpoles were the least efficient predator.

Table 4.4: Summary results of the model fit of the predator experiment

Term	Estimate	Std Error	t Ratio	P-value	95% Confidence interval
Fish	40.98	5.38	7.61	<.0001	51.6 -> 30.4
Boatman	33.59	3.88	8.65	<.0001	41.2 -> 25.9
Dragonfly	28.83	4.91	5.87	<.0001	38.5 -> 19.1
Damselfly	17.68	4.06	4.35	<.0001	25.7 -> 9.7
Scorpion	11.25	4.56	2.47	0.0145	20.2 -> 2.3
Backswimmer	-1.11	10.75	-0.1	0.92	20.1-> -22.3
Beetles	-20.03	5.05	-3.97	0.0001	-10.1 -> -30.0
Spider	-21.27	4.37	-4.87	<.0001	-12.7 -> -29.9
Mayfly	-27.72	5.38	-5.15	<.0001	-17.1 -> -38.3
Tadpoles	-27.99	4.28	-6.53	<.0001	-19.5 -> -36.4

Among the predators studied, fish predation resulted in greatest larval reduction (75.8%) in the experimental basins. However, when fish (75.8%), boatman (69%), and dragonfly nymph (69.5%) reduction rates were compared, there was no significant difference. Similarly, there were no significant differences in reduction

rates between water boatman (69%) and dragonfly nymph (69.5%) basins. Dragonfly (69.5%), damselfly nymph (52.4%), water scorpion and (49.8%) were also similar in predation rates. Water beetles (14.9%), water spiders (12.2%), mayflies (7.3%), and tadpoles (6.9%) were found to be poor predators, with larval reduction rates that were not significantly different from the control basins. Backswimmers (31%) were deemed average because they were neither efficient nor inefficient predators (**Figure 4.13**).



NOTE: Similar letter = not significantly different

Figure 4.13: Predation rates of *An. gambiae* larvae in experimental basins in a laboratory set-up.

4.1.7 Habitat types for life table assessment

Larval samples for the vertical life table experiments were collected from 23 different permanent habitats over the course of the study. This is because permanenet habiats are known to be the preferred habitats for predators. These habitats were classified into four types. These included the 6 replicates of rice paddies, drainage ditches, fish ponds each and five replicates of man- made ponds. The rice paddies contained recently transplanted rice thus were highly flooded. The drainage ditches

were used to drain excess water from the farms while the fishponds were abandoned fishponds. The man-made ponds were formed as a result of human activities like sand and ballast harvesting. There was no correlation between predator increase and *An. gambiae* densities when all habitats were combined (**Figure 4.14A**). ($F_{1,114}$=2.37, , P=0.127). However, there was a strong, positive correlation between increased predator numbers and *Culex* larvae reduction, which was statistically significant (**Figure 4.14B**) ($F_{1,114}$=10.44, P=0.0016).

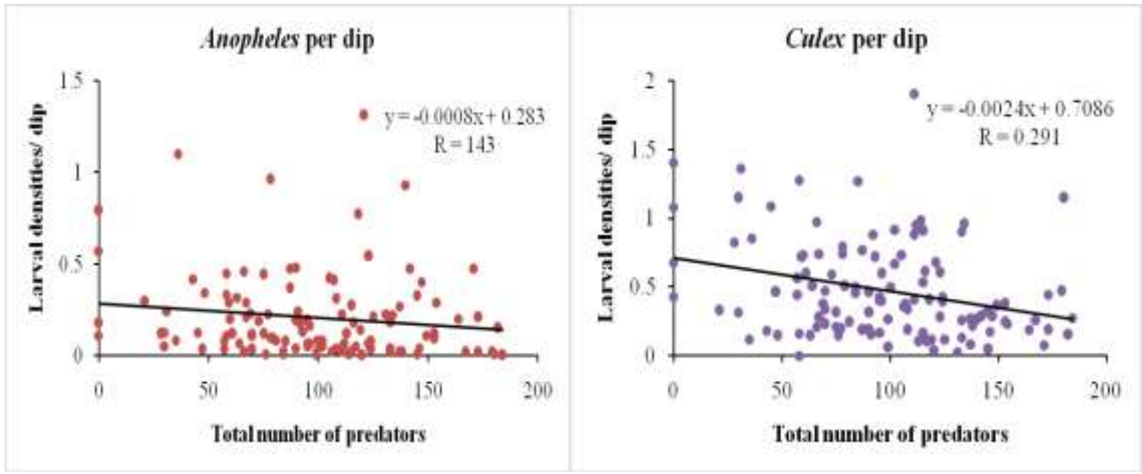
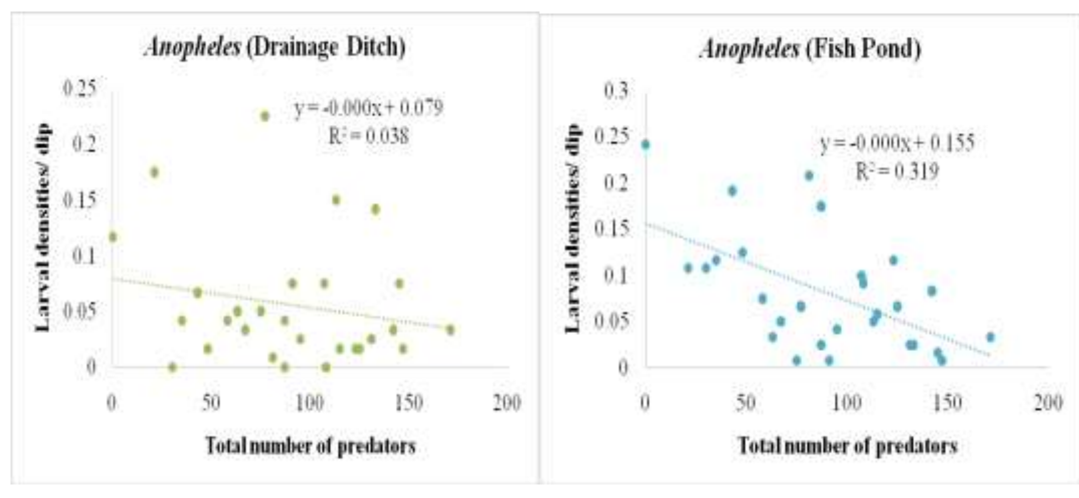
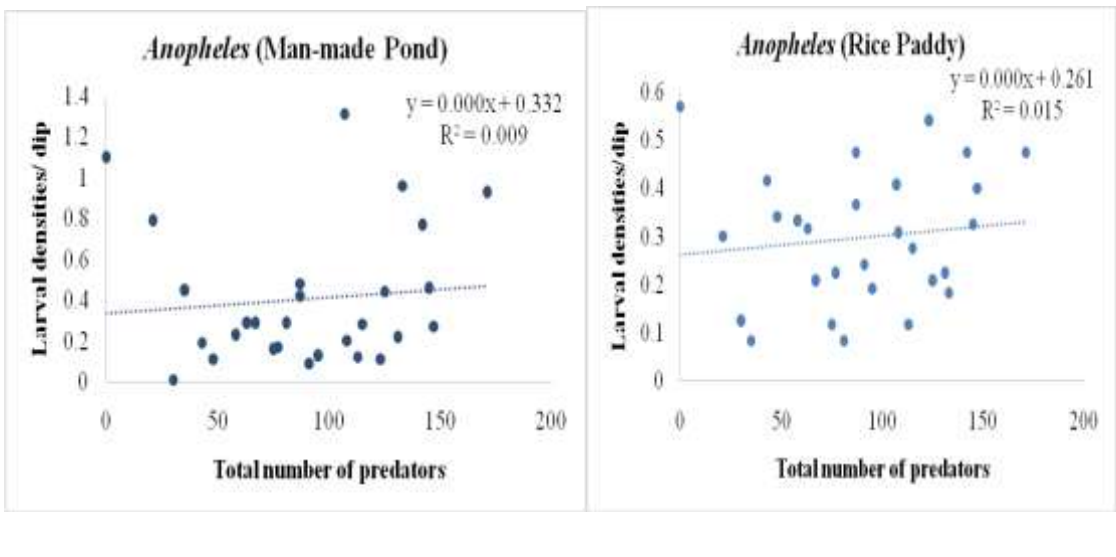


Figure 4.14: Correlation between larval densities and total number of predators for (A) *Anopheles* larvae and (B) *Culex* larvae per dip.

There was observed a non- significant positive correlation in the drainage ditches ($F_{1,114}$=1.03, P=0.32) (**Figure 4.15A**) and a significant positive correlation in the fishponds ($F_{1,114}$=12.20, P=0.002) (**Figure 4.15B**) for *Anopheles* larvae whereby an increase in predators resulted in a decrease in *Anopheles* larval densities. However, a negative correlation which was not significant was observed in man-made ponds ($F_{1,114}$=0.25, P=0.62) (**Figure 4.15C**) and rice paddies ($F_{1,114}$=0.41, P=0.53) (**Figure 4.15D**) resulting in an increase of *Anopheles* larval densities corresponding to an increase in predator densities.



A **B**



C **D**

Figure 4.15: Correlation between larval densities (larvae per dip) and total number of predators for *Anopheles* larvae in (A) drainage ditches, (B) fishponds, (C) man-made ponds, and (D) rice paddies .

For the *Culex* species of larvae, there was observed no correlation between the number of larvae and predators in the drainage ditches ($F_{(1,114)}$ =0.0188, P=0.89) (**Figure 4.16A**), however there was a positive correlation between the number of *Culex* larvae and predator densities in fishponds ($F_{(1,114)}$ =11.2, P=0.003) (**Figure 4.16B**), man- made ponds ($F_{(1,114)}$ =0.604, P=0.44) (**Figure 4.16C**) and rice paddies

($F_{(1,114)}$ =1.63, P=0.21) (**Figure 4.16D**). This correlation was only significant in the fishponds.

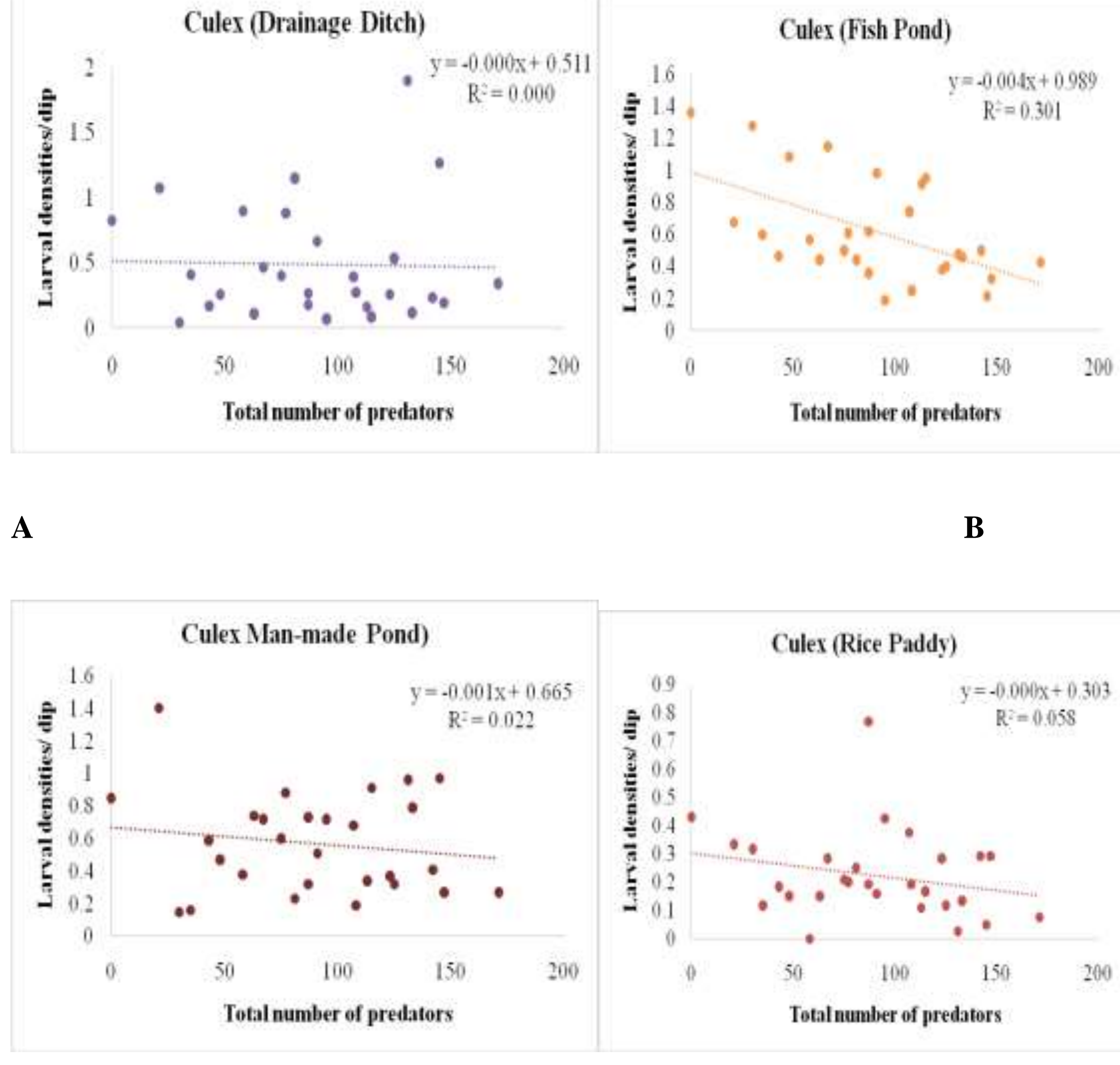
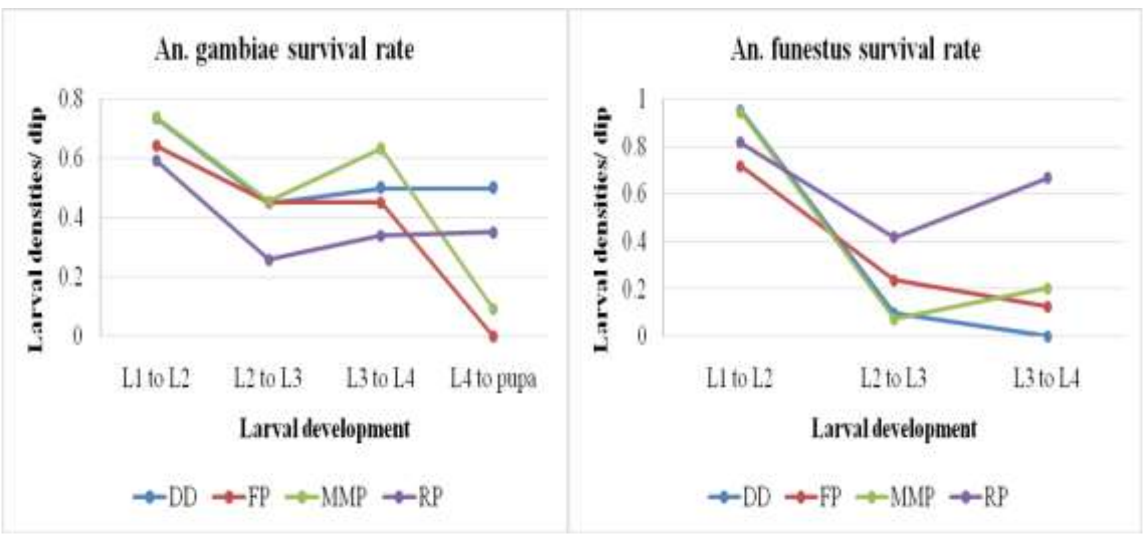


Figure 4.16: Correlation between larval densities (larvae per dip) and total number of predators for *Culex* larvae in (A) drainage ditches, (B) fishponds, (C) man-made ponds, and (D) rice paddies

4.1.8 Survival rates for larval vectors

Anopheles gambiae survived best in drainage ditches, while *An. funestus* thrived best in rice paddies (**Figure 4.17**). In all habitat types, the highest larval stage survival

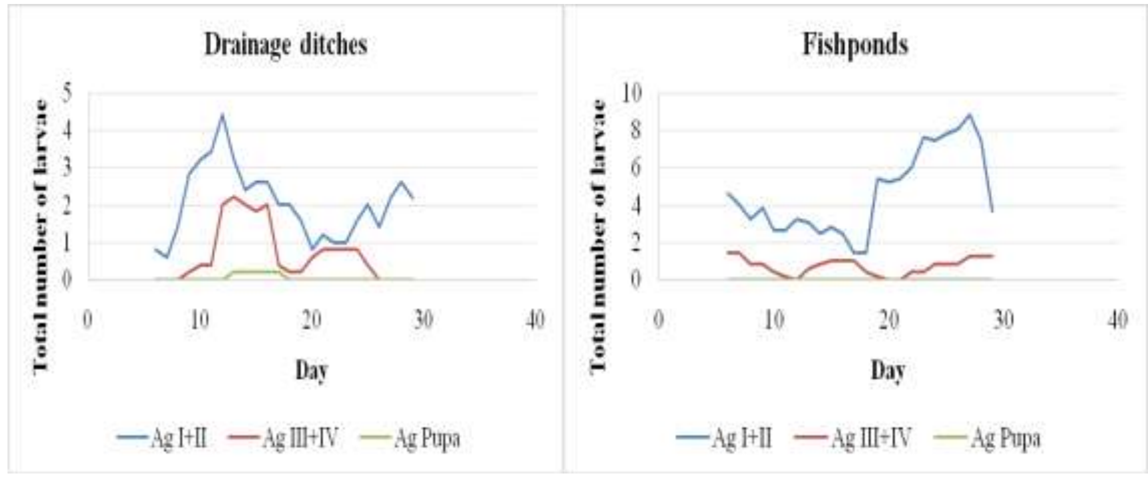
rate was observed in both *An. gambiae* (**Figure 4.17A**) and *An. funestus* (**Figure 4.17B**) species from larva stage I to II, with the lowest being stage IV to pupa for *An. gambiae*. During the study period, no *An. funestus* pupa was collected. The mortality rates of *An. gambiae* immatures was 97.9%, 100%, 98.3%, and 99.2% in drainage ditches fishponds, man- made ponds, and rice paddies respectively while the mortality of *An. funestus* was 100% in all habitat types. The highest stage survival rate was observed in larval stage I to II stages in both *An. gambiae* (**Figure 4.17A**) and *An. funestus* (**Figure 4.17B**) species in all habitat types and the lowest being larval stage IV to pupa for *An. gambiae*. No *An. funestus* pupa was sampled during this study period.



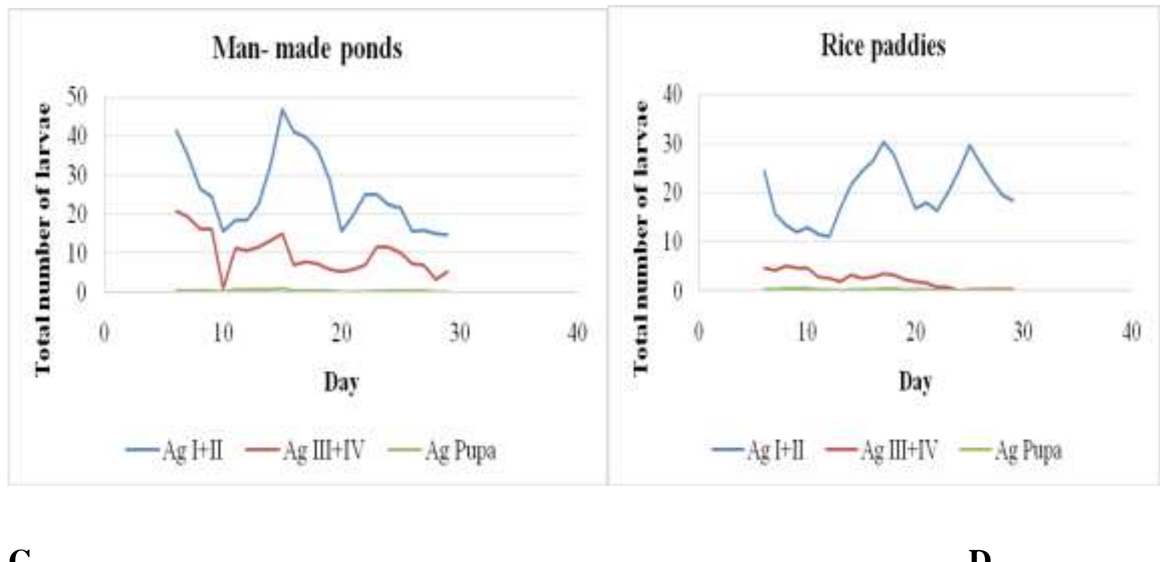
A **B**

Figure 4.17: Mosquito age specific survival rate for (A) *An. gambiae* and (B) *An. funestus* in drainage ditches (DD), fishponds (FP), man-made ponds (MMP), and rice paddies (RP) habitat types.

Furthermore, an increase in younger larval stages resulted in an increase of older larval stages a few days and vice versa (**Figure 4.18**). *Anopheles gambiae* immature larval stages peaked between the 10th and 20th day in drainage ditches, man- made ponds, and rice paddies habitat types as opposed to fish ponds (**Figure 4.18**).



A **B**



C **D**

Figure 4.18: Total number of *An. gambiae* in (A) drainage ditches, (B) fishponds, (C) man-made ponds, and (D) rice paddies using five- day smoothed dynamics

Key:

Ag I+II – *Anophleles gambiae* larval stage I and II

Ag III+IV - *Anophleles gambiae* larval stage III and IV

Ag Pupa - *Anophleles gambiae* Pupa

A 5- day smooth dynamics average of the larvae and pupae in the four habitat types showed that the age distribution of *An. gambiae* s.l. and *An. funestus* larvae fluctuated over the study period. For immature larval stages of *An. funestus*, only fish ponds and rice paddies had similar peak patterns between days 15 and 25, whereas drainage ditches peaked before the 15th day (**Figure 4.19**).

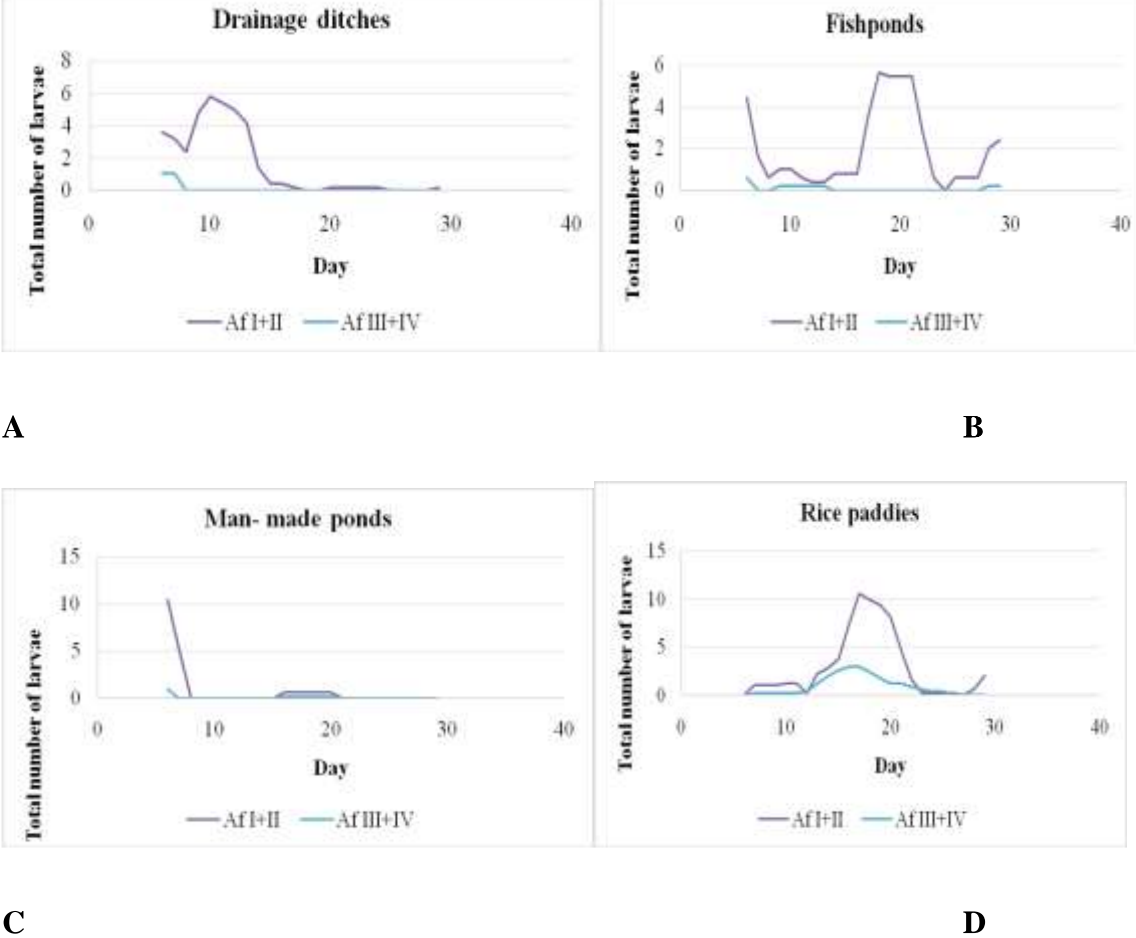


Figure 4.19: Total number of *An. funestus* in (A) drainage ditches, (B) fishponds, (C) man-made ponds, and (D) rice paddies using five- day smoothed dynamics.

Key: Af I+II – *Anophleles funestus* larval stage I and II, **Af III+IV** - *Anophleles funestus* larval stage III and IV, **Af Pupa** - *Anophleles funestus* Pupa

4.2 Bacterial culture results

Samples from the non-irrigated site, both from larval positive and negative habitats failed to show any bacterial culture in MacConkey and EMB since they only selected gram-negative bacteria. Nutrient agar showed growth for both irrigated and non-irrigated larval positive and negative water samples. **Table 4.5** summarizes the cultured samples and their origin and date of collection. The samples were labeled depending on the media in which they were grown. For example sample 1 grown in nutrient agar and EMB was labeled as NA1 and EMB1 respectively. When this sampled was sub-cultured, the labels included letter numbering e,g NA1A, NA1B.

Table 4.5: Origin of samples cultured for MALDI-TOF MS and bacterial sequencing

Sample Number	Origin	Larval Habitat Status	Sampling Month
1	Irrigated area	Positive	November 2021
2	Irrigated area	Negative	November 2021
3	Non-irrigated area	Negative	November 2021
4	Non-irrigated area	Positive	November 2021
5	Irrigated area	Negative	December 2021
6	Irrigated area	Positive	December 2021
7	Non-irrigated area	Negative	December 2021
8	Non-irrigated area	Positive	December 2021
9	Irrigated area	Negative	January 2022
10	Irrigated area	Positive	January 2022
11	Non-irrigated area	Positive	January 2022
12	Non-irrigated area	Negative	January 2022

4.3 MALDI-TOF MS results

A total of eight genera were identified using MALDI-TOF MS. There was observed more diverse genera of bacteria in the irrigated area than the non-irrigated area. *Bacillus* (*Bacillus amyloliquefaciens*) was the only species identified in larval sources in the non-irrigated area. The rest of the identified genera (51) were from the

irrigated area. The dominant genera among the isolates, *Shigella* accounted for 47% (24 samples) followed by *Escherichia* with 25% (13 samples). *Bacillus*, *Citrobacter*, *Brevibacillus*, *Enterobacter*, and *Klebsiella* were observed at a lower frequency of less than 10% (**Figure 4.20**).

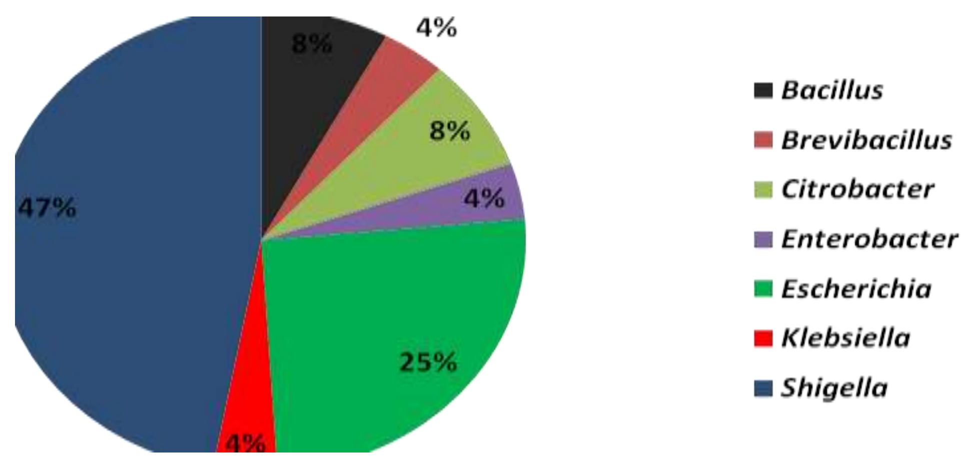


Figure 4.20: Distribution of bacterial genera detected by MALDI-TOF MS from irrigated and non-irrigated larval sources.

Escherichia coli was the dominant identified species (13 samples). This was closely followed by *Shigella sonnei* (11 samples) and *Shigella boydii* (8 samples), *Shigella flexneri* and *Bacillus amyloliquefaciens* (3 samples each) were the least amoung the identified species. *Citrobacter freundi*, *Citrobacter braaki*, and *Klebsiella pneumoniae* were each detected twice (**Figure 4.21**). Others included *Enterobacter cloacae*, *Shigella sp.*, *Enterobacter asburiae*, *Shigella dysentriae*, *Bacillus subtilis*, *Brevibacillusagri*, and *Brevibacillusbrevi* (**Figure 4.21**).

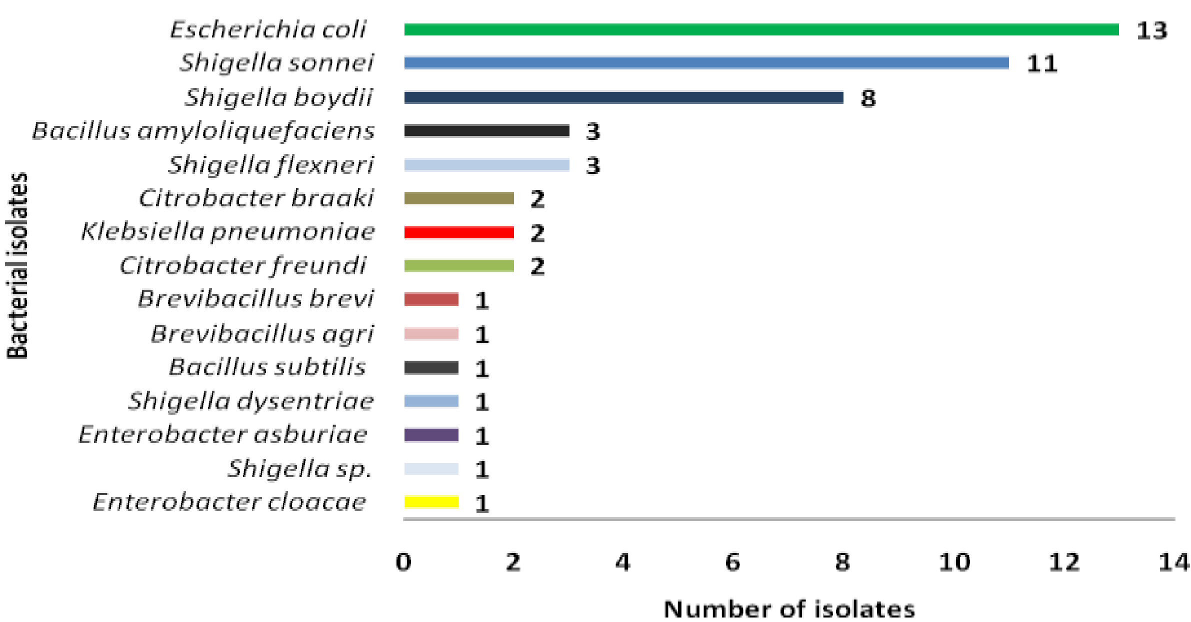


Figure 4.21: Distribution of bacterial species detected by MALDI-TOF MS from larval habitats in the irrigated and non-irrigated areas.

4.4 Bacterial isolates identified on sequencing aquatic larval sources

31 isolates were sequenced. Of these, 65% (20 samples) were *Bacillus*, 13% (4 samples) were *Escherichia*, 10% (3 samples) were *Exiguobacterium*, and 3% (1 sample) each were *Paenibacillus*, *Staphylococcus*, *Citrobacter*, and *Enterococcus* (**Figure 4.22**). The ClustalW results indicated that the aligned sequences had several conserved regions**.**

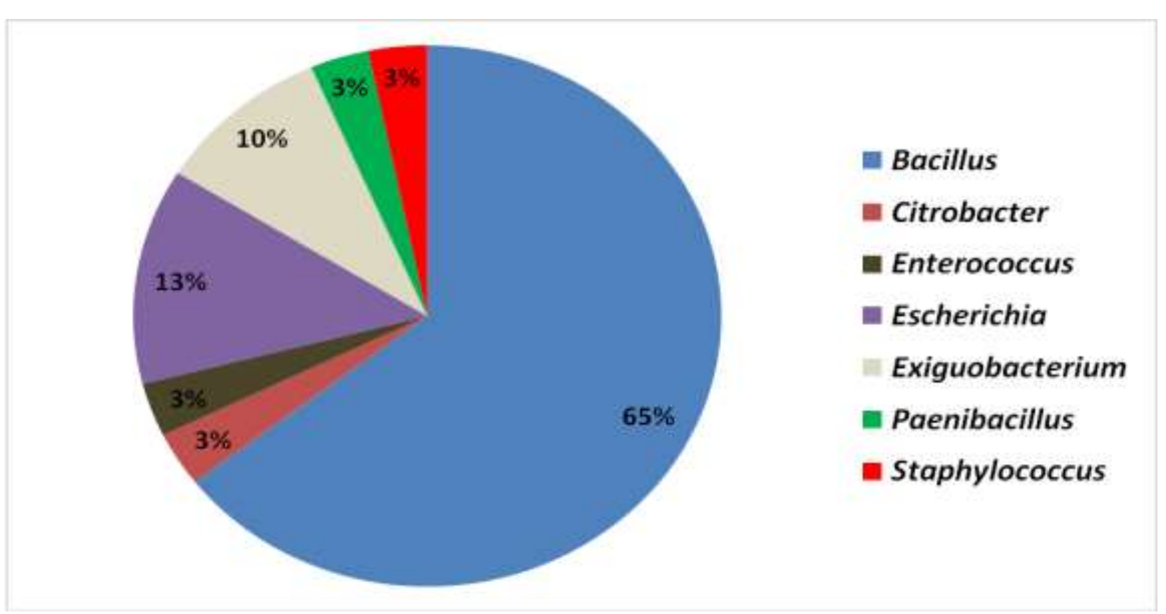


Figure 4.22: Frequency of isolated genera from larval habitats in irrigated and non-irrigated area.

The most predominant *Bacillus* species was *Bacillus velezensis* accounting 25% of the total *Bacillus*, followed by *Bacillus subtilis* accounting for 20%. *Bacillus siralis*, *Bacillus stercoris*, *Bacillus aerius* and *Bacillus cereus* each accounted for 10%. The rest of the identified *Bacillus* species (*Bacillus inaquosorum*, *Bacillus mojavensis*, and *Bacillus tequilensis*) were at a frequency of 5% each. The identity matrix is shown in **Appendix XVII**.

The isolates clustered into 12 clades which were representative of the mentioned genera (**Figure 4.23**). In clade 1, there wree *Bacillus subtilis* (KC441741), *Bacillus mojavensis* (OP218479 and OP482166), *Bacillus tequilensis* (OP435764), and *Bacillus stercoris* (OP521932) clustered with isolate NA10A, NA10B, NA12AA, NA4BB and NASAA. *Bacillus aerius* (OP115492) and isolates EMB6A, EMB6 were in clade 2. Clade 3 comprised of *Robertmurraya siralis* (OK570087), *Bacillus siralis* (MH746088), and isolate NA4A. Clade 4 had *Staphylococcus arlettae* (OP402856) and isolate NA4A while clade 5 had *Enterococcus gallinarum* (OP501807) and isolate NA9. Clade 6 had *Bacillus cereus* (ON860698 and ON430535) and isolate NA3 and MC2 and in Clade 7 there were *Exiguobacterium profundum* (OP793848 and OP263689) and isolates MC1A and NABAA. Clade 8

had *Escherichia coli* (CP102379), uncultured bacterium (JQ265468) and isolate EMB9A while clade 9 had *Escherichia coli* (OP514801) and isolates NABA, NA11A, MC5B, EMB9 and NASB. Clade 10 had *Bacillus inaquosorium* (ON999044), *Bacillus velezensis* (OP554433), *Bacillus subtilis* (KF732994) isolates NA7C, NA2BA, NASAB, NA6B, NA4BA, NA12B, and NA12AB, Clade 11 had *Citrobacter* sp. (OX245674) and isolate MC9 while Clade 12 comprised of *Bacillus siralis* (LC588555), *Paenibacillus dendritiformis* (OX216966) and isolates NA2A, and NA7 (**Figure 4.23**). The tree with the highest log likelihood (-1502.82) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. There was a strong, positive correlation between larval sources in irrigated areas and bacterial species abundance, which was statistically significant (r=0.334, n=44, p=0.027).

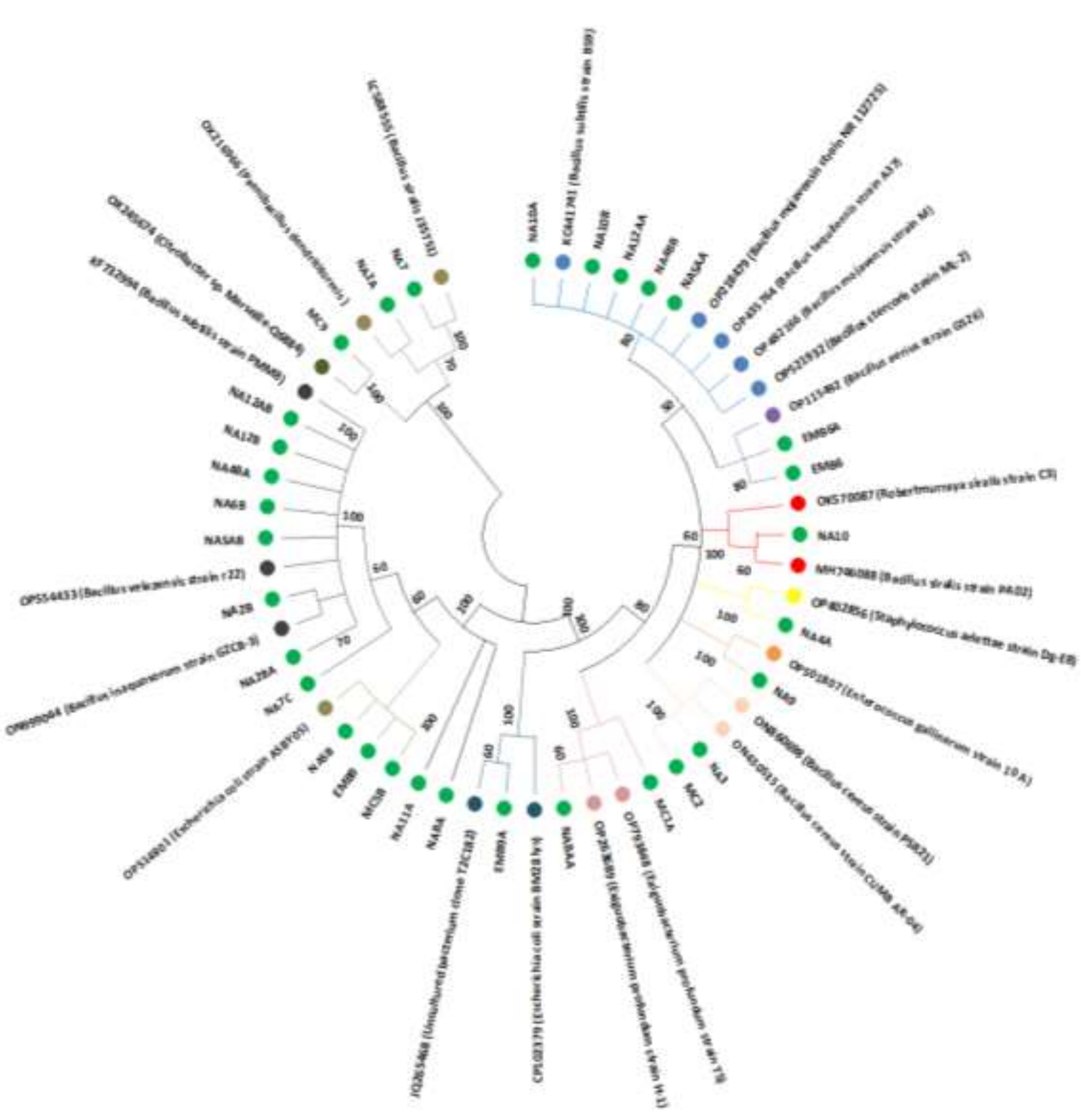


Figure 4.23: Phylogenetic relationship between isolated bacteria and accessions within GenBank.

4.5 Aquatic metabolite analysis

Measurements done for water samples with and without mosquito larvae from the irrigated and non- irrigated areas showed that aquatic habitats had various metabolite composition which varied with the presence or absence of mosquito larvae.

4.5.1 Fatty acids concentration and larval availability

There was varying concentration (0.95%-28.42%) of fatty acids (Palmitic, Palmitoleic, Stearic, Oleic, Linoleic, Linolenic, Arachidonic, Eicosapentaenoic acid (EPA) , Docosahexaenoic acid (DHA), and Nervonic) in larval sites within irrigated and non-irrigated areas with larval presence or absence (**Table 4.6**). Fatty acid concentrations in sites with larvae within irrigated and non-irrigated areas, and sites with no larvae in irrigated and non-irrigated areas did not differ significantly, implying that they had no overall effect on larval presence or absence ($F (3, 36) = 6.732e\text{-}008$, $P>0.99$). At the fatty acid component level, larval presence was observed in irrigated and non-irrigated areas where Stearic concentrations were low (5.6 and 6.3% in irrigated and non-irrigated areas, respectively). Contrary to what was observed at low Stearic concentrations, larval presence was observed at elevated Nervonic concentrations of 6% and 6.2% in irrigated and non-irrigated areas, respectively (**Table 4.6**).

Table 4.6: Fatty acid concentrations in larval sites with and with no larvae within irrigated and non-irrigated areas.

Fatty acids	Irrigated sites with larvae Concentration (%)	Irrigated sites without larvae Concentration (%)	Non-Irrigated sites with larvae Concentration (%)	Non-Irrigated sites without larvae Concentration (%)
Palmitic	28.42	28.28	13.77	22.45
Palmitoleic	16.49	8.74	12.39	12.36
Stearic	5.6	10.76	6.29	14.63
Oleic	14.68	11.9	12.28	26.07
Linoleic	7.07	4.1	9.31	5.33
Linolenic	8.31	3.4	6.86	7.28
Arachidonic	5.99	10.45	10.01	4.92
EPA	3.98	5.27	10.65	2.19
DHA	3.44	13.38	12.22	3.82
Nervonic	6.01	3.72	6.21	0.95
Average fatty acid	10	10	10	10

4.5.2 Phytochemical concentration and larval availability

In both larval breeding sites in irrigated and non-irrigated areas, the crude fat content ranged from 0.01% to 0.18%. The irrigated sites with larvae had a lower content of 0.01% than the same site without larvae (0.18%). The crude fat content (0.01%) was the same in non-irrigated sites with and without larvae. Larval sites within irrigated areas also recorded higher crude protein content (0.13% and 0.16%) in larval sites within irrigated areas with and with no larvae, respectively, compared to those in non-irrigated areas (0.08% and 0.07%). Larvae were found in irrigated areas with low crude protein content (0.13% versus 0.16%) as opposed to what was observed in non-irrigated areas (0.08% versus 0.07%). Similar observation was made on Beta-Carotene (µg/100g) content in irrigated areas (1.26 versus 2.04) and non-irrigated areas (5.2 versus 3.16) as well as total phenol µg (GAE)/100g (**Table 4.7**). Larval presence was evident in sites within irrigated and non-irrigated areas with high total chlorophyll content (1.12 µg/g versus 0.81 µg/g and 3.37 µg/g versus 0.82). Both Tannin and Anti-oxidant contents were low in sites with larvae in irrigated areas and

high in sites with larvae in non-irrigated areas (**Table 4.7**). The varied concentration of each phytochemical component between sites with larvae and with no larvae in irrigated and non-irrigated areas was not significant (**Table 4.7**).

Table 4.7: Phytochemical concentrations in larval sites with and with no larvae within irrigated and non-irrigated areas.

Element	Irrigated sites with larvae	Irrigated sites without larvae	Non-Irrigated sites with larvae	Non-Irrigated sites without larvae
Crude Fat (%)	0.04	0.18	0.01	0.01
Crude Protein (%)	0.13	0.16	0.08	0.07
Beta-Carotene (µg/100g)	1.26	2.04	5.2	3.16
Total Phenol (µg (GAE)/100g)	4.49	5.29	4.55	4.68
Total Chlorophyll (µg/g)	1.12	0.81	3.37	0.82
Tannin (mg (TAE)/100g)	0.1	1.08	3.22	0.28
Anti-oxidant (@0.5ml/ml) % Inhibition	20.38	29.4	26.4	23.65

4.5.3 Sugar concentration and larval availability

Sites with larvae in irrigated areas had high concentration (mg/100ml) of Pectin (0.64 versus 0.50), cellulose (7.72 versus 2.86), and fructose (0.36 versus 0.33) as compared sites with no larvae. Similar trend on pectin, cellulose and fructose was observed in sites with and with no larvae in irrigated areas (**Table 4.8**). The three sugar components were at higher concentration in larval sites within non-irrigated areas with larvae as compared to sites with larvae in irrigated areas (**Table 4.8**). Glucose concentration was however low in sites with larvae in irrigated areas compared to sites with no larvae (0.38 versus 1.16). In non-irrigated areas, glucose concentration was high in sites with larvae as compared to those without (2.67 versus 0.47). Similar observation was made on sucrose concentration in irrigated (0.08 versus 0.16) and non-irrigated areas (0.25 versus 0.09). The varied concentration of

each sugar component between sites with larvae and with no larvae in irrigated and non-irrigated areas was not significant.

Table 4.8: Sugar concentrations in larval sites with and with no larvae within irrigated and non-irrigated areas.

Components	Irrigated sites with larvae	Irrigated sites without larvae	Non-Irrigated sites with larvae	Non-Irrigated sites without larvae
Pectin(mg/100ml)	0.64	0.5	0.77	0.19
Cellulose(mg/100ml)	7.72	2.86	11.17	0.86
Glucose(mg/100ml)	0.38	1.16	2.67	0.47
Fructose(mg/100ml)	0.36	0.33	0.76	0.25
Sucrose(mg/100ml)	0.08	0.16	0.25	0.09

4.5.4 Aquatic mineral concentration and larval availability

High mean concentration of Manganese, Calcium and Copper were observed in sites with larvae in irrigated and non-irrigated areas (**Table 4.9**). Other minerals such as Lead, Iron, Magnesium and Zinc were at near similar concentration in all sites.

Table 4.9: Mineral concentrations in larval sites with and with no larvae within irrigated and non-irrigated areas.

Minerals	Irrigated sites with larvae	Irrigated sites without larvae	Non-Irrigated sites with larvae	Non-Irrigated sites without larvae
Lead (mg/100ml)	5.7	5.41	8.62	9.47
Manganese (mg/100ml)	249.72	153.83	198.04	182.48
Calcium (mg/100ml)	8.46	7.89	9.1	6.51
Copper (mg/100ml)	4.97	3.66	6.25	5
Iron (mg/100ml)	1.16	1.08	1.11	1.16
Magnesium (mg/100ml)	1.12	1.12	1.16	1.12
Zinc (mg/100ml)	0.02	0	0.01	0.01

4.6 Larval chemical exposure tests

4.6.1 Questionnaire survey on use of chemical

Data was collected from agro- vets, households and veterinary and agricultural extension workers in July – August 2018. Questionnaires were distributed in both the irrigated and non- irrigated areas and data collected, 98 were in the irrigated area and 102 in the non-irrigated area. Questionnaires were also distributed to 6 agro- vet shops and 10 veterinary officers/ agricultural extension workers. It was observed that more households in irrigated area (n=96, 46.9%) use pesticides in their farms compared to households in non-irrigated area (n=99, 28.3%) (Chi-square test, X = 6.308, df = 1, P = 0.12). However, more households in non-irrigated area (n=98, 83.7%) used pesticides on livestock than that in the irrigated area (n=98, 68.4%) (Chi-square test, X = 9.179, df = 1, P = 0.02) (**Figure 4.24).**

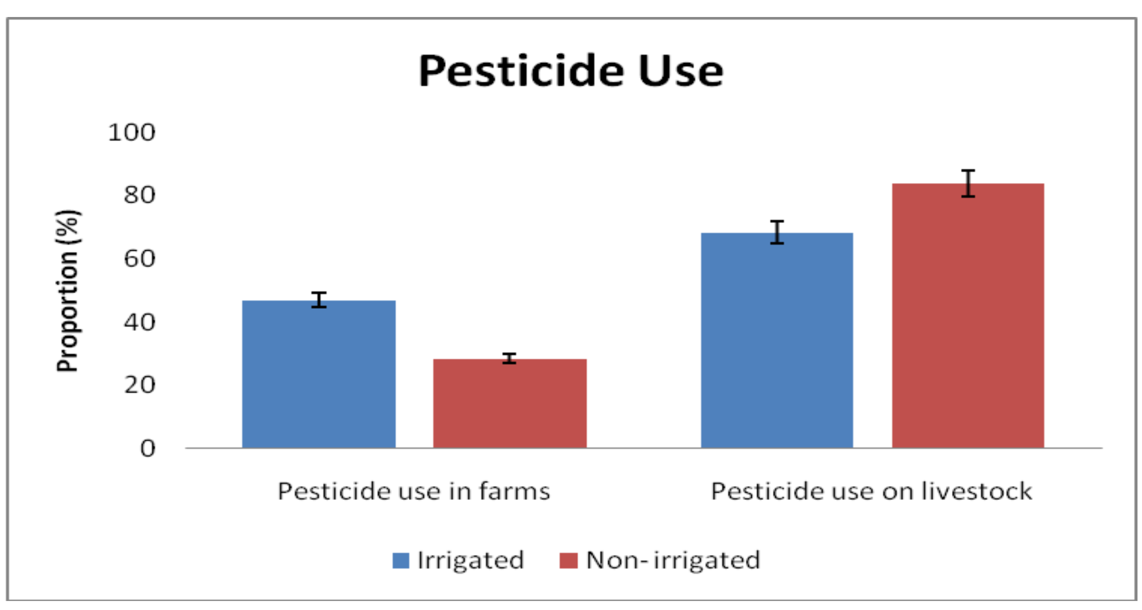


Figure 4.24: Frequency of responses to chemical use in agriculture (farms) and veterinary (animals).

The proportion of the households that used LLITNs, IRS and other commercial insecticides was 91.8%, 84.4% and 51% in the irrigated area respectively and 91.2%, 91.2% and 39.2% in the non- irrigated area respectively (**Table 4.10**).

Table 4.10: Frequency of responses to chemical use in public health, agriculture (farms) and veterinary (animals) use in households in irrigated and non irrigated areas.

Category	Use	Irrigated (n=98)	Non - irrigated (n=102)	P- value
Public Health	LLITNs	91.8 (89.0– 94.6%)	91.2 (88.4– 94%)	0.88
	IRS	84.4% (77.1- 91.6%)	91.2% (85.7- 96.7%)	0.14
	Commercial insecticides	51.0 (41.1– 60.9%)	39.2 (29.7– 48.7%)	0.09
Agricultural/ Veterinary	Veterinary & Agricultural pesticides	80.6 (76.6– 84.6%)	84.3 (80.7– 87.9%)	0.49

There were a higher proportion of households in non-irrigated area (84.3%) that used pesticides in agriculture and veterinary pest control compared to those in the irrigated area (80.6%). There was however no significant difference in the use of public health

(vector control) and agricultural (crops)/veterinary (animals) chemicals between the two zones (T test; df = 6, t- stat = 0.1, p= 0.9). It was also observed that several chemical classes were used in both crop protection and on livestock (**Table 4.11**). These chemical classes included pyrethroids, organophosphates and cabarmates for crop protection and pyrethroids and cabamates for livestock protection.

Table 4.11: Chemical insecticides classes commonly used for crops and livestock pest protection

	Chemical Class	Pesticide Name
Crops	Pyrethroids	Thunder, Profile, Duduthrin
	Organophosphates	Diazonol, Oshothion
	Carbamate	Milraz
Animals	Pyrethroids	Vectocid, Cyperticks
	Carbamate	Sevin

There was no observable difference when the irrigated (75.5%) and the non-irrigated (83.3%) areas were compared in relation to the combined use of public health and agriculture/ veterinary chemicals (T test; df = 10, t- stat = 0.2, p= 0.9). However, a significantly higher use of pyrethroids in the irrigated than the non-irrigated area (Z test; Z- stat = 2.7, p= 0.007) was detected. Households that confirmed the use of chemicals but did not know the chemicals used (unknown classes) were also significantly higher in the non- irrigated area (Z test; Z- stat = -3.2, p= 0.001) (**Table 4.12**).

Table 4.12: Proportion of household that use chemicals and the chemical classes used.

Chemical Class	Irrigated (98)	Non-irrigated (102)	P-value
Use Chemicals	75.5 (67.0, 84.0)	83.3 (76.1, 90.5)	0.17
Pyrethroids**	62.2 (52.6, 71.8)	28.2 (19.5, 36.9)	<0.001
Organophosphates	6.8 (1.8, 11.8)	2.4 (0, 5.4)	0.14
Carbamates	2.7 (0, 5.9)	0	0.09
Other classes	21.6 (13.5, 29.7)	4.7 (0.6, 8.8)	< 0.001
Unknown**	16.2 (8.9, 23.5)	67.1 (58, 76.2)	< 0.001
Don't use chemicals	24.5 (16.0, 33.0)	16.7 (9.5, 23.9)	0.17

** Significant difference between irrigated and non- irrigated area.

The frequency and duration of chemical use on livestock was reported to be higher in the non-irrigated area (**Table 4.13**).

Table4.13: Proportion of farmers using pesticides and the duration since the first use of pesticides in agriculture (crops) and livestock (veterinary) in the irrigated and non- irrigated areas.

Duration	**Irrigated area (n= 59)**	**Non-irrigated area (n= 29)**	**Irrigated area (n= 56)**	**Non-irrigated area (n= 15)**
	Agricultural pesticides:		*Livestock pesticides:*	
<6months	0	24.10%	3.60%	0
6-12months	1.70%	10.30%	3.60%	0
1-3yrs	22%	17.20%	14.30%	0
3-5yrs	6.80%	0%	7.10%	0
5-10yrs	22%	10.30%	23.20%	80%
>10yrs	47.40%	37.90%	48.20%	20%

The active ingredients present in the most commonly used chemicals for crop and animal pest control were analyzed. In summary, it was observed that most farmers use pyrethroids in crop and livestock pest control (**Table 4.14**).

Table 4.14: Summary of questionnaire survey on type and proportion of household use of agricultural and veterinary pesticides by local farmers in the irrigated and non- irrigated area.

Class	Pesticide brand name (n)	Irrigated (95% CI)	Non- irrigated (95% CI)	Active ingredient
Agricultural pesticides				
Pyrethroid	Thunder (99)	33.33% (0.242, 0.435)	17.17% (0.103, 0.261)	Betacyfluthrin
	Profile (99)	20.20% (0.128, 0.295)	9.09% (0.042, 0.166)	Bifenthrin
	Grizzly (99)	2.02% (0.002, 0.071)	0%	Lambdacyhalothrin
Organophosphate	Oshothion (99)	2.02% (0.002, 0.071)	0%	Malathion
	Diazonol (99)	2.02% (0.002, 0.071)	2.02% (0.002, 0.071)	Diazinon
Veterinary pesticides:				
Pyrethroid	Vectocid (73)	5.48% (0.015, 0.134)	0%	Deltamethrin
	Cypertick (10)	30% (0.067, 0.652)	20% (0.025, 0.556)	Cypermethrin
Carbamate	Dusting powder (73)	2.74% (0.003, 0.095)	0%	Carbaryl

4.6.2 Larval susceptibility tests

Diazol and Thunder was observed to produce 100% larval mortality with all dilutions both in larvae from the irrigated and non- irrigated areas. A 98.3% mortality rate was observed when Thunder was in low concentrations (1:10,000,000) in the non-irrigated area and 96% in the irrigated area. Milraz was however observed to 23.2% and 11.2% larval mortality at a concentration of 1:10,000 in irrigated and non-irrigated areas respectively. At a concentration of 1:50,000 there were observed 0% mortality in the basins with Milraz, both in the irrigated and non- irrigated areas.

4.7 Insecticide Resistance

4.7.1 *An. arabiensis* insecticide phenotypic resistance bioassays

A total of 1,657 female mosquitoes were tested for susceptibility against deltamethrin, malathion and DDT in 2018 and 2019 (**Table 4.15**). Nine hundred and fifty-nine (959) females were identified to species from irrigated and 698 from non-irrigated areas and they were all *An. arabiensis*.

Mortality in the positive control Kisumu strain was 100% in all tests while the mortality in the negative control (wild caught mosquitoes which were not exposed to insecticide) ranged between 4.7% - 15.4% in all the tests. Phenotypic resistance to deltamethrin was observed in the non-irrigated areas in 2018 with possible phenotypic resistance in the irrigated areas (**Table 4.15**). Significantly higher mortality was recorded in non-irrigated than the irrigated area in 2018 from deltamethrin exposure (Z-test; z-stat = 5.4, $p<0.00001$). However, in 2019, phenotypic resistance to deltamethrin was observed in both the irrigated and the non-irrigated areas, and the mortalities were comparable in the two areas (78% in irrigated and 83% in non-irrigated area). All the mosquitoes tested on PBO-deltamethrin combination were susceptible (100%) in irrigated and non-irrigated areas (**Table 4.15**).

Table 4.15: The status of phenotypic resistance of *Anopheles arabiensis* in the irrigated and non-irrigated areas in 2018 and 2019.

Insecticide	Year	Zone	N	Mortality (95% CI)	Status
Deltamethrin	2018	Irrigated	324	97.87% (0.960, 0.993)	Susceptible
		Non-irrigated	114	83.86% (0.782, 0.918)	Resistance
	2019	Irrigated	180	78.23% (0.558, 0.704)	Resistance
		Non-irrigated	117	83.25% (0.768, 0.906)	Resistance
Malathion	2018	Irrigated	104	100% (0.965, 1.000)	Susceptible
		Non-irrigated	158	100% (0.977, 1.000)	Susceptible
	2019	Irrigated	158	100% (0.977, 1.000)	Susceptible
		Non-irrigated	111	100% (0.967, 1.000)	Susceptible
DDT	2019	Irrigated	107	98.98% (0.949, 1.000)	Susceptible
		Non-irrigated	123	100% (0.970, 1.000)	Susceptible
PBO+Deltamethrin	2019	Irrigated	86	100% (0.958, 1.000)	Susceptible
		Non-irrigated	75	100% (0.953, 1.000)	Susceptible

CI- Confidence interval

Susceptibility to malathion (100%) and DDT (98.98%-100%) was recorded in both zones in the study site (**Figure 4.25**).

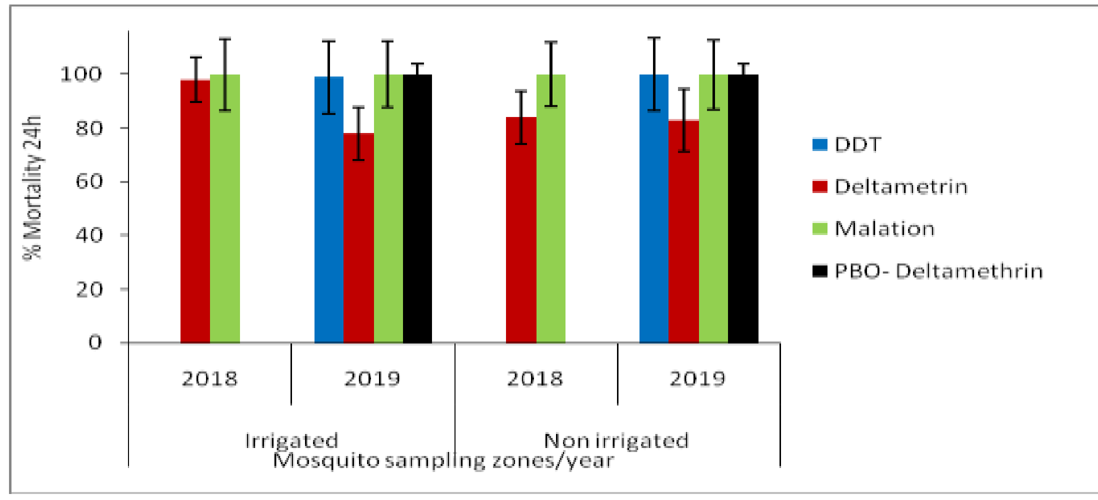


Figure 4.25: Percentage mortality of phenotypic resistance of *Anopheles arabiensis* in the irrigated and non- irrigated areas in 2018 and 2019.

4.7.2 Mechanisms of insecticide resistance

4.7.2.1 Target site mutations

4.7.2.1.1 Frequency of knock- down resistance (*kdr*) alleles.

A total of 317 mosquitoes (both alive (n=38) and dead (n=279) after the bioassays) were tested for the presence of mutation in the voltage- gated sodium channel (*vgsc)* gene. Generally, both *kdr*-east and *kdr*-west mutation were observed in both the irrigated and the non-irrigated areas (**Table 4.16**). The mutation frequency was low in all tests, ranging from 1% to 16%. The *kdr* allele and genotype frequencies were significantly different between irrigated and non-irrigated zones (Pearson Chi-square = 17.804, df=2, P=0.0001 and Pearson Chi-square = 14.848, df=4, P=0.012 respectively). The *kdr* genotypes results show significant deviation from Hardy-Weinberg equilibrium in non-irrigated zones, due to heterozygote deficit (P<0.01), while marginally significant of heterozygote deficit was observed in irrigated zone (P=0.0528). Overall, *kdr* genotype frequencies were not consistent with HWE (Chi-square > 36.7, df = 4, P <0.0001), indicating that *kdr* allele has been under strong selective pressure.

4.7.2.1.2 Frequency of angiotensin-converting enzyme-1 (*ACE-1*) alleles.

Regardless of study area, no mutation in the *ACE-1* gene was detected in 2018 and very low mutation frequency (0.7%) in the non-irrigated area in 2019 after testing 263 *An. arabiensis* samples. Generally, TaqMan enzyme assays revealed very low mutation levels in the non- irrigated area (**Table 4.16**).

Table 4.16: Allele frequency of *vgsc* and *ACE*-1 mutations in *Anopheles arabiensis* in irrigated and non- irrigated areas in Western Kenya in 2018 and 2019.

Year	Zone	*vgsc*				*ACE-1*	
		N	L1014	L1014S	L1014F	N	G119S
2018	Irrigated	81	0.92	0.01	0.07	73	0
	Non-irrigated	72	0.8	0.16	0.04	60	0
2019	Irrigated	76	0.94	0.03	0.03	55	0
	Non-irrigated	88	0.9	0.05	0.05	75	0.007

N: sample size

4.7.2.2 Changes in enzyme metabolism

4.7.3.2.1 Microplate Enzyme assays results

181 samples were tested for changes in enzymes levels. There were 91 samples from the irrigated area and 91 from non- irrigated area. An additional 91 *An. gambiae* Kisumu strain was used as the control group. Protein assays was done to correct for the sizes of the mosquito. The means of the different enzymes for each site was done. A one-way ANOVA revealed a statistically significant difference in mean enzyme activity between groups in all the enzymes tested (oxidases, β-Esterase and Glutathione S-transferases). There were higher oxidase ($F_{(2, 270)} = 10.40$, $p < 0.005$, GST ($F_{(2, 270)} = 10.44$, $p < 0.005$ and β-Esterase ($F_{(2, 270)} = 15.90$, $p < 0.005$) levels in mosquitoes from the irrigated and non- irrigated sites (**Figure 4.26**). Tukey's HSD Test for multiple comparisons found that the enzyme means were significantly different between laboratory strain and the wild- caught mosquitoes in all the enzymes tested. There was a statistically significant difference in the levels of oxidase, GST and esterase enzymes in mosquitoes from the control group and the irrigated (P<0.005, p<0.005, p<0.005) and non- irrigated (p=0.01, p=0.01, p=0.02) area respectively. However, the enzyme levels of oxidase, GST and esterase were not statistically different between the irrigated and non- irrigated area (p=0.76, p=0.76, p=0.75) respectively.

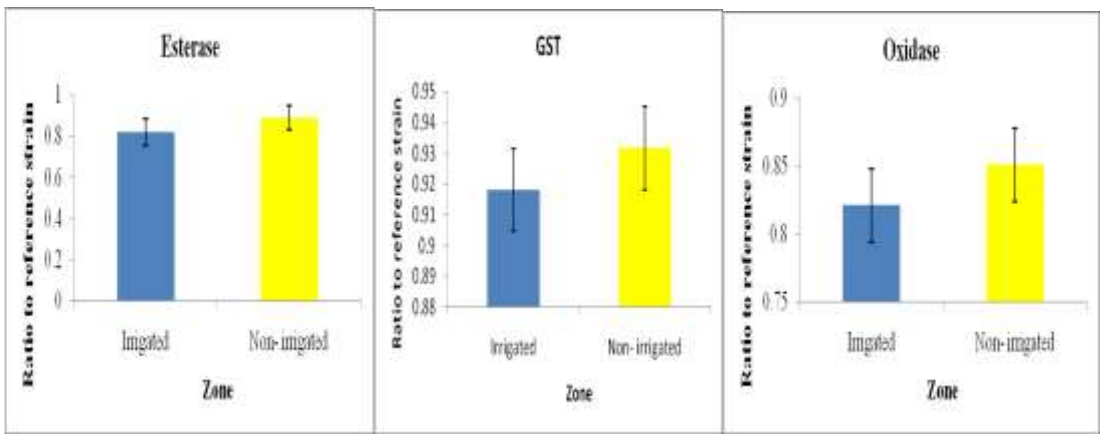
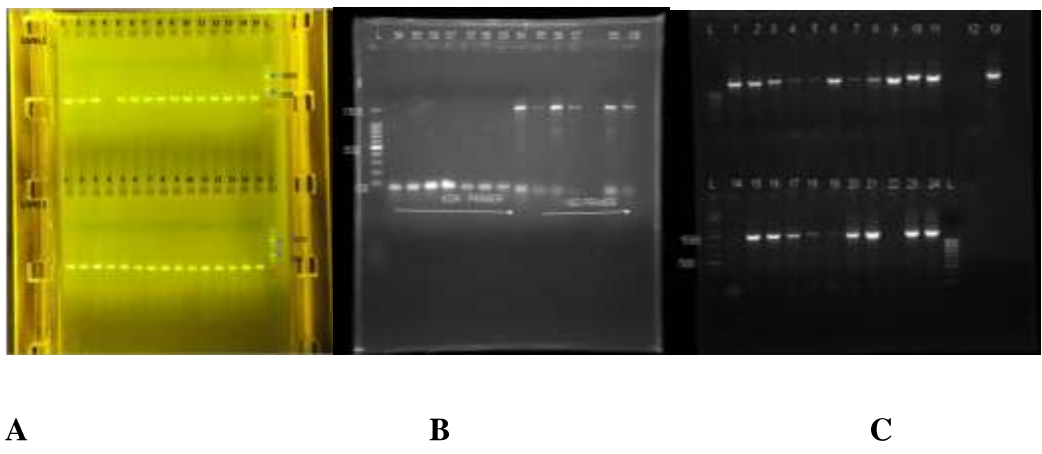


Figure 4.26: Metabolic enzyme activity for *An. gambiae* from irrigated and non-irrigated areas in relation to the reference strain (susceptible Kisumu strain).

4.8 Molecular species identification

In the seasonal larval surveillance, molecular species identification was made on a subset (543 samples) of the *An. gambiae* s.l. sampled during this study. *Anopheles arabiensis* comprised 98.3% of the specimens analyzed, with the remainder being *An. gambiae*. For the adult mosquito productivity surveillance through the emergency traps, all female *An. gambiae* s.l. samples (12) were analysed for species identification. *An. arabiensis* was the only species identified within *An. gambiae* s.l. in the adult mosquito productivity experiment. This was observed as the dominant species in the study area (**Figure 4.27A**).

Primer optimization for 16S and *kdr* was performed to achieve more sensitivity during the PCR process (**Figure 4.27B**). Further, PCR was conducted on isolated bacterial species before sequencing for bacterial species identification to ensure single strains without contamination were sent for sequencing **(Figure 4.27C).**



A B C

Figure 4.27: Gel photos of mosquito species identification (A) kdr and 16S primer optimization (B), and bacterial isolation (C)

CHAPTER FIVE

DISCUSSION

5.1 Discussion

This study was conducted to understand the effects of environmental modifications in the form of concrete based irrigation system on mosquito larval ecology and insecticide resistance in malaria vectors. The current research revealed no significant variations in *An. gambiae* s. l. populations in either ecosystem. Rainfall had a significant influence on the number of larval habitats in non-irrigated areas since more habitats were sampled during the wet season compared to the dry season. Previous research had discovered a significant increase in larval habitats, including temporary pools that were highly productive for *Anopheles* breeding (Nsereko *et al.,* 2020). Contrary to the non-irrigated ecosystem, where larval abundance was associated with seasonal rain, larval abundance in the irrigated ecosystem was associated with irrigation, with rainfall having minimal effect.

Malaria prevalence, however, declined from 2018 as a result of indoor residual spraying (IRS) conducted in the region thus reducing the densities of mosquitoes (Abong'o *et al.,* 2020; Omondi *et al.,* 2022a). This study discovered that environmental changes caused by irrigation canals increased habitat availability, productivity, and stability in the irrigated ecosystem versus the non-irrigated ecosystem. Seasonality was also found to have an effect on habitat availability, productivity, stability, and larval densities in the non-irrigated ecosystem, but not in the irrigated ecosystem. *An. arabiensis* was the most predominant vector in this study area with occurance of *An. funsetus*, *An. coustani* and *An. pharoensis* in lesser proportions. In addition, fish, dragonflies, and water boatmen were observed to be effective *An. arabiensis* larval predators with high larval mortality and low pupal productivity observed in larval habitats. Irrigation was observed to increase the abundance and diversity of bacterial species in larval sources. *Shigella* and *Escherichia* species were the most common genera in larval sources with larvae present in irrigated areas. Furthermore, in both irrigated and non-irrigated areas, high fatty acid concentrations may have an effect on larval production, whereas

chlorophyll, sugar, and manganese promoted growth. The findings from this study suggest that *An. arabiensis* populations in Homa Bay County are still vulnerable to organophosphates, a chemical compound used in IRS. Although this is encouraging, resistance to pyrethroid, a class of chemical compound used in LLITNs, was observed. The susceptibility of these malaria vectors to PBO synergists in pyrethroids suggests that ITN-based vector control interventions is not at risk and can be used in the future. These findings indicate the importance of continuing to monitor larval sources and insecticide resistance status, as these are major challenges to malaria vector control programs. Larval source management, larval habitat dynamics, insecticide use and resistance management will also require collaboration between the agriculture, public health and environmental sectors.

Temporary habitats contributed significantly to larval vector abundance in both ecosystems during the rainy seasons, which have also been reported elsewhere as having high larval survivorship for *An. gambiae* s.l (Gilles & Warrell, 1996; Mwangangi *et al.,* 2010; Nsereko *et al.,* 2020). Due to fewer predators, these habitats have been observed to be preferred *Anopheles* oviposition sites (Munga *et al.,* 2007; Munga *et al.,* 2013; Service, 1977; Silberbush & Blaustein, 2008; Sunahara *et al.,* 2002).*Anopheles* densities were influenced by habitat type in irrigated and non-irrigated ecosystems and seasonality. Seasonality had a greater impact on habitat type availability in non- irrigated areas than in the irrigated area which had more stable aquatic larval habitats. This could be due to the irrigated area's constant availability of water, which was unaffected by seasonal weather changes. The monthly larval sampling revealed that the non- irrigated ecosystem had higher *Anopheles* larval densities than the irrigated ecosystem. The study attributes the observed difference to the non-irrigated ecosystem's limited breeding sites, with concentrated breeding in temporary water pools (animal and human footprints) that serve as communal water collection points.

Overall adult vector productivity, which included factors such as habitat diversity, stability, and availability, demonstrates that permanent and semi- permanent habitats were more productive than temporary habitats in both ecosystems. Adult female vectors' daily productivity from habitats was higher in irrigated ecosystems. Semi-

permanent habitats were observed to predominate the current study site. Furthermore, because *An. arabiensis* was found in a variety of habitats, all aquatic habitats should be considered potential vector breeding sites (Sattler *et al.,* 2005).

Several factors determine malaria vector control strategies, including vector species availability, vector abundance, vector feeding, resting behavior, and the endemic nature of the disease in the targeted area. The abundance of vectors and *Anopheles* breeding sites are critical to reducing malaria transmission and should be targeted to direct interventions (Coetzee *et al.,* 2000; Craig *et al.,* 1999; Eisele *et al.,* 2003). With regard to malaria cases, the relationship between larval densities and adult vectors in irrigated and non-irrigated ecosystems remains contentious (Ijumba & Lindsay, 2001), with some studies correlating larval densities with an increase in malaria cases in irrigated areas (Kibret *et al.,* 2010; Kibret *et al.,* 2017b, 2017c), while others showing no correlation (Ijumba, 1997). Previous research has shown that the rate of adult vector emergence from aquatic habitats is very low (approximately 1-4% of immature stages) (Mukiama & Mwangi, 1989; Munga *et al.,* 2007; Munga *et al.,* 2006), confirming the lack of a relationship between larval densities and malaria cases. Furthermore, the current study found that the number of female *Anopheles* vectors emerging from breeding sites is low as measured by emergence traps.

Several species, including Hemiptera, Coleoptera, fish, amphibians, Odonata, and other Diptera, have been observed to effectively predate on mosquitoes (Acquah-Lamptey & Brandl, 2018; Quiroz-Martínez & Rodríguez-Castro, 2007). For vector control, natural predators may be more effective in larger habitats (Sunahara *et al.,* 2002). Mosquito predators are more prevalent in more expansive, older, and more stable habitats because there is more prey and less chance of the habitat drying up (Sunahara *et al.,* 2002). Habitat preference for oviposition by gravid mosquito species is usually a compromise between competition from other larval species in small habitats and predation in large habitats (Sunahara *et al.,* 2002). According to previous studies, *An. arabiensis* seeks out favorable habitats to improve their chances of survival. (Minakawa *et al.,* 2004) while other mosquito species avoid habitats with predators and competitors (Blaustein *et al.,* 2004; Kiflawi *et al.,* 2003). Some

mosquitoes, however, prefer habitats with conspecific larvae, which may indicate the suitability of the habitat (Sumba *et al.,* 2004b).

According to the findings of this study, fish from the Gambusia genus are effective mosquito larval predators. These outperformed the dragonflies and water boatman. This is consistent with previous research, which found that fish and dragon flies reduced larvae significantly (Quiroz-Martínez & Rodríguez-Castro, 2007; Shaukat *et al.,* 2019). Notonectids, also known as backswimmers, and other bugs in the order Hemiptera have been observed to have predatory habits, sparking interest in their use as biological control agents against mosquito larvae. These are thought to be the most promising (Bassi, 1987; Notestine, 1971). Recent research has shown that these predators become more efficient as prey density increases (Allo & Mekhlif, 2019) and are more effective against late stages of larvae (Buxton *et al.,* 2020). Low prey densities may thus explain the low predation rates observed in this study.

There was a strong, positive correlation between predator numbers and *Culex* larvae reduction. Previous research has shown that mortality at various aquatic stages is caused by a variety of factors such as predation, cannibalism, and environmental factors (Okogun, 2005; Ranasinghe & Amarasinghe, 2020; Shaalan & Canyon, 2009). In the natural setting, our findings show that increasing predator densities resulted in a decrease in *Culex* density. This is in addition to other studies (Zuharah & Lester, 2010). In contrast, there was no effect on *Anopheles* species predator densities. This could be because the habitats chosen were permanent, and previous research has shown that *Anopheles* mosquitoes avoid permanent habitats due to the presence, abundance, and diversity of predators and competitor larvae, suppressing mosquito population densities (Mereta *et al.,* 2013). As a result, the age of the habitat influences habitat productivity (Munga *et al.,* 2013).

Therefore, we observed that fish, dragonflies, and water boatmen were effective *An. arabiensis* larval predators. In larval habitats, high larval mortality and low pupal productivity were also observed. A combination of effective predation and high larval mortality will reduce adult malaria vector abundance and, as a result, malaria transmission in the area (Githeko *et al.,* 2006; Otambo *et al.,* 2022).

In addition, this study revealed the presence of *Bacillus amyloliquefaciens*, a subspecies of the *B. subtilis*, known to be biocontrol against plant pathogens (Asari *et al.,* 2016; El-Hefny *et al.,* 2022; Tran *et al.,* 2020), and the only bacteria identified via MALDI-TOF in the non- irrigated area confirms that *B. amyloliquefaciens* significantly affects the bacterial community diversity and composition in the non-irrigated area. This is similar to studies done by (Yang *et al.,* 2021). The presence of *B. amyloliquefaciens* in non-irrigated long-standing aquatic habitats confirms that the species does not colonize a native bacterial community in a freshwater aquatic environment (Yang *et al.,* 2021; Zhou *et al.,* 2017).

Based on MALDI-TOF MS and sequencing results, larval sources (both larval positive and larval negative aquatic habitats) in irrigated areas had a higher diversity and abundance of bacterial species than sources in non-irrigated areas. Similarly to previous research that linked increased soil bacterial abundance and diversity to irrigation(Frene *et al.,* 2022; H. Li *et al.,* 2021), this study's findings confirm the effect of irrigation on increased bacterial abundance and species diversity, which may influence larval productivity on larval sources or pools. *Shigella* and *Escherichia* were found to be the dominant genera in larval sources with larvae presence in irrigated areas . Except for *Brevibacillus*, some species of the former two and other isolated genera (*Bacillus, Klebsiella, Citrobacter, and Enterobacter*) have been isolated from the gut of *Anopheles* (Chen *et al.,* 2020; Galeano-Castañeda *et al.,* 2019; Gendrin & Christophides, 2013). With the exception of *B. sphaericus*, which reduces vectoral capacity (Yu *et al.,* 2020), species from the five genera have a significant influence on nutrient assimilation, vectoral capacity, and membrane formation in mosquitoes, explaining why their presence in Homa Bay aquatic mosquito larval sources is important (Coon *et al.,* 2014; Minard *et al.,* 2013; Rodgers *et al.,* 2017). Members of the *Bacillus* genera have also been linked to the synthesis of kairomones that attract and stimulate oviposition in vectors such as *Aedes aegypti* and *Aedes albopictus* (Ponnusamy *et al.,* 2008).

Brevibacillus has previously been linked to entomopathogenic activities, with spores produced by *Brevibacillus laterosporus* and *Brevibacillus brevis* being highly toxic to both larvae and adult mosquitoes (Barbieri *et al.,* 2021; Fouda *et al.,* 2022). The

presence of *Brevibacillus brevis* may have an effect on larval densities in larval sources with larvae in irrigated areas in the study site, interfering with their overall larval productivity. This will determine whether the bacterium plays a role in larval reduction or forms bio-larvicidal spores that can be used in larval source management (LSM).

Exiguobacterium profundum was one of the key identified bacteria that were only found in larval present sources in irrigated areas of Homa Bay. The bacterium has been shown to reduce mosquito fecundity, egg hatchability, and larval development, and it may have a negative impact on larval productivity in irrigated areas. (Rajagopal & Ilango, 2021). Although present in potential larval sites devoid of *Anopheles* larvae, *Bacillus subtilis* is well-known for producing the bio-surfactant surfactin, which is a bio- adulticide to *An. stephensi* (Geetha *et al.,* 2012), as well as other secondary metabolites that are bio-larvicides to *Aedes aegypti* (Revathi *et al.,* 2013). The absence of larvae in aquatic habitats with high levels of stearic acid could be due to the nature of this fatty acid. This fatty acid could be originating from live and dead organic matter in the surrounding area. Stearic acid is waxy and slightly soluble in water, forming a thin layer on water surfaces (Acid, 1987). This layer restricts the entry of oxygen into the water. This could be a problem for adult mosquitoes ovipositing because they prefer habitats that will support immature survival. Stearic acid has been found to be toxic to mosquito larvae (Rahuman *et al.,* 2008). Oleic acid, a water-soluble monounsaturated fatty acid, has been shown to benefit other living organisms. However, high concentrations of oleic and linoleic acids have been observed to be lethal to mosquito larvae (Gurunathan *et al.,* 2016; Rahuman *et al.,* 2008). This could account for the higher concentrations of these fatty acids in larval negative habitats. Linoleic acid in adequate concentartions has been shown to help mosquito adult development (Dadd & Kleinjan, 1979).

The nutritional capacity of mosquito larvae is critical for the development of larvae and emerging adult mosquitoes. Inadequate nutrition in larvae may result in developmental failure or have a negative impact on the size and reproductive ability of the resulting adult mosquitoes. In general, high concentrations of simple sugar were associated with the presence of larvae in the habitats. This was observed for

pectin, cellulose, glucose, fructose, and sucrose. These are soluble and food products that mosquito larvae use. Additionally, a high concentration of total chlorophyll was found to support mosquito larvae infestation. This could be due to the presence of algae matter, which serves as food for the immature, allowing them to grow. This is consistent with previous research that found algae to be a source of food for mosquito larvae (Abdelkader & El-Tayeb, 2021; Erzinger *et al.,* 2013; Huzortey *et al.,* 2022; Walker *et al.,* 1988).

Manganese was also found in higher concentrations than other metals throughout larval sites in both irrigated and non-irrigated areas with larval presence. Manganese is known to promote normal mosquito larval development when combined with calcium, chlorine, iron, potassium, magnesium, sodium, sulfur, and phosphorus (Bond *et al.,* 2017; Rivera-Pérez *et al.,* 2017). The majority of the other metals were found in extremely low concentrations, including lead, calcium, copper, iron, magnesium, and zinc. Although copper is required for enzyme function in larval pigmentation, oxidative stress protection, and respiration, high levels of copper are known to be larvicidal (Reza & Ilmiawati, 2020; Reza *et al.,* 2016), and has also been linked to mosquito resistance to insecticides such as lambda-cyhalothrin (Talom *et al.,* 2020).

LLITN coverage in Homa Bay was observed at >91% in both the irrigated and non-irrigated area. This is concurrent with other previous reports in the area (Okoyo *et al.,* 2015). Data collected from households revealed that there is a more use of pesticides in agricultural farms in the irrigated area and more on livestock in the non-irrigated area. Pyrethroid is the most common class used in crop and animal pests control as has also been observed in other regions that practice agriculture (Marcombe *et al.,* 2012; Marcombe *et al.,* 2009; Matowo *et al.,* 2010; Nkya *et al.,* 2013; Ranson *et al.,* 2009). The absence of larval resistance suggests that use of chemicals in public health, rather than in agricultural is the major source of selection pressure as was also observed by (Hemingway, 1983).

This study was conducted in Homa Bay, an area with high insecticide treated net (ITN) coverage (>80%) (National Malaria Control Programme 2015). IRS was also conducted in February/March 2018 and 2019 using the organophosphate insecticide Actellic 300CS thus significantly decreasing the proportion of primary malaria vectors (Abong'o *et al.,* 2020). Results of the adult phenotypic resistance revealed that deltamethrin resistance was present in the irrigated and non-irrigated areas (Orondo *et al.,* 2021). Both *kdr*-east and *kdr*-west mutations were also observed. However, no *ACE-1* mutation was observed in the samples tested from both irrigated and non-irrigated areas (Orondo *et al.,* 2021). Additionally, both areas used chemical-based public health interventions for mosquito biting prevention. However, greater use of pyrethroid agricultural pesticides was observed in the irrigated than the non-irrigated area (Orondo *et al.,* 2021). The metabolic enzyme assays revealed higher oxidase and β-Esterase levels in mosquitoes from the irrigated and non-irrigated areas. Thi indicates that metabolic resistance is also evolving in the area.

With the scaling up of LLITN distribution and usage, an increase in insecticide resistance against the different classes of insecticides has been reported in several regions across sub- Saharan Africa in the malaria vector species (https://anopheles.irmapper.com/). This study showed that *An. arabiensis,* the only vector species tested for phenotypic resistance in the study had developed moderate phenotypic resistance against deltamethrin, belonging to the pyrethroid compounds and is used in most LLITNs. This resistance was observed in both the irrigated and non- irrigated areas. This is similar to reports observed across Africa (Carter *et al.,* 2022; Demissew *et al., 2022)* and specifically in Kenya (Orondo *et al.,* 2021; Owuor *et al.,* 2021). This wide- spread has been attributed to the use of similar chemical classes in public health and agriculture. The presence of phenotypic resistance observed was due to reduced nervous sensitivity in the para- type sodium channel, resulting in knockdown resistance (Soderlund, 2008). The total *kdr* allele frequencies were observed to be higher in the non-irrigated area as compared to the irrigated area. This study revealed a higher frequency of the *kdr* mutation than previous studies (Hemming-Schroeder *et al.,* 2018) with the frequency of *kdr*- west higher in the irrigated area than the non- irrigated area. This could be due to the presence of agricultural pesticides which have previously been linked to the development of *kdr*-

west (Orondo *et al.,* 2021; Sagbohan *et al.,* 2022; Yadouleton *et al.,* 2009) while LLITNs have been linked to the development of *kdr*- east (Mathias *et al.,* 2011; Orondo *et al.,* 2021; Protopopoff *et al.,* 2013)

Previous studies have reported lower *kdr* frequencies than those observed during this study (Hemming-Schroeder et al., 2018). *Kdr* has been observed in the primary malaria vector species including *An. gambiae* s.s (Bass *et al.,* 2007; Safiyanu *et al.,* 2019), *An. funestus* (Irving & Wondji, 2017) and *An. arabiensis* (Carter *et al.,* 2022; Sy *et al.,* 2021). The frequencies of *kdr* were observed to be higher in the irrigated area than the non- irrigated area with *kdr*- west having a higher frequency in this region. This could be as a result of the more frequent use of pesticides in the irrigated areas as compared to the non- irrigated area as has been reported in previous studies (Korti *et al.,* 2021; Sagbohan *et al.,* 2022; Yadouleton *et al.,* 2009).

In the non- irrigated area however, *ACE-1* mutation was observed though in very low frequencies and has been previously associated with carbamate and organophosphate resistance (Binyang *et al.,* 2022; Elanga-Ndille *et al.,* 2019; Essandoh *et al.,* 2013). This is worrying as this area was using Actellic® 300CS, an organophosphate, for IRS during the study period (Omondi *et al.,* 2022a; Orondo *et al.,* 2021). Residuals from the pesticides used for animal pest control could not be dissociated from the observed resistance, as the organophosphates were found to be commonly used for animal pest control. This could be the the source of the observed reistance , however, organophosphate resistance could intensify as a result of its use in public health. The presence of *ACE-1* mutation in low frequencies in the non-irrigated area is a matter that needs to be further investigated, as an increase in this mutation may impact the gains achieved so far with the ongoing IRS program in the region.

In addition to the genetic mutations observed (*kdr* and *ACE*), changes in enzyme levels (oxidases, esterarse and GSTs) were also observed. High levels of detoxifying enzmes were observed in both the irrigated and non- irrigated areas as compared to the susceptible Kisumu strain. This indicated that esterases, GSTs and oxidases could all be contributing to the insecticide resistance observed in the field populations in the area. The levels in the non- irrigated area were slightly higher than those in the

irrigated area. Increased levels of esterases have been observed to confer organophosphate resistance (Hemingway, 1982) and cross resistance to pyrethroids (Brogdon & Barber, 1990; Chareonviriyaphap *et al.,* 1999; Safi *et al.,* 2017; Scott & Georghiou, 1986). Elavation of both oxidase and estarase enymes have been observed in Western Kenya (Ochomo *et al.,* 2013; Vulule *et al.,* 1994) in *An. gambiae* s.s and not *An. arabiensis*. Oxidiases/ monooxygenases/ p450 confer pyrethroid resistance. Presence of increased enzyme levels in this study population of *An. arabiensis* is a cause of concern to vector control. GSTs enzyme levels were also elevated indicating a possible pyrethroid resistance. GSTs have been seen to increase in DDT and pyrethroid resistance (Che-Mendoza *et al.,* 2009; Lumjuan *et al.,* 2005; Lumjuan *et al.,* 2011).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study revealed that environmental modifications caused by irrigation canals increased habitat availability, productivity, and stability in the irrigated ecosystem versus the non-irrigated ecosystem. Seasonality was also found to have an effect on habitat availability, productivity, stability, and larval densities in the non-irrigated ecosystem, but not in the irrigated ecosystem. *An. arabiensis* was the most predominant vector in this study area with occurance of *An. funsetus*, *An. coustani* and *An. pharoensis* in lesser proportions. Fish, dragonflies, and water boatmen were also observed to be effective *An. arabiensis* larval predators. High larval mortality and low pupal productivity were also observed in larval habitats. The combination of effective predation and high larval mortality will reduce adult malaria vector abundance and, as a result, malaria transmission in the area. Additionally, irrigation increased the abundance and diversity of bacterial species in larval sources in the irrigated area. *Shigella* and *Escherichia* species were the most common genera in larval sources with larvae present in irrigated areas. Furthermore, the larvicidal or adulticidal properties of *B. mojavensis, B. tequilensis, B. stercoris*, and *Brevibacillus agri* should be investigated because they clustered with known entomopathogenic bacteria in this study. Furthermore, in both irrigated and non-irrigated areas, high fatty acid concentrations may have an effect on larval production, whereas chlorophyll, sugar, and manganese promoted growth.

More households in irrigated area use pesticides in their farms compared to the non-irrigated area while, more households in non-irrigated area use pesticides on livestock than that in the irrigated area**.** A high proportion of the households in both ecosystems use public health malaria interventions including LLITNs, IRS and other commercial insecticides. It was also observed that several chemical classes were used in both crop protection and on livestock with more use of pyrethroids in the irrigated than the non-irrigated area. When these pesticides were tested for

susceptibility in larval mosquitoes, very low concentrations of were observed to be effective against malaria vector larvae.

This study observed that adult *An. arabiensis* populations in Homa Bay County are still susceptible to organophosphates, a chemical compound class used in IRS. Although this is encouraging, resistance to pyrethroid, a class of chemical compound used in LLITNs, was observed. The susceptibility of these malaria vectors to PBO synergists in pyrethroids suggests that ITN-based vector control interventions have a bright future. Resistance to DDT is a useful indicator of pyrethroid cross-resistance. The absence of DDT resistance is encouraging because it suggests that there is no cross-resistance between pyrethroids and organochlorides. These findings indicate the importance of continuing to monitor insecticide resistance status, as insecticide resistance is a major challenge to malaria vector control programs. Insecticide use and resistance management will also require collaboration between the agriculture andpublic health sectors.

6.2 Recommendations

These findings indicate a) the importance larval source management to control vector populations in the irrigated area. Since semi- permanent habitats predominate at the current study site, the overall adult vector productivity was calculated using some assumptions thus more field surveys and data collection are required to validate this idea. Furthermore, because *Brevibacillus agri* is closely related to *Brevibacillus brevis*, it should be tested for entomopathogenic properties (Shida et al., 1994). The discovery of clustering or grouping with other isolated bacteria such *as B. mojavensis*, *B. tequilensis*, and *B. stercoris* (found in larvae infested larval sources) suggests that these bacteria's larvicidal or adulticidal capability should be investigated. b) Collaboration between different sectors including agriculture andpublic health sectors is essential to maintain the gains achieved in vector control. c) Finally, insecticide resistance monitoring will help understand the evolution, spread and trajectory of insetcicde resistance in malaria vetors in Homa Bay County. With the observed susceptibility of the malaria vectors in Homa Bay to PBO-deltamethrin, the introduction of PBO-impregnated nets in the study area will

probably be effective in malaria vector control.These findings indicate the importance of continuing to monitor insecticide resistance status, as insecticide resistance is a major challenge to malaria vector control programs.

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APPENDICES

Appendix I: Ethical approval letter

MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 11th September, 2019

TO: Dr. Harrysone Atieli
Department of Public Health
School of Public Health and Community Development
Maseno University
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00456/17

RE: Environmental Modification in Sub-Saharan Africa: Changing Epidemiology, Transmission and Pathogenesis of *Plasmodium falciparum* and *Plasmodium vivax* Malaria. Proposal Reference Number: MSU/DRPI/MUERC/ 00456/17

This is to inform you that the Maseno University Ethics Review Committee (MUERC) considered your valued application for extension of ethics approval of your study. The Committee commended the progress made and granted an **approval for continuation** of the study effective this 11th day of September, 2019 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 10th September, 2020. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 15th August, 2020.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 15th August, 2020.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to the MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

MASENO UNIVERSITY SECRETARY

Dr. Bernard Guyah
Ag. Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Appendix II: Ethical clearance from Ministry of Health

MINISTRY OF HEALTH

Telegrams: "MOH" Homa Bay
Telephone: 21039
When replying please quote

Homabaychc@gmail.com

Ref: MOH/CTY/GEN/VOL.III/302

MINISTRY OF HEALTH,
HOMA-BAY COUNTY
P.O. BOX 52
HOMABAY

9th January, 2018

To:

Dr. Harrysone Atieli, PhD
Project Manager, ICEMR,
Dear Sir/Madam,

RE: AUTHORITY TO CONDUCT MALARIA RESEARCH IN HOMABAY COUNTY

Following your request to conduct malaria research in Homa bay county for a study entitled '*Environmental Modifications in sub-Saharan Africa: Changing Epidemiology, Transmission and Pathogenesis of Plasmodium falciparum and P. vivax Malaria* ,' you are hereby authorized to proceed with the exercise for the duration and under the conditions permitted by the University of California, Irvine Institutional Review Board (UCI IRB) dated March 15, 2017 and the Maseno University Ethical Review Committee dated 11th September, 2017 ref, MSU/DRPI/MUERC/00456/17

You will be required to adhere to the hospitals norms and regulations during the data collection period. You are also expected to communicate your findings to the Directors' office at the end of the research.

Wish you all the best as you plan the study and am looking forward for future collaborations.

MINISTRY OF HEALTH
DIRECTOR OF MEDICAL SERVICES
09 JAN 2018
Sign.............
P.O. Box 52-40300 HOMA-BAY

Dr Gordon Okomo
County Director of Health
Homabay County

Cc: SC MOH – Homa Bay Township, Rangwe, Rachuonyo North,

Appendix III: Questionnaire for households

<u>Questionnaire for Households</u>

Introduction

This ICEMR project is aimed at identifying the common insecticide used in mosquito control in the households and how these insecticides subsequently affect mosquito and their contribution to malaria transmission. In addition, we aim to identify the households where the government indoor residual spraying (IRS) was conducted in 2018, 2019 and 2020. This information will assist to identify the effect of IRS to mosquitoes. I would like to request for your voluntary participation and to take part in this survey by responding to a few questions stated in this questionnaire.

Interviewer: _______________ **Date of Interview: _____________**

Name of respondent: _____________ **Gender: ___________________**

Cluster Number: _______________

1. Function of the respondent: 1= Household head
   2=Others (specify) _____________
2. How do you protect yourself from mosquito bites and how often do you use the method

Prevention method	**Frequency of use**
Mosquito nets	
Insecticide spraying	
Physically killing	
Others (specify) __________	
None	

3. Was IRS spraying ever done in your house? 1 = Yes

   2 = No

4. If "NO", Why?

   ____

   ____

   ____

5. When was IRS done?

Year	Door Number	Card Number	Reason IRS wasn't done
2018			1) 2) 3) 4)
2019			1) 2) 3) 4)
2020			1) 2) 3) 4)

Any other information

____

____

____

____

Appendix IV: Questionnaire for agro- vet shops

<u>**Questionnaire for Agro-vet shops**</u>

Introduction

This ICEMR project is aimed at identifying the common agricultural pesticides used in the control of pests and diseases in the farms and how these pesticides subsequently affect mosquito immature stages and their contribution to malaria transmission. I would like to request for your voluntary participation and to take part in this survey by responding to a few questions stated in this questionnaire.

Interviewer: ______________ **Date of Interview: _________**

Name of respondent: ____________ **Gender: __________________**

Shop name: __________________ **Shop location: __________________**

6. Function of the respondent in the shop:
   1= Owner
   2 = Manager
   3 = Sales personnel
   4 = Others (specify)____________
7. Which agricultural pesticides are sold in this shop
   ____
   ____
   ____
   ____
   ____
8. Which pesticide is frequently bought to be used in the farms for the following infestations

Infestation	Pesticide
Fungal	
Insects (e.g. Beatles	
Virus	
Worms	
Bacteria	

9. Which diseases commonly affect the following crops

Crop	Disease (Fungal/ Beetles/ Virus/ Worms/ Bacterial)
Maize	
Millet	
Vegetables	
Fruits	
Green grams	
Rice	
Beans	
Others	

10. Do you advise on the following areas when the pesticides are bought:
    - Storage Yes ☐ No ☐
    - Constitution Yes ☐ No ☐
    - Usage Yes ☐ No ☐
    - Disposal Yes ☐ No ☐
    - Expiration date Yes ☐ No ☐

11. Please answer the following

Pesticide used	Which crop is treated	Supplier	Approximate dosage	Frequency of application	Chemical have been used since

Appendix V: Questionnaire for crop farmers

<u>Questionnaire for Farmers - Crops</u>

Introduction

This ICEMR project is aimed at identifying the common agricultural pesticides used in the control of pests and diseases in the farms and how these pesticides subsequently affect mosquito immature stages and their contribution to malaria transmission. I would like to request for your voluntary participation and to take part in this survey by responding to a few questions stated in this questionnaire.

Interviewer: ______________ **Date of Interview: ______________**

Name of respondent: ____________ **Gender: __________________**

Village: __________________

1. Function of the respondent in the farm: 1= Owner
   2 = Manager
   3 = Farm worker
   4 = Others

2. What do you farm? (Tick appropriately)

Crop	**Response**	**Crop**	**Response**
Maize		Green grams	
Millet		Rice	
Vegetables		Beans	
Fruits (Watermelon, Bananas, Mangoes)		Others (specify)	

3. Are your crops affected by pests and diseases? Yes ☐ No ☐

4. Which pests / diseases commonly affect your crops

Crop	**Disease (Fungal/ Beetles/ Virus/ Worms/ Bacterial)**
Maize	
Millet	
Vegetables	
Fruits (Watermelon, Bananas, Mangoes)	
Green grams	
Rice	
Beans	
Others	

5 a) Do you use pesticides in your farm? ☐ ☐

b) If so please answer the following

Pesticide name	Where do you buy it	Form of the pesticide (powder, granules, liquid)	How is the pesticide constituted	Mode of application (Spraying, direct)	Approximate dosage for application	Frequency of application	Crop treated	Duration of pesticide use (<6months; 6-12months; 1-3yrs; 3-5yrs; 5-10yrs; >10yrs)

6. How do you dispose the excess chemical and the empty containers? _______________
7. Where do you wash or clean the equipments used? _____________________________
8. Do you think the chemical gets into the water (rivers/ lake)? _____________________
9. If you do not use pesticides, how do you control pests and weeds __________________

Appendix VI: Questionnaire for livestock farmers

Questionnaire for Farmers - Animals

Introduction

This ICEMR project is aimed at identifying the common insecticides used in the control of pests and diseases on animals and how these chemicals subsequently affect mosquito immature stages and their contribution to malaria transmission. I would like to request for your voluntary participation and to take part in this survey by responding to a few questions stated in this questionnaire.

Interviewer: ______________ **Date of Interview: ______________**

Name of respondent: ____________ **Gender: __________________**

Cluster: __________________

1. Function of the respondent in the farm: 1= Owner
   2 = Manager
   3 = Farm worker
   4 = Others (specify) ___________

2. What livestock do you keep? (Tick appropriately)

Animals	**Response**	**Animals**	**Response**
Cattle		Poultry (chicken, ducks, turkey)	
Goats		Cat	
Sheep		Dog	
Donkey		Others (specify)_______________	

3. Are your animals affected by pests? Yes ☐ No ☐

4. Which pests commonly affect your animals

Animals	**Pests (Ticks, Mites, Flees)**
Cattle	
Goats	
Sheep	
Donkey	
Poultry (Chicken, ducks, turkey)	
Cat	
Dog	
Others	

5. a) Do you use insecticides on your animals Yes ☐ No ☐

b) If so please answer the following

Insecticide name	Form of the insecticide (powder, granules, liquid)	How is the insecticide constituted	Where applied (On the animal, In the animal shade)	Approximate dosage for application	Frequency of application	Animal treated	Duration of insecticide usage (<6months; 6-12months; 1-3yrs; 3-5yrs; 5-10yrs; >10yrs)

10. Where do you apply the insecticide
    a) In the animal shade
    b) Within the compound but outside the animal shade
    c) Outside the compound
    d) In the cultivated/ farm lands
    e) Along the river bed or lake shore
    f) Others (specify)_____________________________
11. How do you dispose the excess chemical and the empty containers? _______________
12. Where do you wash or clean the equipments used? _____________________________
13. Do you think the chemical gets into the water (rivers/ lake)? _____________________

If you do not use insecticide, how do you control pests __________________

Appendix VII: Questionnaire for veterinary officers/ agricultural extension workers

Questionnaire for Vets/ Ext workers

Introduction

This ICEMR project is aimed at identifying the common insecticides used in the control of arthropods on farm animals and how these chemicals subsequently affect mosquito immature stages and their contribution to malaria transmission. I would like to request for your voluntary participation and to take part in this survey by responding to a few questions stated in this questionnaire.

Interviewer: _______________ Date of Interview: _________

Name of respondent: _____________ Gender: ___________________

Area of duty (Location): ___________

1. Are you:
   1= Self- employed
   2 = Government/ county appointed
   3 = Other (Specify) __________________

2. Which animals do you normally treat of arthropods and ecto-nematods

Crop	**Common arthropods/ ecto-nematods**
Cattle	
Goats	
Sheep	
Donkey	
Poultry (Chicken, Ducks, Turkey)	
Cats	
Dogs	
Others (Specify)	

3. Do you: 1= Treat the animals personally
   2= Advice on the treatment
   3= Both
4. Are the animals: 1= Brought to your clinic for treatment
   2= Treated in the farms
   3= Both
5. Where do you apply the insecticide
   a) In the animal shade
   b) Within the compound but outside the animal shade
   c) Outside the compound
   d) In the cultivated/ farm lands
   e) Along the river bed or lake shore
   f) Others (specify)______________________________

6. Please answer the following

Chemical name	Form of the chemical (powder, granules. liquid)	How is the chemical constituted (none, diluted)	Mode of application (spraying, dusting)	Where applied (on animal, in animal shade, injected)	Approximate dosage	Frequency of application	Animal treated	Chemical have been used since

7. How do you dispose the excess chemical and the empty containers? _______________
8. Where do you wash or clean the equipments used? ______________________________
9. Do you think the chemical gets into the water (rivers/ lake)? _____________________

Appendix VIII: Emergent traps field form

EMERGENT TRAP FIELD DATA ENTRY FORM

Habitat No: ___________________ **Habitat Type: __________________________**

Coordinates- Lat: _______________ **Long: __________________** **Alt: ________________** **Acc: __________**

Date	Time	Vegetation Cover	Shade	Temp	Aquaflour Measurements						Distance to the nearest house	Habitat Size (M)			Dips	*Anopleles gambiae* Larvae				
					Survey A			Survey B				L	W	D		L 1	L 2	L 3	L 4	P
					Read 1	Read 2	Read 3	Read 1	Read 2	Read 3										

Anopleles funestus Larvae					*An. coustani*	*Culex*	Others	Number of predators											*An. gambiae* Adults		*An. funestus* Adults		*An. coustani* Adults		*Culex* adults		Other mosquitoes	
L1	L2	L3	L4	P				Fish	Amphibians	Annelids	Arachnids	Coleoptera	Diptera	Ephmeroptera	Molluscs	Odonata	Hemiptera	Other	M	F	M	F	M	F	M	F	M	F

Appendix IX: Predation rates laboratory form

EXPERIMENT ON PREDATION RATES

Predators	Date	Replicate	#Mosquitoes	# Predators	#Day 1	#Day 2	#Day 3	#Day 4	# Day 5	#Day 6	# Day 7

Appendix X: Vertical life table field form

Vertical life table experiment

Habitat No: ___________________ Habitat Type: ________________________ Location: ________________________

Date	**Time**	**Vegetation Cover**	**Shade**	**Distance to the nearest house**	**Size**	**Dips**	**Temp**	***An. gambiae* larvae**					***An. funestus* larvae**					***An. Coustani* larvae**	***Culex***	**Other mosquitoes**
								L1	**L2**	**L3**	**L4**	**P**	**L1**	**L2**	**L3**	**L4**	**P**			

Predators	**Date**																		
Amphibians																			
Fish																			
Arachnids																			
Annelids																			
Coleoptera																			
Diptera																			
Emphmeroptera																			
Molluscs																			
Odonata																			
Others																			

Appendix XI: Larval and water sampling field form

LARVAL HABITATS FORM

Date	Cluster	Habitat No.	Latitude	Longitude	Altitude	Accuracy	Habitat Type	Land Use	Vegetation Cover (%)	Shade (%)	Habitat Substrate	Distance from the House (m)	Irrigation Type	Crop	Habitat Length (m)	Habitat Width (m)	Habitat Depth (m)

Water Flow (F/S/NA)	**Water Clarity**	**Water Sample Collected (Y/N)**	**No. of Dips**	***An. gambiae* 1/2**	***An. gambiae* 3/4**	***An. gambiae* Pupae**	***An. funestus* 1/2**	***An. funestus* 3/4**	***An. funestus* Pupae**	***An. coustani* 1/2**	***An. coustani* 3/4**	***An. coustani* Pupae**	***An. pharoensis* 1/2**	***An. pharoensis* 3/4**	***An. pharoensis* Pupae**

Other *Anoph*	***Aedes***	***Culex***	**Other Mosq**	**Predators (Y/N)**	**Tadpoles**	**Fish**	**Amphibians**	**Annelids**	**Arachnids**	**Coleoptera**	**Diptera**	**Ephmeroptera**	**Hemiptera**	**Molluscs**	**Odonata**	**Other Predators**

Appendix XII: Phenotypic resistance laboratory data form

Molecular Entomology Laboratory

Resistance Bioassay Data Form

Experiment

Start Time: **Stop Time:**

Collection Site: _____________ Insecticide: ________

Bioassay Date: ___________ Sample: __________________

Time in mins	-ve control	+ve control	T1	T2	T3	T4	T5	T6	TOTAL
	Number of Mosquitoes Knocked Down								
10									
20									
30									
40									
50									
60									
80	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Sample Size									
Mortality 24h									

Appendix XIII: Microplate enzyme assay laboratory data form

MICROPLATE ENZYME ASSAYS PLATE

Plate name:::: **Date:** **Extracted by:**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Appendix XIV: Species identification laboratory data form

MOLECULAR ENTOMOLOGY LABORATORY

PCR ASSAY LAB DATA SHEET

USER NAME________________________________ DATE____________________

PURPOSE: ____

PROGRAM Name: _____________________________

PCR Cycle: Denature- ____; Anneal-____; Extend -____ Auto extend-____ No. of Cycles: ____

	1X	**X______**
Molecular H_2O		
TAQ POLYMERASE		
PRIMERS		
DNA TEMPLATE		

Lane 1		Lane 2		Lane 3		Lane 4	
Specimen ID	**Score**	**Specimen ID**	**Score**	**Specimen ID**	**Score**	**Specimen ID**	**Score**
1.		1.		1.		1.	
2.		2.		2.		2.	
3.		3.		3.		3.	
4.		4.		4.		4.	
5.		5.		5.		5.	
6.		6.		6.		6.	
7.		7.		7.		7.	
8.		8.		8.		8.	
9.		9.		9.		9.	
10.		10.		10.		10.	
11.		11.		11.		11.	
12.		12.		12.		12.	
13.		13.		13.		13.	
14.		14.		14.		14.	
15.		15.		15.		15.	
16.		16.		16.		16.	

Appendix XV: Molecular assays (*kdr* and *ACE-1*) laboratory data form

ENTOMOLOGY KDR/TAQMAN ENZYME PLATE FORM

DATE:…………….. **PLATE NUMBER:…………** **TEST:…………..….**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Appendix XVI: Larval bioassay laboratory data form

ICEMR LARVAL MORTALITY FORM

BASIN NO.	DATE	ZONE	TIME	CHEMICAL	DILUTION RATIO	NO. OF MOSQUITOES	MORTALITY AT 24HRS	MORTALITY AT 48HRS

Appendix XVII: Identity matrix table

Identity matrix table showing the frequency of relationship or association between isolated bacteria and GenBank accession numbers.

GENBANK ACCESSION	MC9	NA7	NA2A	EMB9A	NA9	NA4A	NA10	MC2	NA3	NA5AA	NA10A
OX245674 (*Citrobacter* sp. Marseille-Q6884)	100.00	57.05	59.34	27.14	30.77	32.08	31.75	32.94	32.94	32.35	32.35
LC588555 (*Bacillus siralis* J35TS1)	57.05	99.91	70.23	27.04	28.60	31.04	32.83	33.48	33.43	32.78	32.60
OX216966 (*Paenibacillus dendritiformis*)	59.56	70.65	99.55	25.83	28.27	31.46	31.51	32.09	32.09	31.34	31.34
CP102379 (*Escherichia coli* strain BM28 lys)	27.14	27.04	25.63	99.70	57.31	56.80	70.11	56.90	56.90	55.21	54.96
JQ265468 (Uncultured bacterium clone T2C182)	27.14	27.04	25.63	100.00	57.31	56.55	70.11	56.90	56.90	55.21	54.96
OP501807 (*Enterococcus gallinarum* strain 10 A)	30.77	28.60	28.57	57.44	99.78	81.03	86.10	86.61	86.61	83.48	84.58
OP402856 (*Staphylococcus arlettae* strain Dg-E8)	32.08	31.04	31.46	56.55	80.92	100.00	87.72	91.11	91.28	89.26	89.02
MH746088 (*Bacillus siralis*	31.75	32.39	31.51	70.11	86.54	88.46	99.26	91.12	91.12	92.01	91.72

strain PA02)											
OK570087 (*Robertmurraya siralis* strain C3)	31.75	32.39	31.51	70.11	86.54	88.46	99.26	91.12	91.12	92.01	91.72
ON430535 (*Bacillus cereus* strain CUMB AR-04)	32.94	33.48	32.09	56.90	86.50	91.11	90.39	100.00	100.00	92.20	93.26
ON860698 (*Bacillus cereus* strain PSR21)	32.94	33.43	32.09	56.90	86.50	91.28	90.39	100.00	100.00	92.23	93.26
OP435764 (*Bacillus tequilensis* strain A37)	32.35	32.60	30.97	54.96	84.47	89.02	90.98	93.26	93.26	99.81	100.00
KC441741 (*Bacillus subtilis* strain B59)	32.40	32.87	31.02	55.02	83.43	88.87	91.05	92.47	92.47	99.77	99.86

GENBANK ACCESSION	MC9	NA7	NA2A	EMB9A	NA9	NA4A	NA10	MC2	NA3	NA5AA	NA10A
OP521932 (*Bacillus stercoris* strain ML-2)	32.54	32.93	31.16	55.21	83.59	89.26	91.27	92.37	92.40	99.84	99.81
OP482166 (*Bacillus mojavensis* strain M)	32.15	32.83	30.78	54.96	83.37	89.12	90.98	92.19	92.23	99.49	99.62
OP218479 (*Bacillus mojavensis* strain NR 112725)	32.35	32.92	30.97	55.21	84.35	88.81	91.27	93.28	93.28	99.44	99.42
OP514801 (*Escherichia coli* strain ASBY05)	31.95	26.08	28.06	32.23	38.95	39.11	38.06	39.79	39.79	39.27	39.53
OP793848 (*Exiguobacterium profundum* strain T5)	30.56	32.38	29.46	55.58	78.68	80.84	83.28	84.51	83.42	84.01	85.16
OP263689 (*Exiguobacterium profundum* strain H-1)	30.56	32.38	29.46	55.58	78.68	80.84	83.28	84.51	83.42	84.01	85.16
KF732994 (*Bacillus subtilis* strain PMM8)	33.06	26.59	31.47	31.67	38.20	38.86	39.66	38.81	38.76	39.76	40.00
OP554433 (*Bacillus*	33.06	26.96	31.47	31.67	38.20	38.94	39.66	38.90	38.84	39.64	39.80

velezensis strain r22)											
ON999044 (*Bacillus inaquosorum* strain GZCB-3)	33.06	29.12	31.47	31.45	39.23	38.82	40.06	39.41	39.41	39.78	39.96
OP115492 (*Bacillus aerius* strain GS26)	32.21	31.71	30.83	56.80	85.38	88.62	92.75	91.89	91.89	93.51	94.22

GENBANK ACCESSION	NA10B	NA4BB	NA12AA	MC5B	NA5B	EMB9	MC1A	NABAA	NABA	EMB6
OX245674 (*Citrobacter* sp. Marseille-Q6884)	32.35	32.54	32.25	31.42	31.28	31.95	30.75	30.56	30.56	32.21
LC588555 (*Bacillus siralis* J35TS1)	32.83	32.70	32.90	23.73	23.54	26.08	32.68	32.30	32.55	32.18
OX216966 (*Paenibacillus dendritiformis*)	31.34	31.53	31.25	28.45	28.51	28.32	30.02	29.83	29.83	31.40
CP102379 (*Escherichia coli* strain BM28 lys)	54.96	55.21	55.09	31.31	31.38	32.23	55.34	55.34	55.34	56.80
JQ265468 (Uncultured bacterium clone T2C182)	54.96	55.21	55.09	31.31	31.38	32.23	55.34	55.34	55.34	56.80
OP501807 (*Enterococcus gallinarum* strain 10 A)	83.48	83.89	83.59	36.36	35.91	39.08	78.35	78.57	78.57	86.59
OP402856 (*Staphylococcus arlettae* strain Dg-E8)	88.83	88.25	89.03	38.18	38.43	39.11	80.50	80.06	80.76	90.48
MH746088 (*Bacillus siralis* strain PA02)	91.72	92.01	91.86	37.47	37.59	38.06	82.54	83.28	83.28	93.49
OK570087 (*Robertmurraya*	91.72	92.01	91.86	37.47	37.59	38.06	82.54	83.28	83.28	93.49

siralis strain C3)										
ON430535 (*Bacillus cereus* strain CUMB AR-04)	92.42	92.83	92.28	38.42	37.66	39.79	84.01	83.79	84.92	93.31
ON860698 (*Bacillus cereus* strain PSR21)	92.42	92.83	92.31	38.42	37.61	39.79	82.93	83.06	84.46	93.31
OP435764 (*Bacillus tequilensis* strain A37)	99.81	99.80	99.52	38.42	38.51	39.53	84.59	85.07	85.07	94.60
KC441741 (*Bacillus subtilis* strain B59)	99.86	99.65	99.49	38.27	38.24	39.33	82.86	83.32	83.32	94.66
OP521932 (*Bacillus stercoris* strain ML-2)	99.82	99.80	99.58	38.22	38.31	39.27	83.52	83.39	83.93	95.26
OP482166 (*Bacillus mojavensis* strain M)	99.44	99.40	99.92	38.42	38.38	39.53	83.45	83.18	83.87	94.57

GENBANK ACCESSION	NA10B	NA4BB	NA12AA	MC5B	NA5B	EMB9	MC1A	NABAA	NABA	EMB6
OP218479 (*Bacillus mojavensis* strain NR 112725)	99.43	99.80	99.91	38.22	38.29	39.27	84.89	85.10	85.35	94.55
OP514801 (*Escherichia coli* strain ASBY05)	39.27	39.27	39.40	100.00	100.00	100.00	38.32	38.45	38.45	39.63
OP793848 (*Exiguobacterium profundum* strain T5)	83.36	84.66	84.04	38.25	37.21	38.58	98.17	99.65	99.84	87.35
OP263689 (*Exiguobacterium profundum* strain H-1)	83.36	84.66	84.04	38.25	37.21	38.58	99.54	99.82	99.84	87.35
KF732994 (*Bacillus subtilis* strain PMM8)	39.66	39.93	39.74	81.74	82.55	81.90	37.70	38.28	39.02	40.68
OP554433 (*Bacillus velezensis* strain r22)	39.51	39.93	39.79	81.74	82.55	81.90	38.25	38.38	39.10	40.51
ON999044 (*Bacillus*	39.81	40.00	40.06	80.49	80.44	81.60	40.00	40.28	40.09	40.59

inaquosorum strain GZCB-3)										
OP115492 (*Bacillus aerius* strain GS26)	93.31	94.29	93.21	39.46	40.00	39.63	84.55	84.96	84.96	100.00

GENBANK ACCESSION	NA2BA	NA12B	NA4BA	NASAB	NA6B	NA12AB	NA2B	NA7C	NA11A	EMB6A
OX245674 (*Citrobacter* sp. Marseille-Q6884)	32.85	33.06	33.06	33.26	33.06	32.96	33.06	34.59	33.13	32.21
LC588555 (*Bacillus siralis* J35TS1)	26.59	26.96	26.96	29.12	26.96	27.04	28.94	30.35	26.37	31.71
OX216966 (*Paenibacillus dendritiformis*)	31.47	31.66	31.66	31.57	31.66	31.76	31.27	32.16	31.73	31.20
CP102379 (*Escherichia coli* strain BM28 lys)	31.45	31.67	31.67	31.41	31.67	31.78	31.45	31.76	31.78	56.80
JQ265468 (Uncultured bacterium clone T2C182)	31.45	31.67	31.67	31.41	31.67	31.78	31.45	31.76	31.78	56.80
OP501807 (*Enterococcus gallinarum* strain 10 A)	38.30	38.30	38.30	40.52	38.30	38.30	39.34	42.97	38.98	85.49
OP402856 (*Staphylococcus arlettae*	39.03	39.08	39.00	39.09	38.82	39.03	38.45	40.64	39.25	88.62

strain Dg-E8)										
MH746088 (*Bacillus siralis* strain PA02)	39.66	39.66	39.66	40.13	39.66	39.66	40.06	41.95	42.61	93.49
OK570087 (*Robertmurraya siralis* strain C3)	39.66	39.66	39.66	40.13	39.66	39.66	40.06	41.95	42.61	93.49
ON430535 (*Bacillus cereus* strain CUMB AR-04)	38.98	39.03	38.95	39.72	38.78	38.98	39.04	42.37	40.03	91.89
ON860698 (*Bacillus cereus* strain PSR21)	38.93	38.98	38.90	39.72	38.73	38.93	39.04	42.37	40.03	91.89
OP435764 (*Bacillus tequilensis* strain A37)	40.00	39.80	39.93	40.40	39.80	39.88	39.59	41.74	41.18	94.22
KC441741 (*Bacillus subtilis* strain B59)	39.87	39.55	39.65	40.29	39.55	39.63	39.49	41.61	40.93	93.36
OP521932 (*Bacillus stercoris* strain ML-2)	39.93	39.77	39.70	40.32	39.65	39.72	39.41	41.37	40.89	93.51

GENBANK ACCESSION	**NA2BA**	**NA12B**	**NA4BA**	**NASAB**	**NA6B**	**NA12AB**	**NA2B**	**NA7C**	**NA11A**	**EMB6A**
OP482166 (*Bacillus mojavensis* strain M)	40.00	40.00	39.93	40.71	39.87	39.95	39.78	41.77	41.23	93.10
OP218479 (*Bacillus mojavensis* strain NR 112725)	39.79	39.97	39.90	40.51	39.84	39.92	39.60	41.15	41.14	94.05
OP514801 (*Escherichia coli* strain ASBY05)	81.60	81.90	81.90	81.90	81.90	81.90	81.60	82.79	82.20	39.63
OP793848 (*Exiguobacterium profundum* strain T5)	38.19	38.63	38.51	40.56	38.45	38.48	39.82	40.24	38.91	85.16
OP263689 (*Exiguobacterium profundum* strain H-1)	37.86	38.47	38.51	40.56	38.45	38.48	39.82	40.24	38.91	85.16
KF732994 (*Bacillus subtilis* strain PMM8)	99.50	100.00	100.00	100.00	100.00	99.91	99.34	95.26	90.32	41.18

OP554433 (*Bacillus velezensis* strain r22)	99.62	100.00	100.00	100.00	100.00	99.91	99.37	95.58	90.57	41.18
ON999044 (*Bacillus inaquosorum* strain GZCB-3)	99.56	99.79	99.79	99.78	99.79	99.69	99.59	93.10	89.81	41.25
OP115492 (*Bacillus aerius* strain GS26)	41.00	41.18	41.18	41.38	41.18	41.27	41.05	42.23	42.60	100.00