Larval ecological adaptations, water quality and aquatic bacterial communities in *Anopheles gambiae* s.l. : prospects for improved rearing techniques towards release programmes

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Abstract

The sibling species *Anopheles gambiae* s.s. and *Anopheles coluzzii* are the most important vectors of human malaria in sub-Saharan Africa. They are thought to be undergoing speciation with gene flow with rare viable hybrids but are reproductively isolated by assortative mating and ecological divergence. As such important vectors, they are the focus of novel control strategies based on mosquito releases. One of the known drivers of their ecological speciation is their divergent larval ecological adaptation that is possibly linked to rice domestication in Africa. The potential impact of such divergence has never been integrated into mosquito rearing to improve alternative vector control methods such as Sterile Insect Technique (SIT) and Genetically Modified Mosquitoes (GMM), that are needed to accelerate the progress towards malaria elimination. These innovative vector control methods depend on mass rearing of millions of mosquitoes in a manner that is both efficient and economic, to produce mosquitoes that are of adequate quality, able to favourably compete with wild populations. In this thesis, we investigated the phenotypic plasticity of these sibling species to typical stressors in the rice field ecosystem with a focus on ammonia in their larval habitat. Experiments were conducted in small containers and in contrasted microcosms to test the direct effects of mineral water and increasing ammonia concentrations on larval development and to highlight divergent reaction norms between the sibling species. We also evaluated the use of zeolite to improve larval water quality management in *An. gambiae* s.l. insectary. To further understand the dynamics of the nitrogen cycle in larval rearing trays that led to larval mortality, we characterised their bacteria communities using 16S rRNA gene sequencing. Functional filters were applied to identify candidate bacteria species beneficial and detrimental to larval development and these were validated by qPCR. Our results suggest that genotype-by-environment interactions associated with rice domestication event in

Africa are indeed an important driver of the eco-speciation between the sibling species. *An. coluzzii* was more tolerant to ammonia and rice-field like conditions supporting the idea that this may have driven its speciation from the ancestral *An. gambiae* s.s. We show that mineral water is beneficial for improved mosquito yield and phenotypic quality of adult mosquitoes in the insectary and this can be used to improve rearing protocols for these species. For the first time, we demonstrated that zeolite can be used to improve rearing results for *An. gambiae* s.l., providing a water conserving alternative for rearing mosquitoes for mass release programmes, especially in arid regions. Furthermore, the ensuing analyses of bacterial communities larval trays is also a novel endeavour which led to the identification of 1031 bacteria species and of several key species with various opportunities for further improvement of larval rearing towards mass release purposes and/or for novel direct vector control. In conclusion, we have made modest contributions towards the control of these malaria vectors and the fight to eliminate this multifaceted disease. It is therefore important that policy makers in malaria endemic countries ensure that policy reformations in irrigational agriculture and urbanization consider the impact of policy on these disease vectors that are of immense public health importance.

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Abbreviations

ACT- Artemisinin-based Combination Therapy

AIs- Active Ingredients

ATSB- Attractive Toxic Sugar Baits

AuN- Gold Nanoparticles

CAEP- Centre for Applied Entomology and Parasitology

Cas9- CRISPR-associated 9 endonuclease system

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

DDT- dichloro-diphenyl-trichloethane

DNA- Deoxyribonucleic Acid

EOs- Essential oils

FAO/IAEA- Food and Agriculture Organisation/International Atomic Energy Agency

GDP-Gross Domestic Product

GluCl- Glutamate-gated Chlorine

GMAP- Global Malaria Action Plan

gRNAs- guide RNAs

HDR- Homology-Directed Repair

HGDs- Homing-based Gene Drives

HTS- High-throughput Sequencing

IIT- Incompatible Insect Technique

IRSS- Institut de Recherche en Sciences de La Sante

IPTp - Intermittent Preventive Treatment for pregnant women

IRS- Indoor Residual Spraying

ITNs- Insecticide Treated Nets

IVCC- Innovative Vector Control Consortium

IVM- Integrated Vector Management

Kdr- knock down resistance mutations

LLINs- Long-Lasting Insecticide Treated Nets

LSM- Larval Source Management

MDA- Mass Drug Administration

NCBI- National Center for Biotechnology Information

OTUs- Operational Taxonomic Units

PBO- Pyrethroid synergist-Piperinyl Butoxide

PCR-Polymerase Chain Reaction

pgSIT- precision-guided Sterile Insect Technique

qPCR-Quantitative PCR/Real time PCR

RBM- Roll Back Malaria

RDTs- Rapid Diagnostic Tests

RNA- Ribonucleic Acid

RO- Reverse Osmosis

RTS,S/AS01-Mosquirix- antimalarial vaccine

SIT- Sterile Insect Technique

SR- Spatial Repellents

UF- Ultra-filtration

USD- United States Dollars

WHO- World Health Organisation

WHO- GTS- WHO's Global Technical Strategy

WHOPES- WHO Pesticide Evaluation Scheme

Acknowledgements

I am immensely grateful to my Lord and Saviour Jesus Christ who has granted me the strength, grace, resources and the will, to complete this PhD research. I know this programme will not have been possible without his power that works in me, and I am forever grateful that I have him in my life.

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My PhD journey has been a very interesting one with intriguing turns, providing wonderful training opportunities one of which was my placement at the Vector Functional Genomics and Microbiology Laboratory, in the Institute of Biotechnology, São Paulo State University "Júlio des Mesquita Filho" (UNESP), Botucatu campus, Brazil, where I conducted the 16S rRNA gene sequencing for my chapter 7, in 2018. I want to thank Prof Jayme Souza-Neto, the Keele-UNESP coordinator, for help with organising this placement. To all the laboratory staff and students at UNESP, Bianca Carlos, Kamila Voges, Bruno Tinoco Nunes, Vanessa Calvarh, Mariana Porto, Pedro Affonso, Barbra and Felipe, thank you for making my time in Brazil both effective and immensely pleasurable. I was amazed at the enthusiasm and candour with which you received me and for providing all the assistance I needed to complete the sequencing within the short time I had to be in the Brazil. I am also grateful to my host Terezinha, who shared her home and family with me and made me temporarily forget that I had left

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CHAPTER 1

Introduction

1.1. The global burden of malaria.

Malaria is a major public health concern; according to the 2019 World Malaria Report, there were 228 million cases and 405,000 deaths occurring worldwide in 2018 (WHO, 2019). In the same year, sub-Saharan Africa accounted for 93% of global malaria incidence, South-East Asia for 3.4% and the Eastern Mediterranean region for 2.1% (WHO, 2019). Almost 85% of this global malaria burden was carried by 19 countries in sub-Saharan Africa and India. Six countries accounting for over 50% of these cases include Nigeria (25%), the Democratic Republic of Congo (12%), Uganda (5%), Côte d'Ivoire, Mozambique and Niger (4% each) (WHO, 2019).

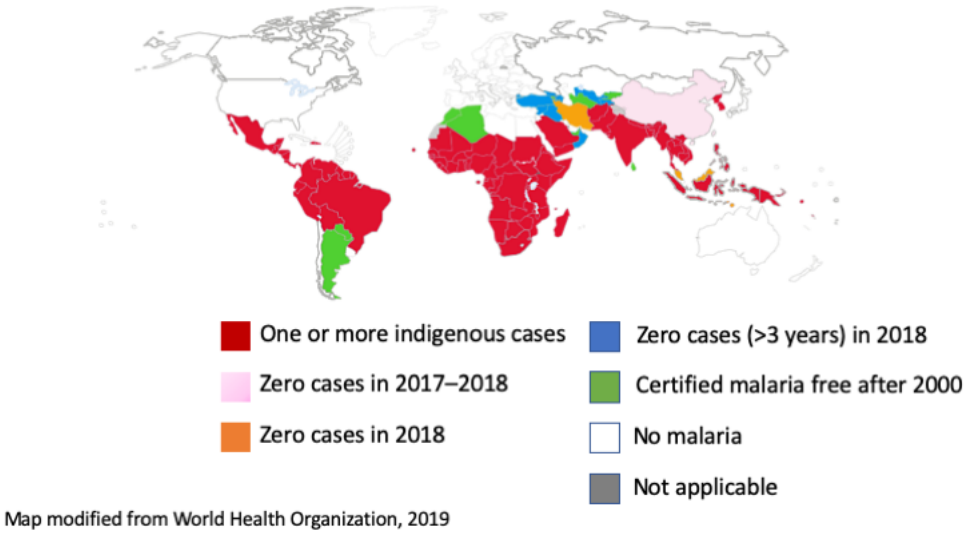

Figure 1.1: Countries with indigenous cases in 2000 and their status in 2018.
Countries with zero indigenous cases over at least the past 3 consecutive years are considered to be malaria-free (blue). In 2018, both China and El Salvador reported zero indigenous cases (pink) for the second consecutive year and Iran (Islamic Republic of), Malaysia and Timor-Leste reported zero indigenous cases for the first time (orange). Malaria cases were still common in many countries in the Americas, Africa and Asia (red) (WHO, 2019).

The malaria scourge continues to strike hardest against the most vulnerable in the society, with children under 5 years old accounting for 67% (272,000) of all malaria deaths worldwide (WHO, 2019). Nearly 85% of malaria related deaths in 2018 occurred in 20 countries in Africa and India (WHO, 2019). Aside from the mortality associated with severe malaria, it causes significant morbidity especially in pregnant women. Malaria in pregnancy compromises the health of the woman, resulting in maternal anaemia which puts her at a greater risk of death. It also negatively impacts on the health of the unborn child, resulting in premature births, low birth weight, and is also a major contributor of neonatal and infant mortality (Bardají et al., 2017). In 2018, 11 million pregnant women were infected with malaria resulting in 872,000 infants with low birthweight (WHO, 2017; WHO, 2019). In addition to neonatal and perinatal risks, the disease can also impact on the growth and intellectual development in children (Chima, et al., 2003; Guyatt & Snow, 2001; Stevens et al., 2013).

Malaria also has a major economic impact on these developing countries that are endemic for this disease. Bearing the heaviest burden of the disease, they suffer direct economic losses such as household expenditures in the purchase of insecticides, treatment, transport to a health facility, government's spending on insecticides, drugs, treated nets and healthcare facilities. Further indirect losses are incurred through loss of tourism and productivity (Benelli & Beier, 2017). When there is sustained investment in health and malaria in particular the potential of human capital to generate growth is unlocked. It has been demonstrated that a 10% reduction in malaria associates with 0.3% rise in annual GDP (Gross Domestic Product). At the family level, reducing malaria protects the household income from lost earnings due to illness and costs of seeking care (WHO, 2019).

From a baseline of 2015, WHO's Global Technical Strategy (GTS) aimed for a reduction in malaria morbidity, incidence and mortality rates by 40% in 2020 and elimination in at least 10 countries that were malaria endemic in 2015 and to prevent reintroduction in these countries that have achieved elimination (WHO, 2015). Remarkable progress has since been made, as many countries are moving towards zero indigenous malaria cases. In 2018, countries reporting fewer than 10,000 cases increased from 46 in 2017 to 49, which represent marked progress from the 40 countries reported in 2010. Likewise, 27 countries reported fewer than 100 indigenous cases in 2018, an increase from the 25 in 2017 and 17 in 2010. Similarly, malaria mortality has reduced drastically from 585,000 in 2010 to 416,000 in 2017 and 405,000 in 2018, representing the impressive gains that have been made towards malaria control in the past decade. Global progress in malaria reduction is further evidenced by Paraguay and Uzbekistan receiving the award for elimination of malaria in 2018, Algeria and Argentina in 2019, while China, El-Salvador, Iran, Malaysia and Timor-Leste reported zero indigenous cases (WHO, 2019). Following the September 2018 "Malaria Challenge" by WHO for greater investment in research and development of novel transformational tools, technologies and approaches to accelerate progress in reducing the impact of malaria, Ghana, Kenya and Malawi recently introduced the world's first malaria vaccine into selected areas (Asante et al., 2019; WHO, 2019). In 2018, there was an increase of 18 million USD for basic research and product development for malaria compared to 2017 (WHO, 2019). There was a 47% increase between 2010 and 2018, in the number of patients suspected of having malaria and tested with either rapid diagnostic tests (RDTs) or microscopy (WHO, 2019). The use of RDTs alongside increase in the supply and distribution of Artemisinin-based combination therapy (ACT) and long-lasting insecticide treated nets (LLINs) have helped to reduce malaria mortality and morbidity drastically from 2010 to 2018 (WHO, 2019).

Despite these impressive achievements, the global malaria challenge remains enormous, and there has been a stall in progress towards reduction of malaria morbidity, incidence and mortality rates, and in some cases, a standstill, since 2016. More worrisome is the rise in malaria cases across some high-burden countries in Africa. Although the global malaria incidence declined from 71 to 57 cases per 1000 population at risk from 2010 to 2018, the lower case mark of 57 was achieved in 2014 and has remained at similar levels through to 2018 (WHO, 2019). The rate of malaria mortality reduction was slower between 2016 to 2018, than between 2010 to 2015. Further, global investments in malaria control and elimination in 2018 was estimated at 2.7 billion USD, representing a reduction from the 3.2 billion USD invested in 2017 and also falling short of the 5 billion USD estimated to be required to achieve the GTS target (WHO, 2019). Insecticide resistance amongst other factors is indicted for this change in trajectory towards malaria elimination (Hancock et al., 2018). In the last decade, 73 out of 81 malaria endemic countries have reported insecticide resistance to at least 1 of the 4 insecticide classes, an increase of 5 countries from 2017 to 2018. This illustrates how fragile are the gains made in our quest to control, and ultimately eradicate the disease. The pace of progress towards malaria control and elimination needs to be improved to meet the GTS deadlines, and also to preserve the currently available malaria interventions. The Global Malaria Report for 2019 recommended improved interventions in these areas: affordable and people oriented health services, accurate surveillance and response systems, strategies tailored to local malaria settings and increased financing for malaria research (WHO, 2019). To improve malaria surveillance, there was a recommendation for the facilitation of the collection and use of entomology and vector control data to inform decision-making processes at country level. Insecticide resistance monitoring, adult mosquito surveillance

and identification and mosquito larval habitat monitoring were amongst the intervention area highlighted for surveillance (WHO, 2019).

1.2. Malaria transmission in Africa

Malaria is a complex disease caused by five *Plasmodium* species, *Plasmodium falciparum, Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi* (Bronner, et al., 2009). *Plasmodium falciparum* is the most prevalent malaria parasite in sub-Saharan Africa, accounting for 99.7% of malaria cases in the region (WHO, 2019). There have been reports of partial resistance to Artemisinin-based combined therapy (ACT) drugs mostly used for treatment of malaria in the African region (Agnandji, et al., 2011a; 2011b). An antimalarial vaccine RTS,S/AS01-Mosquirix, which only provides transient protection against *Plasmodium falciparum* malaria in infants, has been developed following 30 years of research and clinical trials by GlaxoSmithKline Biologicals (Agnandji et al., 2011c; Benelli & Beier, 2017). This was achieved through a partnership with the PATH Malaria Vaccine Initiative with support from Bill and Melinda Gates Foundation and African research centres (Asante, et al., 2019; Benelli & Beier, 2017). On the World Malaria Day, April 2019, this malaria vaccine, was introduced into the routine health systems in Malawi, Kenya and Ghana as a pilot implementation study to evaluate the impact of the vaccine on mortality, cerebral malaria and meningitis in a larger population (Asante et al., 2019)

All human malaria is transmitted by female mosquitoes of the genus *Anopheles* (Coetzee, et al., 2000). Of the 450 *Anopheles* species, only ~100 are considered as disease vectors (Cohuet, et al., 2010; Wiebe et al., 2017). The malaria parasite requires two hosts, the mosquito vector and the human host to complete its developmental cycle (Figure 1.2). Most vector control measures (insecticides, biological control, larvicides, insecticide

treated nets, sterile insect techniques) are typically designed to interrupt the disease cycle by preventing the transfer of malaria-causing parasites to humans (Lees, et al., 2015). In the absence of effective vaccines and the evolution of resistance to artemisinin by *Plasmodium* malaria parasites, vector control continues to be the most cost-effective line of defence against malaria (Benelli & Beier, 2017; Lees et al., 2015).

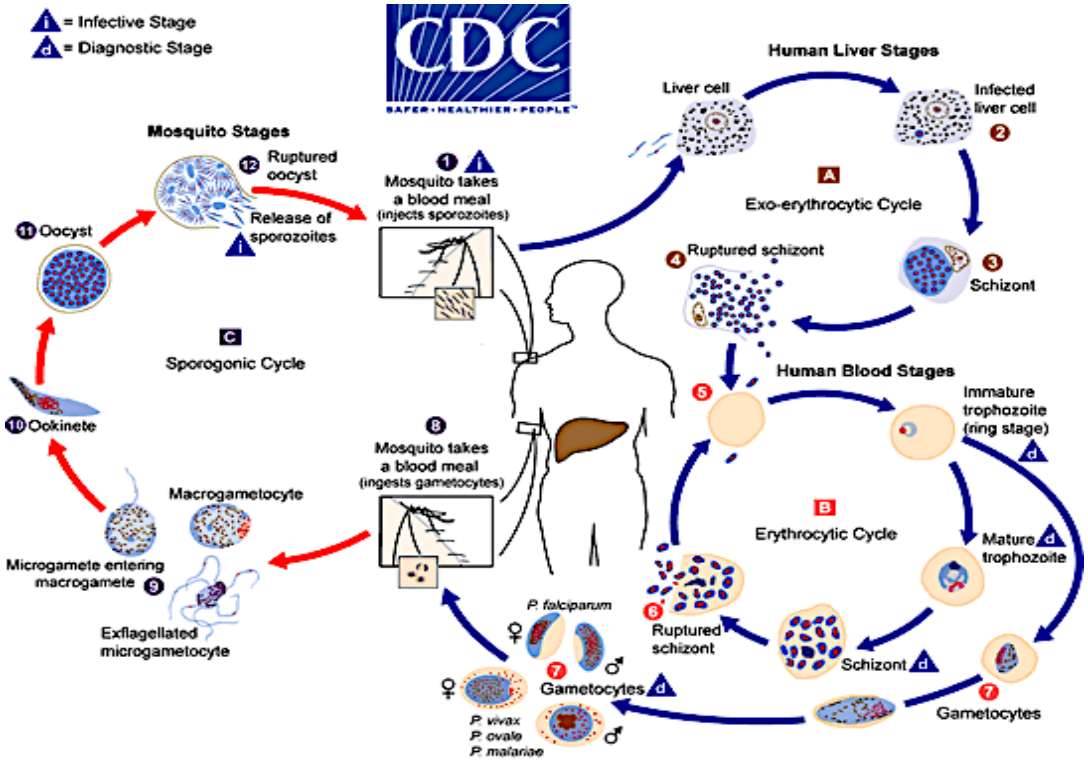

Figure 1.2. Life cycle of *Plasmodium* spp. The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (1). Sporozoites infect liver cells (2) and mature into schizonts (3), which rupture and release merozoites (4). (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver (if untreated) and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony (A)), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony (B)). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). Some parasites differentiate into sexual erythrocytic stages (gametocytes) (7). Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (8). The parasites' multiplication in the mosquito is known as the sporogonic cycle (C). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10) which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites (13) into a new human host perpetuates the malaria life cycle (Schaeffer, 2011)

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1.2.1. *Anopheles gambiae* s.l. complex

Malaria control through vector control tools is particularly complicated in Africa because malaria transmission on the continent is sustained by several vector complexes. The most important in terms of vectorial importance is the *An. gambiae* complex comprising of 8 morphologically indistinguishable sibling species (Lanzaro & Lee, 2013; Lindsay, et al., 1998). The complex is made up of three brackish-water species namely *Anopheles bwambae*, *Anopheles. melas*, and *Anopheles merus* and six freshwater species that include *Anopheles arabiensis*, *Anopheles amharicus* (previously referred to as*, Anopheles quadriannulatus* species B*)*, *Anopheles quadriannulatus*, *Anopheles gambiae s.s.* (formerly S molecular form of *An. gambiae* s.s.), *Anopheles coluzzii* (formerly M molecular form of *An. gambiae* s.s.) and the recently discovered *An. fontenillei* (Barrón et al., 2019; Huestis & Lehmann, 2014). All sibling species are successful malaria vectors except the two-freshwater species, *An. quadriannulatus* and *An. amharicus* whose adults typically feed exclusively on animals (Coetzee et al., 2013; Hunt et al., 1998). The newly discovered *An. fontenellei* are assumed zoophagic because they were found in forested areas of Gabon, Central Africa, lacking in human hosts. Although no blood-fed mosquitoes were captured, it is possible they could feed on humans as CO_2 based traps and Human-landing catches were sucessful. (Barrón et al., 2019). Two of the brackish water species *An. merus* and *An. melas* have limited coastal distribution (east and west coasts of Africa respectively) due to larval adaptation to salt-water larval habitats (Coetzee et al., 2013; Hunt, et al.,1998; Lanzaro & Lee, 2013). The third brackish-water species, *An. bwambae*, occurs only in association with the hot springs in Semliki Forest National Park, in eastern Uganda (Lanzaro & Lee, 2013). Three freshwater species, with the broadest geographic distribution, *An. gambiae* s.s., *An. coluzzii*, and *An. arabiensis*, are the most important vectors of human malaria, transmitting malaria over vast ranges

of sub-Saharan Africa (Coetzee et al., 2000; Gillies & Coetzee, 1987; Lanzaro & Lee, 2013)

1.2.2. Morphology and life cycle of *Anopheles gambiae* s.l.

Morphology

Adult *Anopheles gambiae* s.l. in comparison with other anophelines are medium-sized mosquitoes with an average wing length of 2.8 to 4.4mm. Their body colour is variable, ranging from light brown to grey, with characteristic pale spots of yellow, white or cream scales on the wing veins arranged in blocks (Gillies & Coetzee, 1987). Males usually possess plumose antennae with females having non-plumose antennae (Holstein, 1954). Palps of adult females are about as long as the proboscis and usually lie closely alongside the palps and may be marked, particularly the apical half, with broad and narrow rings of pale scales. The palps in males are swollen at the ends and may have apical rings of pale scales (Service, 1969). Adult *An. gambiae* s.l. usually rests with the body at an angle to the surface, with proboscis and abdomen in a straight line (Ward, 2008). *An. gambiae* s.l. eggs are brownish, 0.47 - 0.48mm long, boat-shaped, and typically have a pair of air-filled sacs known as floats (Ward, 2008).

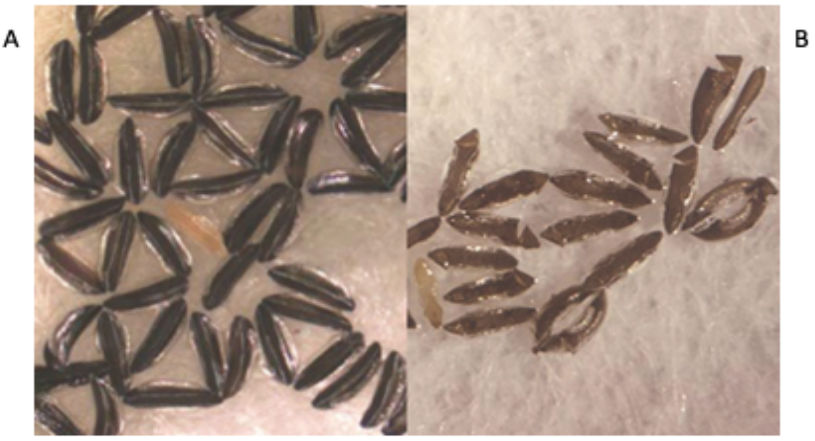

Figure 1.3: Eggs of *Anopheles stephens*i (A) unhatched, (B) hatched, showing singly laid *Anopheles* eggs with floats on both sides, and the split created by emerged larvae (Schaeffer, 2011).

They are laid singly on water and are unable to withstand desiccation (Ward, 2008). Larvae of *An. gambiae* s.l. typically lie parallel to the water surface, a position that facilitates filter feeding. They possess brown sclerotized tergal plates on the 1st to 8th segments of their abdomen as well as mature palmate hairs on the thorax, serving as floats (Holstein, 1954). On the 8th segment of their abdomen, they have pectens (teeth-like sclerotized structures) that are unique to species (Ward, 2008). Side view of *An. gambiae* s.l. pupae present a comma-shaped organism with dorsally placed, short and broad respiratory trumpets. A combined head and thorax form the cephalothorax, abdomen consist of 10 hairy segments (Ward, 2008). Also distinctive is the presence of short peg-like spines located laterally close to the distal margins of the abdominal segments (Ward, 2008).

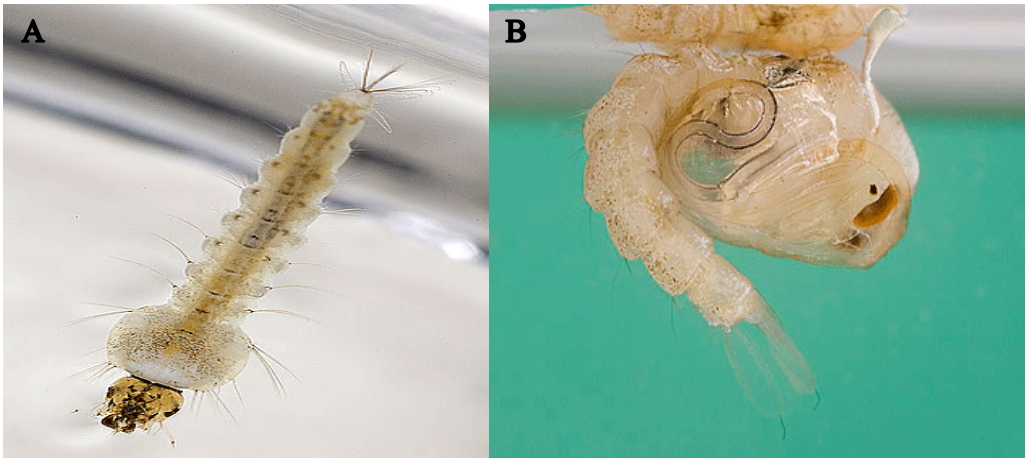

Figure 1.4: A- Larva and B- Pupa of *Anopheles gambiae* s.l. (Schaeffer, 2011)

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Life-cycle of *Anopheles gambiae* s.l.

Under optimal conditions (25°C to 26°C, 70-80% relative humidity, and availability of nutrients), the development of *An. gambiae* s.l. comprising of four life stages: egg, larvae, pupae and adult, takes about 8 to 10 days (Coluzzi, 1964). Both sexes feed on nectar from plants but only females blood-feeds on vertebrates. There exists a gonotrophic life cycle

from a blood meal to oviposition, though multiple blood meals in a gonotrophic cycle have been reported (Takken, et al., 1998). The adult female lays between 50-200 small brownish/blackish boat-shaped eggs on the surface of the water after mating and blood-feeding. Females mate only once in their lifetime while males mate several times (Diabate & Tripet, 2015). In the tropics, viable eggs hatch within 2-3 days, taking 4-7 days or longer in temperate regions. Larvae undergo four moults within 6-9 days at mean temperatures of 25-28°C. The larval stages are elongated by changes in temperature, nutrient and competition (Ward, 2008). The pupal stage is a non-feeding period that develops after the fourth larval instar. The adult mosquito emerges when the pupal skin splits dorsally, the new mosquito inflates its wings, grooms its head appendages and flies away. Twenty-four hours after emergence, males become ready for mating which is often preceded or accompanied by swarming in which the females seek out males in the swarm (Diabate & Tripet, 2015; Holstein, 1954). Most anopheline males die after mating (Diabate & Tripet, 2015). Females usually take a blood meal that is required for ovarian development, leading to maturation and oviposition of a batch of eggs (Gillies & Coetzee, 1987)

Populations of the members of the *An. gambiae* s.l. complex that develop in freshwater strongly depend on rainfall. Their densities increase typically at the onset of the rainy seasons, reaching their peak in mid-season and decline as water levels stabilize and aquatic predators are established (Gillies & Coetzee, 1987). There is usually heavy mortality at the larval developmental stages due to predators, disease, drought and flooding. All of these environmental factors have the potential of drastically reducing the percentage of eggs that successfully develop into adults (Huestis & Lehmann, 2014; Roux et al., 2013; Simard et al., 2009).

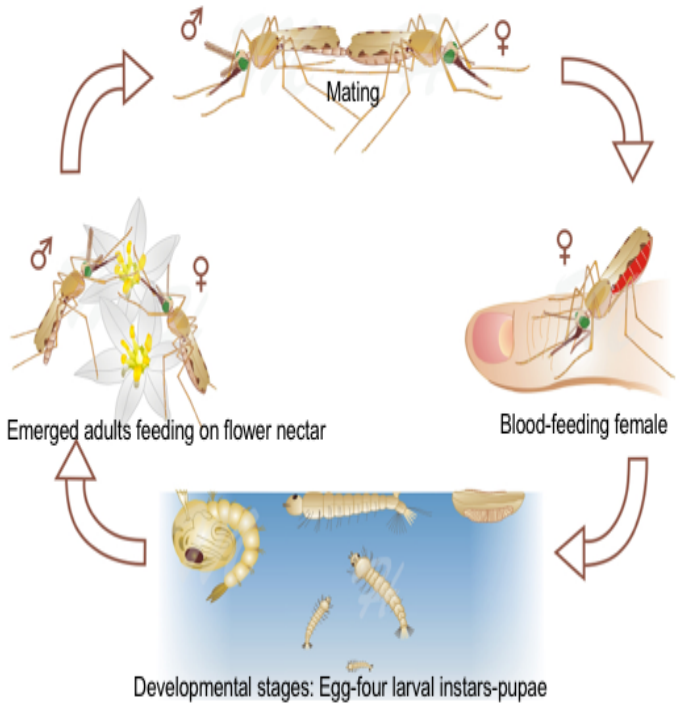

Figure 1.5: The life cycle of *Anopheles gambiae* s.l. (modified from Gathany, 2007)

1.2.3. Ecology of *Anopheles gambiae* s.l.

Immature stages

Adult females lay 50-200 brownish or blackish eggs per oviposition. Eggs are laid singly in water and are not resistant to desiccation. They hatch within 2-3 days to larvae though this may take up to 2-3 weeks in colder regions (Davidson, 1977). Larvae are typically thought to prefer small collections of water exposed to the sun, however, they have sometimes been found breeding in unlikely places like grassy swamps, backwaters of streams, irrigation ditches, cultivated rice fields. It is difficult to attribute a definite breeding place to *An. gambiae* s.l.. "It is likely to breed in almost any water that happens to be available" (Holstein, 1954)

Of entomological and epidemiological importance is the close association of some members of the *An. gambiae* s.l. complex breeding sites to human activity. The physicochemical properties of larval breeding sites typical of *An. gambiae* has a broad

range, with pH ranging from 5.8 to 9.0 (optimum pH at 7.8), temperature between 20°C and 30°C, and larvae density reducing as water becomes more acidic (Davidson & Jackson, 1962)

Adult stage

They are nocturnal, mostly active at dusk, preferring to feed and rest indoors (Gary, 2005), while some species are exophilic, resting in a variety of natural shelters such as vegetation, crevices of trees, termite mounds, and feed on livestock and wild animals (Ward, 2008). The degree of anthropophilism and zoophilism varies according to species (Knols & De Jong, 1996). The blood-feeding females usually feed when their host is less defensive (sleeping), a pattern indicative of adaptation to host defensive behaviour; the mosquito is, therefore, able to feed for long periods and sometimes expels serous fluid (Gary, 2005). Female *An. gambiae* s.s., *An. coluzzii* and other anthropophilic members of the complex are host specific, attracted to the body heat, carbon dioxide emission, and skin microflora of their human host (Knols & De Jong, 1996).

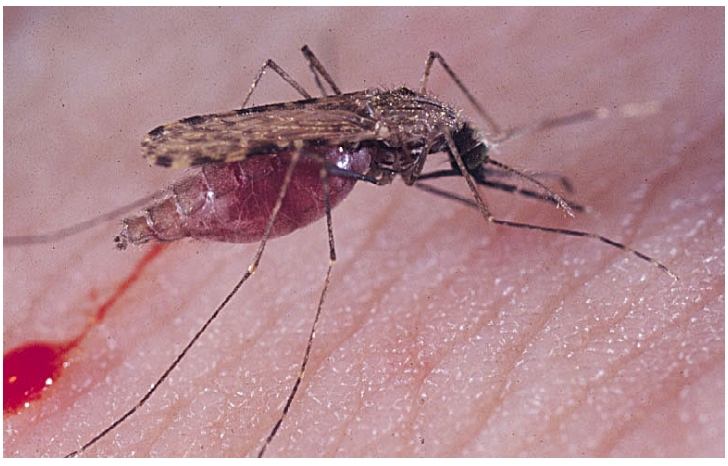

Figure 1.6: Adult female *Anopheles gambiae* s.l. blood-feeding on a human host (Cornel, 2020)

1.2.4. Distribution of the sibling species *Anopheles gambiae* s.s. and *An. coluzzii.*

For clarity, sibling species henceforth refer to *An. gambiae* s.s. and *An. coluzzii*. Anopheline mosquitoes are found everywhere except Antarctica, however, the sibling species co-occur over large areas of sub-Saharan Africa and do not exhibit intrinsic post-mating barriers to reproduction (Aboagye-Antwi et al., 2015). Whilst *An. gambiae* s.s. is widespread throughout the region, extending across the continent into Madagascar (della Torre et al., 2005) and is presumed ancestral, *An. coluzzii* spans from northern Senegal (West Africa), East-Central Africa and Angola (Southern Africa), with an exceptional individual found in the Zambezi valley in Zimbabwe (della Torre, et al., 2005; Gimonneau et al., 2010). A species distribution modelling study and several larval habitat correlational studies reveal that the geographical range of *An. coluzzii* corresponds with the xeric savannahs and coastal fringes of West Africa with its sibling *An. gambiae* s.s. found mostly in inland rural areas (Diabate et al., 2005; Edillo et al., 2006; Gimonneau et al., 2014; Kudom, 2015; Tene Fossog et al., 2015).

1.3. Vector control

In 1998, the Roll Back Malaria (RBM) initiative was launched as a partnership of malaria endemic countries, United Nations agencies, bilateral development agencies, the research community, the private sector, non-governmental organisations, foundations and the media (WHO, 2008). The objective of RBM was to halve the malaria burden in countries participating in the initiative. The technical strategy of RBM was based on early detection and prompt treatment of malaria cases, the detection and control of malaria epidemics, mosquito control and the prevention of malaria in pregnancy (WHO, 2008). The RBM partnership developed the Global Malaria Action Plan (GMAP) to support malaria endemic countries by providing a global framework around which actions and efforts

towards malaria control and eradication can be directed (WHO, 2008). The primary tools for malaria prevention as highlighted in the GMAP are, long-lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS) and intermittent preventive treatment for pregnant women (IPTp). Other vector control measure such as larviciding and environmental management are implemented following integrated vector management (IVM) protocols to ensure interventions were used as appropriate (WHO, 2017). Although these methods have been very effective in the reduction of mortality and morbidity over the past decade, 50% of the countries with ongoing malaria transmission which were on track towards critical targets for reduction in mortality and morbidity, have recorded a stall in progress (WHO, 2017). This current trend brings to the forefront the research for new vector control methods to complement the existing IVM techniques.

1.3.1. Insecticide resistance

Malaria prevalence in Africa has declined over the last two decades and these gains are, in a large part due to the widespread implementation of insecticide-based vector control measures (Bhatt et al., 2015; WHO, 2019). As efforts to control malaria were intensified, the selection pressure on mosquito vectors to develop resistance to insecticide also increased (Ranson & Lissenden, 2016). In this same period, there has been increases in insecticide resistance that have the potential to derail or even reverse the progress made in reducing malaria transmission (Hemingway et al., 2016a). Under four broad insecticide classes (pyrethroid, carbamates, organophosphates, and organochlorines), there are only 12 available insecticides (all insect neurotoxins) that have the WHO Pesticide Evaluation Scheme (WHOPES) approval for use in malaria vector control (WHO, 2017). To manage these limited resources, WHO has encouraged countries to develop insecticide management plans based on local data, however, these data are sparse or non-existent in

many places (WHO, 2019). According to the World Malaria Report 2019, an increase of 5 countries from 2017 – 2018 reporting resistance for all the main classes of insecticides in 26 countries, evidenced rapid development of insecticide resistance in African malaria vectors which threatens the continued efficacy of important vector control methods that rely on a limited number of insecticide classes (Hancock et al., 2018; WHO, 2019)

Three categories of mechanisms that confer resistance to neurotoxic insecticides in malaria vectors include alterations to metabolic genes or pathways (metabolic resistance), target site mutations (phenotypic resistance) and cuticular thickening (mechanical resistance) (Edi et al., 2014; Hancock et al., 2018). Metabolic resistance is very common and results primarily from the amplification or up-regulation of detoxification enzymes (especially esterases, P450 monoxygenases and glutathione S-transferases) and can confer high levels of resistance (Edi et al., 2014; Ibrahim, et al., 2016; Mitchell et al., 2012). Whilst some enzymes have been linked to resistance to a specific insecticide class, others confer resistance across several insecticide classes (Antonio-Nkondjio et al., 2017; David, et al., 2013; Edi et al., 2014; Ibrahim, et al., 2016; Mitchell et al., 2012). Changes to insecticide target sites are also prevalent and are frequently associated with phenotypic resistance (Kawada et al., 2011; Kwiatkowska et al., 2013; Reimer et al., 2008). For instance, acetylcholinesterase target site alteration caused by a mutation to the Ace-1 gene typically confers cross-resistance to organophosphate and carbamate insecticides (Edi et al., 2014; Essandoh, et al., 2013). Cross resistance to pyrethroids and the organochlorine (DDT) arise from knockdown resistance (*kdr*) mutations in the *Vgsc* gene which encodes the para voltage-gated sodium channel target site (Hancock et al., 2018). Currently, there is less evidence for cuticular thickening as a mechanism of resistance (Balabanidou et al., 2016; Wood, et al., 2010; Yahouédo et al., 2017).

Pyrethroid resistance (the only approved insecticide class for LLINs) is now ubiquitous on the African continent with resistant *An. gambiae* s.l. populations found in Kenya, much of Tanzania, Zambia, Zimbabwe and Democratic republic of Congo (Kanzaa et al., 2013). Although populations of *An. gambiae* s.l. fully susceptible to pyrethroids were found in Angola, Madagascar and Mozambique in 2015, they are becoming increasingly outnumbered by resistant populations (Ranson & Lissenden, 2016). In 2019, the World Malaria Report recorded pyrethroid and organochlorine resistance in at least one malaria vector in more than 67% of the sites tested. Pyrethroid resistance was highest in sub-Saharan Africa and East Mediterranean region. Resistance to carbamates and organophosphates was less prevalent and was detected in 31% and 26% of the tested sites in the 2019 report respectively (WHO, 2019).

To counteract the rapid emergence of resistance and sustain progress made towards malaria control and elimination targets, new insecticides are urgently needed. It is also critical that future insecticide based approaches are not dependent on a single active ingredient in the way we relied on pyrethroids since 2000. To achieve this, a product development partnership, the Innovative Vector Control Consortium (IVCC), was set-up to deliver three novel public health insecticides by 2022 (Hancock et al., 2018; Hemingway et al., 2016b; Ranson & Lissenden, 2016). These new insecticides will allow for combination and rotation strategies that will optimise performance and reduce the likelihood of resistance development (Ranson & Lissenden, 2016). Thus, manufacturers, donors, control programmes, the WHO and other stakeholders must work together to implement resistance management strategies to maximise the duration of efficacy of these novel chemistries (Ranson & Lissenden, 2016). There is also the hope that additional insecticide classes will become available via the repurposing and

reformulating of agricultural insecticides for use in vector control especially targeted at IRS (Ranson & Lissenden, 2016).

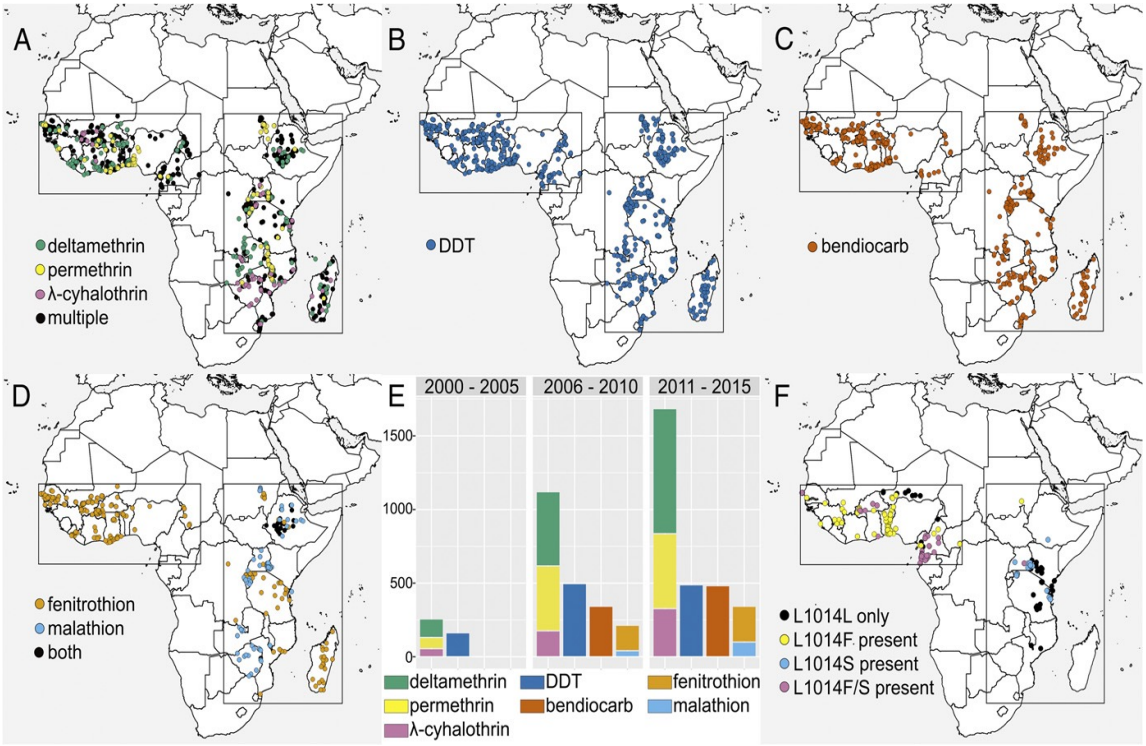

Figure 1.7: The spatial-temporal distribution of insecticide resistance from 1183 locations in 38 African countries located in the West and East African regions. (A) Pyrethroid bioassays. (B) Organochlorine bioassays. (C) Carbamate bioassays. (D) Organophosphate bioassays. (E) The number of bioassay records for each time period. The keys in A – D correspond to the insecticides shown in E. (F) The locations of sample collections used to calculate Vgsc allele frequency data. The mutations L1014F and L1014S present at each location are shown (Hancock et al., 2018).

1.3.2. Current trends in malaria vector control

There has been an evolution of malaria vector control methods from the rudimentary environmental control, single chemical control approach, to Integrated Vector Management. In 2017, the World Health Assembly welcomed the global vector control response 2016 – 2030 and adopted the resolution to promote an integrated approach to the control of vector borne diseases, primarily building on the concept of IVM (WHO, 2019-Vector control guidelines). IVM is simply defined as the "rational decision-making process for the optimal use of resources for vector control"(WHO, 2008). It involves the

use of sound principles of management, allowing the full consideration of the determinants of disease transmission and control. In line with the IVM concept, there is strong advice against vector management plans that rely on any one form of control such as the use of chemical insecticides in isolation, to ensure the value of such methods are not lost by biological phenomena such as insecticide resistance. Rather, the development and effective combination of several locally adapted vector control approaches, is encouraged as the best approach to reduce the malaria burden in an environmentally sustainable fashion. Currently, IVM strongly suggest making use of the full range of control methods available as well as regular local assessments of disease transmission dynamics and interaction with public and private stakeholders. (WHO, 2019-Vector control guidelines).

Indoor Residual Spraying (IRS) and Long-Lasting Insecticidal Nets (LLINs) are the two most widely used control tools for *Anopheles gambiae* s.l. control. In some areas, these tools are synergistically combined with larval source management (Benelli & Beier, 2017). In the light of insecticide resistance and potentially negative effect of chemical insecticides on human and environmental health, sustained efforts are required to develop new and/or complementary control techniques for *Anopheles gambiae* s.l. control. Policy improvements in these core malaria vector interventions and supplementary interventions are discussed below.

Insecticide Treated Nets (ITNs)

WHO recommends ITNs (which should be LLINs in some settings) as a core vector control intervention for use in protecting populations at risk of malaria, including areas where malaria has been eliminated or transmission has been interrupted with the risk of reintroduction still remaining (Keating et al., 2011; WHO, 2019-Vector control guidelines). The use of ITNs have played a major role in the reduction of malaria cases

since 2000 (Paaijmans & Huijben, 2020). Sixty-eight percent of the overall malaria reduction observed between 2000 and 2015 can be attributed to the use of ITNs (Bhatt et al., 2015). Factors contributing to its success includes, its effectiveness as a physical barrier, containing an insecticide that kills susceptible mosquitoes upon and after contact, and the excito-repellent effect of pyrethroids (Paaijmans & Huijben, 2020). ITNs are most effective where the principal malaria vector(s) mosquitoes bite predominantly at night after people have retired under their bed-nets. There are two main ITN classes currently recommended by WHO; pyrethroid-only nets (including LLINs) and recently developed LLINs that contain pyrethroid synergist-piperinyl butoxide (PBO) as well as other nets containing multiple active ingredients (N'Guessan et al., 2014; Tiono et al., 2015; WHO, 2019-Vector control guidelines). Pyrethroid-only nets include both conventionally treated nets that rely on periodic re-treatment of nets by dipping into an insecticide formulation, and factory-treated LLINs made of netting materials with insecticide incorporated within or bound around the fibres (Pinder et al., 2015; WHO, 2019-Vector control guidelines). LLINs are defined as retaining their effective biological activity for at least 20 standard washes under laboratory conditions and 3 years of recommended use under field conditions (WHO, 2019-Vector control guidelines). WHO prequalified pyrethroid-PBO nets are conditionally recommended for deployment instead of pyrethroid-only LLINs where the principal malaria vector(s) exhibit confirmed pyrethroid resistance of intermediate level, that is conferred by a monooxygenase-based resistance mechanism (Strode et al., 2014; WHO, 2019-Vector control guidelines). The use of these nets may help maintain the personal and community benefits of LLINs in areas of pyrethroid resistance until novel public health insecticides become available (Hemingway et al., 2016a; Paaijmans & Huijben, 2020; Ranson & Lissenden, 2016). PBO acts by inhibiting certain metabolic enzymes (e.g. mixed-function oxidases) within

the mosquito responsible for the detoxification or sequestration of insecticides before they can have a toxic effect on the mosquito (Strode, et al., 2014). Therefore, compared to a pyrethroid-only net, a pyrethroid-PBO net should lead to increased killing effect on malaria vectors that express such resistance mechanisms.

In the light of pyrethroid resistance, the long-wait for novel insecticides and environmental concerns with the testing of novel chemistries, there is a call for the removal of insecticides in LLINs. Proposers argue that mosquito nets without insecticides can provide personal and community protection when used alongside other interventions and technologies (Paaijmans & Huijben, 2020).

Indoor Residual Spraying (IRS)

This is the application of a residual insecticide on resting surfaces of potential malaria vectors, such as internal walls of houses, eaves and ceilings of houses or domestic animal shelters, where vectors might come in contact with the insecticide (WHO, 2019-Vector control guidelines). When implemented correctly, IRS has been shown to be a powerful vector control intervention (also regarded as a core vector intervention tool by WHO), reducing adult mosquito density and longevity and thus malaria transmission (WHO, 2019-Vector control guidelines). Insecticide formulation for IRS fall under three main modes of actions: a) sodium channel modulators (pyrethroids and organochlorines), b) acetylcholine inhibitors (organophosphates and carbamates) and c) nicotinic acetylcholine receptor competitive modulators (neonicotinoids) (Feachem, 2018). For optimal effectiveness, residual efficacy needs to continue for at least 3 months after application of the insecticide to the substrate (WHO, 2017). For IRS to be considered an appropriate intervention, the majority of the vector populations have to be endophilic, endophagic, and susceptible to the insecticide used. Further, people at risk of malaria

transmission have to mainly sleep indoors at night and malaria transmission has to be such that the population can be protected by one or two rounds of IRS per year. To avoid high transportation and logistical costs, structures to be sprayed should not be scattered over wide areas (WHO, 2019-Vector control guidelines).

Recent review indicates that end-users of IRS modify their wall surfaces post-spraying and this has the potential to reduce actual IRS coverage, effectiveness and impact. Clear guidelines are needed to monitor IRS acceptability and/or coverage both before and after spraying for the monitoring and evaluation of malaria programmes (Opiyo & Paaijmans, 2020).

Larval source management (LSM)

This involves the management of water bodies that are potential larval habitats for mosquito vector populations in order to prevent the completion of development from immature stages (eggs, larvae, pupae) to adult mosquitoes (WHO, 2019-Vector control guidelines). Four major LSM approaches implemented for malaria vector control include: a) **Habitat modification**: which involves a permanent alteration to the environment such as land reclamation (Raghavendra, et al., 2011). b) **Habitat manipulation**: involves a recurrent activity to discourage mosquito development such as flushing of streams (Raghavendra, et al., 2011; 'WHO, 2019-Vector control guidelines). c) **Larviciding**: involves the regular application of biological or chemical insecticides to water bodies to kill mosquito larvae, such as *Bacillus thuringiensis* serovariety *israelensis*, *Bacillus sphaericus*; and botanical larvicides (Benelli & Beier, 2017). d) **biological control**: the introduction of natural predators into water bodies to help suppress mosquito larval populations. Mosquito larval populations can be controlled by a number of aquatic predators including cyclopoid copepods, *Toxorhynchites* mosquitoes, backswimmers,

water bugs, fishes, crabs, tadpoles, and odonate young instars (Benelli & Beier, 2017; Bowatte, et al., 2013; Kalimuthu, et al., 2014).

These LSM approaches are regarded as supplementary vector intervention tools to be implemented in synergy with IRS and LLINs (WHO, 2019-Vector control guidelines). As a general recommendation, environmental management (habitat modification and manipulation) should where feasible, be implemented as the primary strategy to reduce the availability of mosquito larval habitats. Application of chemical and/or biological larvicides to water bodies for malaria prevention should be supplementary and used in areas where high coverage with a core intervention has been achieved. For cost effective applications, aquatic habitats should be few, fixed and easily located and accessed (WHO, 2019-Vector control guidelines). To achieve the ambitious goal to reduce malaria burden by 90% by 2030 as outlined in the Global Technical Strategy for Malaria 2016-2030, innovation is required (WHO, 2015). Some novel (such as Gene Drive Technology) concepts for malaria vector control that are currently under research and development are discussed below. Also discussed below are older vector control concepts such as Sterile Insect Technique (SIT) that are being revisited and improved by researchers in the light of increasing insecticide resistance to provide additional alternatives to the use of chemical insecticides or reduce the human, environmental or non-target organism interactions with chemical insecticides, especially in tackling outdoor malaria transmission where LLINs or IRS are not effective.

Use of ivermectin for malaria vector control

Ivermectin has a promising potential for use in reducing malaria transmission complementing local vector control programmes (Chaccour, et al., 2017). It is an anti-parasitic medicine that has been widely employed in single-dose community programmes

for the control of *Onchocerciasis*, lymphatic filariasis, strongyloidiasis and scabies (Merck & Co, 2009). Ivermectin has been licensed for human consumption for almost 30 years and its safety has been accessed in over 70 trials (Chaccour, et al., 2017). Ivermectin acts as a feed-through insecticide; killing blood-feeding *Anopheles* mosquitoes that feed on the human host with residual traces of ivermectin left in the bloodstream following treatment with a standard oral dose of ivermectin (Chaccour et al., 2013; Foy, et al., 2011). This endectocidal property of ivermectin makes Mass Drug Administration (MDA) with ivermectin a potential tool to reduce malaria transmission by increasing the mortality of malaria vectors biting treated individuals. Such interventions will be particularly effective on those mosquitoes only partially affected by LLINs and IRS due to behavioural or physiological resistance, hence, covering any spatial and temporal gaps left by core vector control interventions (Chaccour et al., 2013; Foy, et al., 2011).

The mechanism of action of ivermectin in invertebrates (including mosquitoes) involves the blocking of synaptic transmission by binding to glutamate-gated chlorine (GluCl) channels in nerve and muscle, resulting in hyperpolarisation, paralysis and death (Merck & Co, 2009; Meyers et al., 2015). The recently characterised GluCl in *An. gambiae* s.l. are predominantly expressed in some organs involved in motor and sensory systems, which explains the paralytic and other effects (reduced fertility, knockdown, lesser flight performance and reduced biting) of the drug on mosquitoes, even at sub-lethal concentrations (Butters et al., 2012; Gardner, et al., 1993; Kobylinski et al., 2010; Meyers et al., 2015). Several studies have shown that ivermectin might inhibit *Plasmodium* sporogony and could have an effect on liver schizonts as seen in vitro and confirmed in a mouse model (Da Cruz et al., 2012; Kobylinski, et al., 2012). In *An gambiae* s.l. laboratory populations, the gene for the GluCl can be expressed in four isoforms, with only one isoform being insensitive to ivermectin. There is little knowledge

on the expression of these channels in wild mosquito populations. There has been no report of resistance to ivermectin in mosquitoes and reduced fertility reported in mosquitoes ingesting sub-lethal doses of the drug could help delay the possibility of resistance (Gardner et al., 1993).

Although ivermectin has been used as an oral drug for treating human diseases for over three decades, for applications as a vector control measure, further safety evaluation is needed when used at higher doses or with longer exposure and for vulnerable groups such as pregnant women and children under 15kg (Chaccour, et al., 2017). Additionally, its safety when used in combination with antimalarial drugs and other drugs commonly used in malaria endemic areas such as antiretrovirals, tuberculosis drugs and other antihelminthics will need to evaluated (Chaccour, et al., 2017).

Plant-based substances with insecticidal and repellent effects.

Eco-friendly insecticides being developed to serve as an alternative to chemical insecticide in mosquito control include plant-based essential oils (EOs), which have shown high effectiveness and low toxicity on non-target organisms (Pavela & Benelli, 2016). Further studies on encapsulation of EOs, will allow for their persistence and lasting efficacy, making them commercially viable as a replacement to traditional chemical larvicides (Pavela, et al., 2016). EOs have also been shown to be effective deterrents of oviposition against malaria-transmitting mosquitoes and research is ongoing to improve formulation for commercialization (Prajapati, et al., 2005). Due to their chemical instability, volatility, low water solubility and oxidation, EOs are limited in their use as alternative vector control tools. Incorporating EOs in nano-formulations could provide a solution to this problem. Nano-solutions have a high dissolution rate,

dispersal uniformity, increased water solubility and bioavailability on application (Pavela & Benelli, 2016).

Additionally, plant-borne compounds have been used to synthesise insecticidal gold nanoparticles (AuN) using different plants and fungi that are effective against mosquito larvae at very low dosages (Naresh Kumar et al., 2013; Soni & Prakash, 2012) (One study even demonstrated that application of AuN biosynthesised using *Cymbopogan citratus* (lemon grass) against *An. stephensi* larvae improved predator efficiency in *M. aspericornis* in a copepod based vector control programme (Murugan et al., 2015).

For many poor African communities, the use of mosquito repellent plants is the only efficient vector control method available to them. The preservation of the ethnobotanical knowledge of these plants previously passed orally from one generation to the other is an area that requires more research. Three major methods applied by the locals for the use of these plants are: production of repellent smoke from plants, hanging plants inside the house or sprinkling leaves on the floor, the use of plant oils or juices. There is a need to further investigate and preserve this knowledge for the future generation (Pavela & Benelli, 2016; Pavela et al., 2016).

The eave tube concept

The house and the peri-domiciliary domain are closely linked to malaria transmission; up to 70% of infectious disease transmission occurs indoors (WHO, 1997). It is therefore unsurprising that the core malaria interventions (LLINs and IRS) are house-based. African housing is currently undergoing changes in design with traditional huts being replaced with modern materials. This presents an opportunity to integrate malaria vector control measures such as the ‘eave tube concept’ into the new housing designs (Knols et

al., 2016). The eave tube concept involves the modification of ventilation gaps under the eaves of houses in rural African societies for the control of mosquito populations (Benelli & Beier, 2017). Plastic tubes containing contact insecticide are inserted under the roofline of houses with the rest of the opening sealed (Figure 1.8).

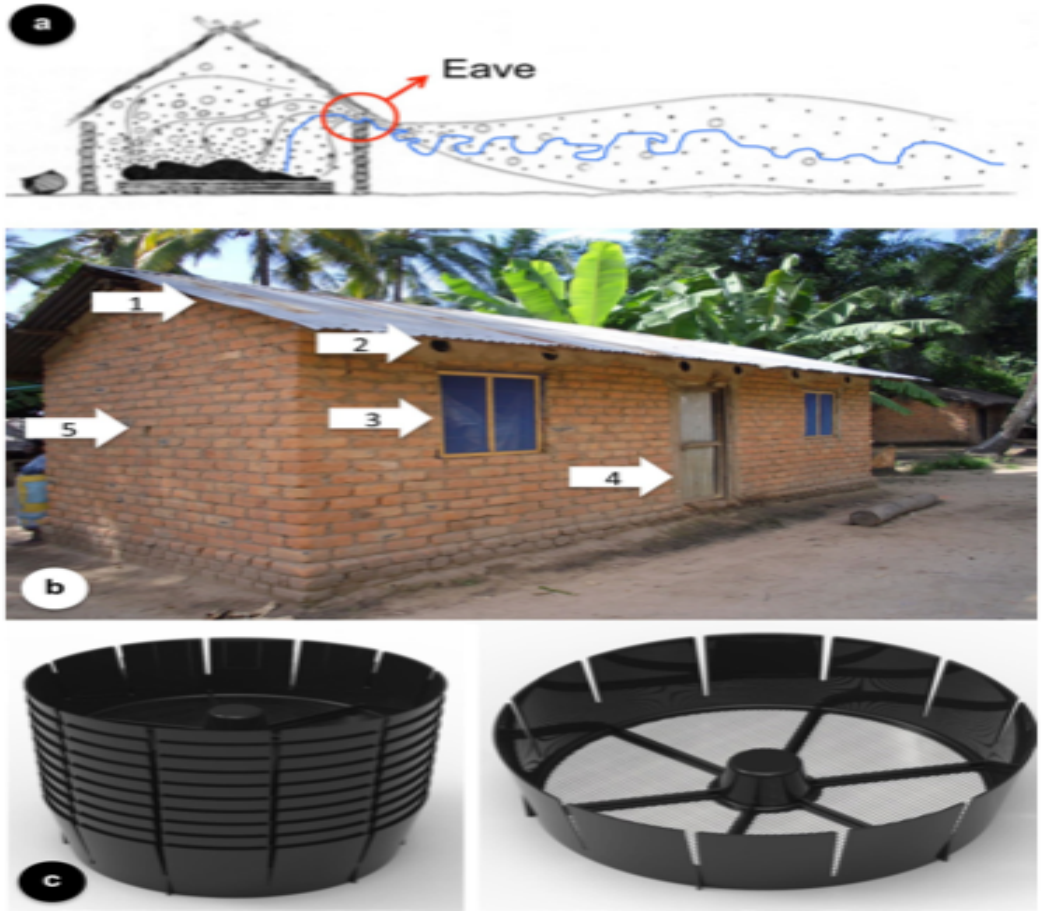

Figure 1.8: The eaves concept. **a** African anophelines fly up-winds in host odour plumes (blue line) and enter the house through the gap between the roof and the walls- the eave (red circle). **b** A house in southern Tanzania fitted with eave tubes and rendered mosquito proof through fitting of mosquito screening and sealing of the eaves. The house modifications comprise: 1 closing the eaves, 2 installation of eave tubes, 3 fitting of window screening, 4 repairs of the door (where necessary), and 5 closing of cracks and holes. **c** Eave tube inserts fitted with insecticide-treated nets that fit inside PVC pipes (Knols et al., 2016).

Mosquitoes are attracted to the house by the odour of the human host and come in contact with insecticide (Knols et al., 2016; Sternberg et al., 2016). This "attract and kill" method of insecticide delivery will complement IRS and LLINs because: it minimizes human

contact with insecticides, allows for the application of novel insecticides and even a combination of insecticides, reduces the amount of insecticides applied and is an environmentally safe manner of insecticide application, does not compromise the comfort of house occupants, operates passively without active engagement from house occupants, operates without electricity or mosquito attractants and is easy to mass-produce and install (Knols et al., 2016; Waite, et al., 2016).

Attractive Toxic Sugar Baits (ATSB)

Both male and female mosquitoes use plant sugar as an energy source (Manda et al., 2007). *An. gambiae* s.s females exhibit a discriminatory preference for plants containing high glucose and fructose. Successful feeding from such plants correlates with higher survival and egg laying rates in this species (Manda et al., 2007). The application of ASTB for malaria vector control takes advantage of this behaviour to control mosquito populations by using the combination of a concentrated sugar-based food source, an olfaction stimulant and an oral insecticide to lure and kill mosquitoes at a bait station (Beier, et al., 2012; Müller, et al., 2010). Although ASTB has mostly been successfully implemented outdoors with both male and females feeding on them, several studies have demonstrated that indoor usage of ATSB has the potential to supplement mosquito nets for controlling *Anopheles* mosquitoes (Allan, 2011; Beier et al., 2012; Stewart et al., 2013). The method has been described as being highly effective, target specific and environmentally friendly (Benelli & Beier, 2017).

Insecticide-treated clothes

Research is ongoing to improve the application of insecticides to clothing or other items for protection against insect vectors. Insecticide-treated clothing has long been used by

the military and in recreational activities for personal protection against bites from a variety of arthropods such as ticks, chigger mites, sandflies and mosquitoes (U.S. Environmental Protection Agency, 2009). Permethrin and the insect repellent DEET are the commonly used active ingredients (AI) to treat clothing either by home or factory dipping (Siler-Marinkovic, et al., 2006). New technologies for impregnating clothing with insecticides and repellents such as microencapsulation and polymer coating are now available. These new technologies may prolong the activities of insecticides on clothing, and help overcome the inevitable reduction in efficacy that results from washing, ultraviolet light exposure and the normal wear and tear of the fabric (Banks, et al., 2014; Faulde, et al., 2009; Faulde & Uedelhoven, 2006; Siler-Marinkovic et al., 2006).

Although the use of microencapsulation and polymer coating technologies to impregnate clothing with permethrin are more expensive than dipping, manufacturers claim that their efficacy lasts up to 70 and 100 washes respectively (Faulde & Uedelhoven, 2006; Vaughn & Meshnick, 2011). These new technologies also provide the added benefits of allowing for a specified rate of release, lower absorption rates into the skin, and an additional environmental benefit by reducing the impact of washing insecticidal treated clothing (Banks et al., 2014; Rossbach, et al., 2010; Siler-Marinkovic et al., 2006)

Spatial repellents (SR) to reduce human-vector contact

The overdependence on pyrethroids in both public health and agriculture, the resultant development of insecticide resistance and the poor understanding of vector ecology has limited the efforts to reduce malaria burden with focus on LLINs and IRS (Benelli & Beier, 2017). Broadening the scope of active ingredients (AI) discovery to include screening criteria that allow for the identification of compounds that exploit behavioural

modifications as a means of disease reduction is required to mitigate insecticide resistance and maximise the limited arsenal of AIs currently available (WHO, 2019-Vector control guidelines). Spatial or area repellents (also known as deterrents) are chemicals that work in the vapour phase to prevent vector-human contact by disrupting the normal behavioural patterns of the mosquito vector within a designated area or safe zone, thus making the space unsuitable for the insect (Figure 1.9). This concept of the use of SRs to discourage an arthropod from entering a space occupied by a human host thus reducing human-vector encounters, resulting in the elimination or reduction of the risk of pathogen transmission to either the insect or humans has been demonstrably effective (Grieco et al., 2007; Hill, et al., 2007; Killeen & Smith, 2007; Lindsay, et al., 2002; Ogoma et al., 2010; Galbadon, 1949; Roberts, et al., 2000).

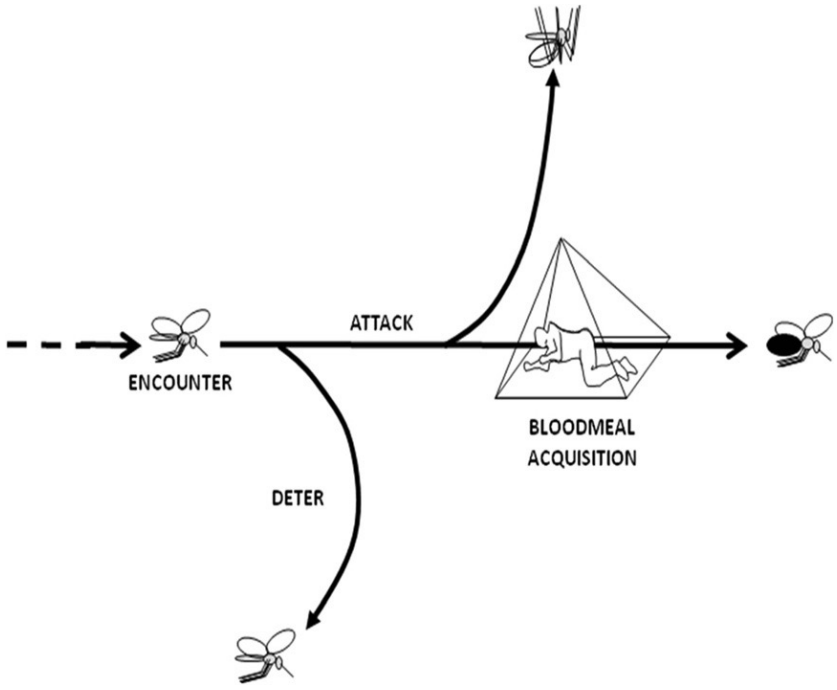

Figure 1.9: Spatial repellency concept: the fundamental choice between killing mosquitoes and repelling them (Achee et al., 2012).

Multiple rationales supporting the use of sub-lethal AIs over conventional lethal AIs include: 1. delay the onset of insecticide resistance for AIs used in LLINs and IRS by minimising the intensity of selection pressure from contact mediated toxicity mechanisms as well as the potential reduction of toxic effect on humans and non-target organisms; 2. effectiveness for outdoor protection, an attribute where ITNs and IRS have little impact; 3. usefulness in addressing other components of vector behaviour such as pre, during, and post-host seeking behaviours- these are critical behavioural sequences that can be disrupted to prevent blood-feeding which is necessary for disease transmission and thereby strengthen integrated vector control strategies; 4. employability against multiple vectors, behaviours and species (not limited to species that rest and feed indoors); 5. usefulness against economically important insects especially agricultural pests, where market forces will drive the cost of AI discovery and development (Achee et al., 2012).

Other advantages of the use SRs for malaria vector control include long-term effects on mosquito life histories that can reduce malaria transmission by forcing *Anopheles* mosquitoes to either feed on non-human hosts or to search more broadly for alternative blood and oviposition sites, thereby reducing vector survival, feeding frequency and reproductive rates (Killeen & Smith, 2007). Vector populations that survive SRs show permanent or semi-permanent disruption of host seeking and blood feeding behaviour (Hao, et al., 2008). It is also likely that the longer a vector remains exposed to harsher and more demanding outdoor conditions (greater risk of predation, physiologically stressful conditions, excessive energy expenditure during host seeking or in identifying resting and oviposition sites), the more likely it will die (Griffin et al., 2010; Killeen & Smith, 2007). This reduction in host-contact/feeding success could ultimately lead to reduced overall numbers and survival of older mosquito populations that transmit mature infectious stage parasites, thereby suppressing transmission at community level

without resort to adulticidal strategies (Killen & Smith, 2007; Achee et al., 2012). SRs could represent a subtle method of achieving malaria vector mortality and population reduction without chemically induced selection pressures, potentially increasing the sustainability of existing and novel chemical interventions (Achee et al., 2012).

SRs can either be delivered as a single consumer product or integrated into a community-based vector control programme to enhance IRS and ITNs interventions. Traditional consumer product where spatial repellents have been used include, mosquito coils, aerosols, nets and curtains. SRs can also be integrated innovatively into consumer products that increase the market value of such products by combining end-user needs/wants with vector control, thereby ensuring compliance and sustainability. For instance, products that provide utility or beautification such as decorative mats and items used for interior decoration of homes can be imbedded with SRs (Achee et al., 2012). SR products could also be adapted to exterior areas of houses or within peri-domestic environments to cover for outdoor-biting vectors or spaces where physical structures are absent, to tackle residual transmission (Ferguson et al., 2010).

Transgenic approaches

Transgenic approaches involving the use of gene drive systems has the potential to provide revolutionary solutions to key public health issues like malaria (Champer et al., 2016; Hamilton, 1967). Most gene drive systems are based on naturally occurring 'selfish' genetic elements that function by increasing in frequency with each generation even without conferring a fitness advantage upon their host, thus forcing non-Mendelian inheritance patterns (Alphey, 2014; Alphey et al., 2013; Burt, 2014; Hurst & Werren, 2001; Sinkins & Gould, 2006; Werren, 2011). The introduction of novel genes into the genome of mosquito species using the RNA guided CRISPR-Cas9 (clustered regularly

interspaced short palindromic repeats-CRISPR-associated 9) endonuclease system presents many possibilities for transgenic vector control strategies (Champer et al., 2016; Mali, et al., 2013). The CRISPR-Cas9 system which can be used in conjunction with small guide RNAs (gRNAs) to cleave specific sequence of any target genome has been adapted to modify the genes of mosquitoes (Basu et al., 2015; Gantz et al., 2015; Hammond et al., 2016; Kistler, et al., 2015). The Cas9 endonuclease has several important characteristics that make it attractive for engineering gene drives: first, it can be utilized to induce cleavage of DNA followed by homology-directed repair (HDR) from a co-delivered DNA cassette, thus enabling precise genomic manipulation (Gantz & Ethan, 2015); second, it has the capacity to cleave mRNA, thus broadening its potential for use in developing gene drives (O'Connell et al., 2014; Price, et al., 2015); third, it can be multiplexed for simultaneous Cas9-mediated targeting of multiple sequences, permitting increased cleavage rates and reduced evolution of resistance alleles (Gao & Zhao, 2014; Xie, et al., 2015); and finally, available bioinformatics tools can be used to minimise off-target cleavage and improve species specificity (Xie, et al., 2014).

Transgenes can potentially be used to overcome the evolutionary disadvantages of certain desirable traits (e.g. refractory/resistant to *Plasmodium*) and thus spread such traits more quickly throughout wild populations or suppress mosquito populations altogether (Alphey, 2014; Alphey et al., 2013; Burt, 2014; Sinkins & Gould, 2006; Werren, 2011). However, traditional transgenes that mediate refractoriness may have a fitness cost on the insects that carry them placing them at a competitive disadvantage with wild populations which are large and dispersed over wide areas (Champer et al., 2018, 2017). To overcome this challenge, CRISPR methods have been used to accelerate the development of Homing-based Gene Drives (HGDs) in *An. gambiae* s.l. mosquitoes that can spread linked genes that mediate disease refractoriness into wild populations at

greater than Mendelian frequencies (Figure 1.10) (Champer et al., 2018, 2017; Gantz et al., 2015; Hammond et al., 2016; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Li et al., 2020). Several studies have demonstrated that the release of HGDs linked with effector genes that inhibit mosquito pathogen transmission may lead to the replacement of disease-susceptible mosquitoes with disease-resistant counterparts, thereby reducing pathogen transmission (Buchman et al., 2019, 2020; Isaacs et al., 2011; Jupatanakul et al., 2017). Other studies have demonstrated that HGDs targeting genes that affect the fitness of female mosquitoes could also lead to gradual population declines and even elimination (Kyrou et al., 2018; Windbichler et al., 2011; Windbichler, et al., 2008).

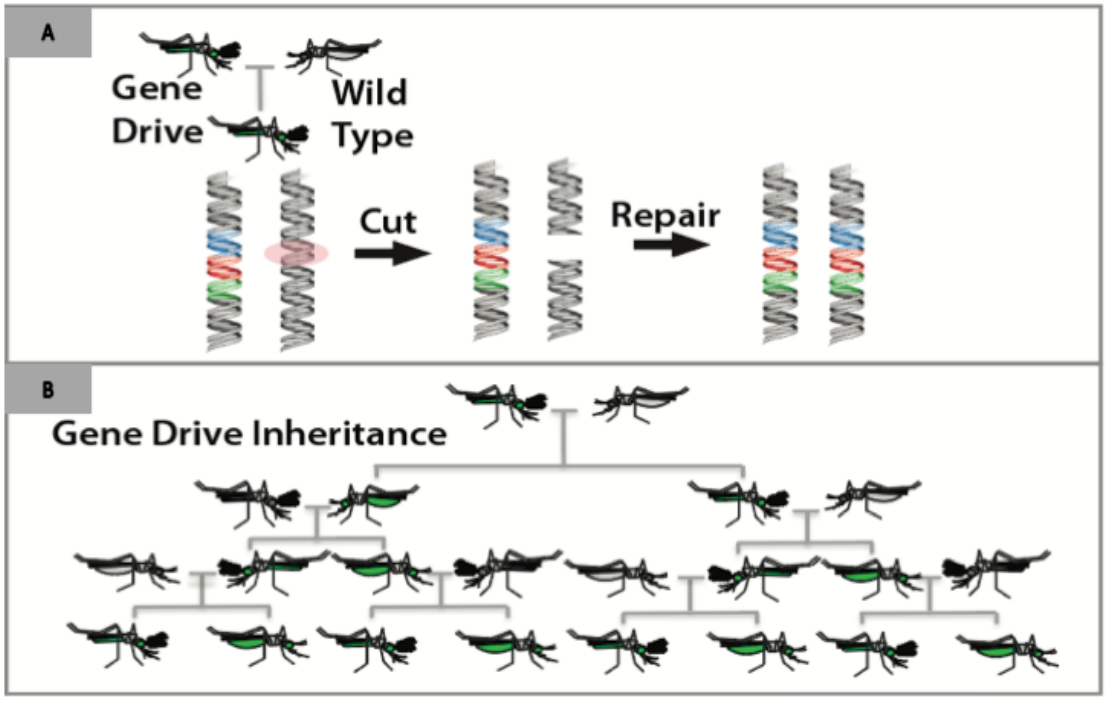

Figure 1.10: Expression of Homing-based Gene Drive (HDG) transgenic method. (A)-HDGs convert heterozygotes to homozygotes using a cut/repair process. (B) green denotes individuals with the gene drive, grey denotes wild type mosquitoes (modified from Akbari, 2020).

Transgenesis thus has potential for mosquito vector population suppression, and modification, providing an environmentally friendly, sustainable and self-perpetuating

approach for malaria prevention and control and in the future could replace core vector interventions (IRS and LLINs).

Wolbachia for malaria vector control

They are a group of endo-parasitic, vertically transmitted, maternally inherited bacteria, present in a wide range of arthropods and nematodes (Serbus, et al., 2008). Originally discovered in mosquito populations in 1924, *Wolbachia* have the ability to selfishly promote their existence in subsequent generations by manipulating and interfering with their host's reproductive capabilities (Hertig & Wolbach, 1924). Certain strains of *Wolbachia* cause reproductive abnormalities in various insect hosts such as cytoplasmic incompatibility, thelytokous parthenogenesis, the feminisation of males and male mortality (Burt, 2014). In some cases, *Wolbachia* infections have resulted in speciation, reduced vector competence and the shortening of the host's lifespan (Bian et al., 2013a; 2013b; Blagrove, et al., 2012; McMeniman et al., 2009; Kambris & Al, 2009; Moreira et al., 2009; Rasgon, et al., 2009; Sinkins, 2013; Werren, 2011). Experimental infection of *Ae. aegypti* with the wMel strain of *Wolbachia* known to significantly inhibit malaria and dengue transmission has been successful in invading wild *Aedes aegypti* populations in Australia, despite the mild fitness cost associated with the infection (Hoffmann et al., 2011; Walker et al., 2011).

The use of Wolbachia to control mosquito vector of malaria is promising but these intracellular parasites are yet to be genetically engineered. However, the RNA guided endonucleases provide flexibility that can allow for the development of improved *Wolbachia* strains with enhanced disease refractoriness and reduce host fitness cost, which will allow for more rapid propagation of desirable traits into mosquito populations (Champer, et al., 2016)

Sterile Insect Technique (SIT)

Dating back to the mid-1930s, SIT involves the mass rearing and release of large numbers of sterile males to control and eradicate insect populations (Bushland et al., 1955; Klassen, 2005). Sterile males mate with wild females leading to a reduction in the reproductive output of females which result in depletion or elimination of the target population (Burt, 2014; Bourtzis, et al., 2016; Lees et al., 2015). This method of insect pest control was largely abandoned partly because traditional SIT methodologies relied on sterilization via radiation which destroys the DNA of the sterilised organism and substantially reduces its overall fitness and mating competitiveness (Kandul et al., 2019). A comeback to SIT has been necessitated by the pressure from mosquito-borne disease burden and the increase of insecticidal resistance. The possibilities of using modern biotechnology to sterilise mosquitoes or alter them have also renewed interests in SIT as a viable vector control tool (Lees et al., 2015). To overcome these challenges (fitness cost and mating competitiveness), microbe-mediated *Wolbachia*-based incompatible insect technique (IIT) was introduced to induce fertility (Panagiotis & Bourtzis, 2007; Sinkins, 2004). Other SIT-like systems that have also been used to control insect pest populations include release of fertile male insects carrying a dominant lethal gene that specifically kill females (female-specific RIDL) and autosomal-linked X-chromosome shredders (Fu et al., 2010; Thomas, et al., 2000; Windbichler et al., 2008)

To further improve the effectiveness of SIT, a novel CRISPR-based precision-guided sterile insect technique (pgSIT) has been developed using *Drosophila* as the model insect (Kandul et al., 2019). The pgSIT technique mechanistically relies on a dominant genetic technology that enables simultaneous sexing and sterilization of mosquitoes to facilitate the release of eggs into the environment, resulting in only male progeny that are 100% sterile (Kandul et al., 2019). This was achieved by mass producing

two strains of *Drosophila*, one expressing the CRISPR-associated protein 9 (Cas9) endonuclease and the other expressing two guide RNAs (gRNAs). One gRNA targeted a female viability gene and the other, a male fertility gene. Following the crossing of the two strains of *Drosophila*, the only surviving progenies were 100% sterile males which can be directly deployed (Figure 1.11) (Kandul et al., 2019). This development is particularly efficient for field applications as the release of eggs eliminates the difficulty of manual sexing and sterilisation of males, thus saving time and effort and increasing scalability (Akbari, 2020). There are ongoing efforts to transfer this technology to mosquito control (Kandul et al., 2019).

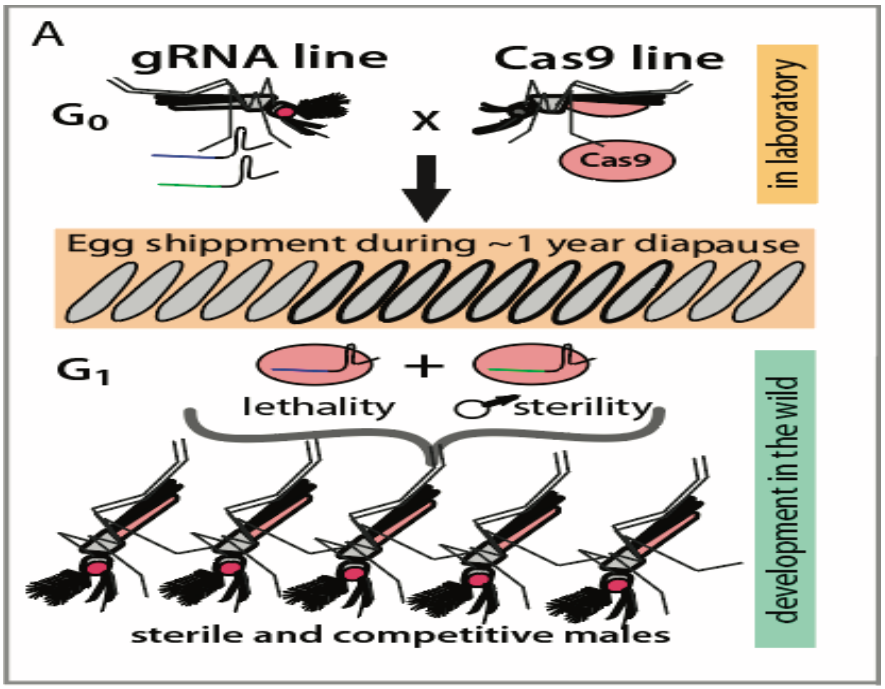

Figure 1.11: Precision guided sterile insect technique (pgSIT). pgSIT relies on mass rearing two separate strains: the first expresses two guide RNAs (gRNAs) designed to target female viability and male fertility genes, the second expresses CRISPR-associated protein 9 (Cas9) endonuclease. When crossed, the only surviving progeny are sterile males, which can be repeatedly released as eggs into the environment, resulting in population suppression, as they compete with wild males for females (modified from Akbari, 2020).

The Joint FAO/IAEA Programme and their collaborators have been at the forefront of the research for repackaging SIT for implementation, fuelled by enthusiasm from many countries for the development of SIT against the mosquito. Other aspects of the "SIT package" undergoing development include mass-rearing equipment, diet and rearing protocols for *Anopheles* and *Aedes* species (Lees et al., 2015). To ensure adequate performance and competitiveness after release, quality management of sterile male mosquitoes is at the front-burner of ongoing research in mosquito SIT development (Lees et al., 2015)

1.4 Processes of divergent ecological speciation between *An. gambiae* s.s. and *An. coluzzii.*

1.4.1. Genetic basis for speciation amongst the sibling species *Anopheles gambiae* s.s. and *coluzzii.*

Speciation is the process by which new living things are thought to develop from existing ones during evolution (Feder, et al., 2012). This process promotes biodiversity, which is positive in the context of environmental conservation, however, for the epidemiology of infectious diseases, speciation creates a complex that challenges ongoing disease control measures (Lehmann & Diabate, 2008). Three major geographic modes of speciation exist in nature, they include allopatric (geographically isolated by e.g. physical barriers such as mountains and subsequently develop physical and genetic differences in their populations due to varied selective pressures, genetic drift and mutations), parapatric (partial separation leading to reduced fitness due to reduced heterogeneity in their gene pool and subsequent adaptations that may reduce interbreeding) and sympatric (occupying same geographical space yet diverging) modes of speciation (Butlin et al., 2012).

Understanding the genomic process behind sympatric speciation, which implies the evolution of new species from a single ancestral species within the same geographical region is fundamental to our understanding of biodiversity and particularly those of disease vectors (Aboagye-Antwi et al., 2015; Butlin et al., 2012; Feder et al., 2012). The discovery of distinct genomic signatures can aid in the understanding of the genetic and ecological conditions that facilitate the emergence and divergence of two gene pools from an originally panmictic (randomly mating) population (Aboagye-Antwi et al., 2015; Butlin et al., 2012; Feder et al., 2012). For sympatric speciation with gene flow to occur between populations, it is necessary that divergent selection acting on locally adapted genes overcomes the homogenising effects of migration and recombination (Butlin et al., 2012; Feder et al., 2012). Genomic features such as chromosomal inversions and peri-centromeric regions that suppress recombination and link together genes for pre-mating isolation and ecological adaptation genes are predicted to promote sympatric speciation (Coyne & Orr, 2004; Feder et al., 2012; Reiseberg, 2001; Servedio, 2009).

The sibling species were formally known as the 'M' (now *An. coluzzii*) and 'S' (now *An. gambiae* s.s.) molecular forms of *An. gambiae* s.s. in reference to diagnostic genetic differences in their ribosomal DNA regions (Coetzee et al., 2013). They co-occur over large areas of West Africa and do not exhibit intrinsic post-mating barriers to reproduction (Diabaté, et al., 2007; Tripet, et al., 2005). The sibling species are morphologically similar and share the same resources including vertebrate hosts, adult resting sites and freshwater and larval habitats (Reidenbach et al., 2012). Across much of their sympatric range, they are uniquely divergent as a result of strong assortative mating with occasional hybrids and resultant low levels of genetic introgression (>1%) leading to speciation (Dabire et al., 2013; Lee et al., 2013; Tripet et al., 2001; Weetman, et al., 2012). Adults of both species have similar feeding and resting habits and mating occurs

at dusk in swarms. Swarm site segregation is thought to contribute to assortative mating, but the occurrence of mixed swarms at various frequencies points towards additional conspecific recognition mechanisms such as flight tones (Dabire et al., 2013; Diabaté et al., 2006; Gibson, et al., 2010; Pennetier, et al., 2010). There are however large hybrid pockets in the westernmost extreme of the *An. gambiae* s.l. range, specifically in the coastal countries of Guinea Bissau (>20%), The Gambia (7%) and 3% in Senegal, with high levels of genetic introgression (Caputo et al., 2011; Lee et al., 2013; Marsden et al., 2011; Weetman et al., 2012).

On the genetic basis of sympatric speciation, several ground-breaking studies have shown that the sympatric speciation in these two incipient species probably involved the divergence of a few 'islands of divergence' that possibly contain clusters of speciation genes and are located in areas of low recombination (Turner, et al., 2005; White, et al., 2010). These putative 'islands of speciation' include 3 pericentromeric islands of divergence located on the X, 2L and 3L chromosomes as well as smaller islands located in the vicinity of inversion breakpoints (Turner, et al., 2005; White, et al., 2010). In central West Africa, perfect linkage disequilibrium between the X, 2L and 3L islands was found in samples from sympatric populations of *An. coluzzii* and *An. gambiae* s.s. (White et al., 2010). The result suggests a pattern of speciation (between *An. gambiae* s.s. and *An. coluzzii*) where there is very low gene flow between the sibling species and the possibility that the pericentromeric islands of divergence were merely 'incidental rather than instrumental' to the speciation process (White et al., 2010). This pattern was further reinforced by a subsequent study that suggested divergence at many loci across the genome and a more advanced stage of sympatric speciation (Reidenbach et al., 2012). However, other recent studies have revealed a different genomic differentiation pattern between the sibling species, showing that the linkage disequilibrium between the

pericentromeric islands breaks down to various degrees in areas with higher introgression (Caputo et al., 2011; Lee et al., 2013). Considering both patterns together, the comparative genomics data would therefore support a model of genomic divergence in which pericentromeric divergence islands play a major role in speciation in the face of varying levels of gene flow (Aboagye-Antwi et al., 2015; Lee et al., 2013; Weetman et al., 2012). A more recent study on laboratory recombinant strains of the sibling species show close associations of assortative mating genes with the X-island of speciation, thus supporting the hypothesis that pericentromeric regions can create linkage disequilibrium and hence, protect associations between genes of pre-mating isolation and ecological adaptation and facilitate the onset of sympatric speciation (Aboagye-Antwi et al., 2015; Coyne & Orr, 2004; Feder et al., 2012; Servedio, 2009). This further lends support to the model of speciation involving pericentromeric suppression in these sympatric incipient species (Aboagye-Antwi et al., 2015)

1.4.2. Water quality of larval breeding sites of the sibling species, larval habitat divergence and eco-speciation .

Immature life stages of mosquitoes are aquatic, occurring in varying water qualities depending on mosquito species (Mamai, et al., 2016). In nature, larvae of both species are filter feeders, depending on nutrients in the water for survival. Although larval stages may be collected at the same habitats, the sibling species differ markedly in their choice of larval habitats; with *An. coluzzii* being associated with longer-lasting breeding sites resulting from human activities . In West African savannahs, these tend to be irrigated habitats such as rice fields, reservoirs, abandoned mines and quarries, and drainage ditches. In the central African rainforest areas, they tend to be urban pools, which are sometimes polluted with organic waste. *An. gambiae*, on the other hand, usually reaches

its population peak during the rainy season, preferring more ephemeral habitats that are rain-dependent (Della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008). This habitat segregation amongst the larval stages of these species is assumed to be a driver of speciation (ecological speciation) among both species (Costantini et al., 2009; Gimonneau et al., 2010). Currently, the strongest evidence that adaptation to rice fields may have played such a role in the speciation of *An. coluzzii* and *An. gambiae* s.s., stems from larval transplantation experiments conducted in Burkina Faso, which showed that in the absence of predation, the sibling species outcompeted each other in their respective natural habitat. *An. gambiae* s.s. behaved as surface feeders, mainly thrashing at the water surface and foraging through floating vegetation, while *An. coluzzii* larvae mimicked benthic feeders spending a significantly greater amount of time browsing at the bottom of the container and diving more frequently than the former (Gimonneau et al., 2012). This behaviour is probably directly linked to its success in both habitats under predator pressure. In the presence of predators however, developmental success was significantly higher in *An. coluzzii* (Diabaté et al., 2008). Larvae of *An. gambiae* developed faster than *An. coluzzii* in temporary water collections when predators are few, inversely, larvae of *An. coluzzii* outcompetes those of *An. gambiae* in predator rich environments (Gimonneau et al., 2010; Tene Fossog et al., 2015). The success of *An. coluzzii* breeding in rice fields may have originated from earlier association with rice field domestication in Africa (Li et al., 2011; Reidenbach et al., 2012). Environmental changes drive speciation by introducing evolutionary novelty and emergence of adaptive variation through natural selection (Schluter, 2001).

In the laboratory, mosquitoes are routinely reared in deionized water containing little or no minerals (Suchismita Das, et al., 2007; Tchigossou et al., 2018). A good understanding of larval divergent preference between the sibling species could form the

basis to improve rearing protocols for each sibling species. Water quality used in rearing *Anopheles* mosquitoes is vital for oviposition, adult emergence, and larval survival (Akpodiete, et al., 2019; Tchigossou et al., 2018). Physiochemical parameters such as ammonia, nitrates, nitrites, and biotic factors such as the decomposition of organic matter by microorganisms will likely impact on the development and survival of *An. gambiae* s.s. and *An. coluzzii* in rearing facilities (Tene Fossog et al., 2013, 2012). With the return of "SIT against mosquitoes" to mainstream research as well as other novel genetic approaches (transgenes), there is a need to develop efficient and economical methods to produce large numbers of sterile or GM mosquitoes of high phenotypic quality (Mamai et al., 2017).

Ammonia tolerance in the sibling species

Ammonia is a by-product of biological waste production in aquatic ecosystems. It is excreted as the end-product of protein metabolism and may be toxic if allowed to accumulate (Hargreaves 1998). Ammonia levels are typically higher in more permanent and eutrophic larval breeding sites, such as rice fields which is the preferred habitat of *An. coluzzii* (Ishii, et al., 2011)

Acute toxicity studies focused on plastic responses of the sibling species to high concentrations of ammonia have been conducted and results showed higher ammonia tolerance in *An. coluzzii* compared to *An. gambiae* s.s, (Tene Fossog et al., 2013, 2012). These studies were aimed at describing the sibling species along gradients of urbanization and not on eco-speciation as a result of adaptation to rice field domestication. The concentrations of ammonia used in these studies were however far higher than what would be obtained from the pre-fertilization rice field ecosystem. To understand the impact of ammonia as an environmental trigger for selection in the rice paddy, it is

therefore important to study ammonia tolerance throughout the larval, pupal stages and its effect on adult emergence using ammonia concentrations that are typically found in the rice-field.

Bacterial communities in *An. gambiae* s.l. insectary

Microbial communities have significant ecosystem functions which include primary production, decomposition and nutrient cycling (Zak, et al., 2003). Those found in ephemeral aquatic ecosystems play important roles in the growth and development of several aquatic insects including mosquitoes (Gimnig et al., 2002; Kaufman et al., 2006). Mosquito larvae are filter feeders, feeding mainly on microorganisms and detritus (Muturi, et al., 2017), and their internal microbial communities are influenced by biotic and abiotic factors such as temperature, diet, competition, soil substrate, and predator exposure (Charan, et al., 2013). The development, survival and behaviour of mosquitoes are influenced by their microbiome throughout their life cycle (Coon, et al., 2014; Dickson et al., 2017; Onchuru et al., 2016; Ponnusamy et al., 2008). Likewise, mosquito larvae via their feeding behaviour can alter the composition and phenotypic quality of the microbial communities of their habitats (Kaufman, et al., 1999; Pernthaler, 2005; Wallace & Merritt, 2004). Other important roles played by mosquito microbiome include assistance with digestion, stimulation of immune function and providing resistance to colonisation by foreign and potentially harmful microbes and viruses (Cirimotich, et al., 2011).

For most holometabolous insects like mosquitoes, conditions of larval growth, biotic and abiotic, affect adult life-history traits (Dickson et al., 2017). The effect of bacterial communities in larval development sites on mosquito development is largely unknown. Understanding how this carryover effect works in a laboratory (insectary)

environment for *Anopheles gambiae* s.l. will have a major impact on rearing protocols. The identification of the microbial communities in mosquito larval trays characterised by stagnant water, a bacteria influenced nitrogen cycle (nitrification-denitrification) directly linked to uneaten food and metabolic by-products (ammonia) and how this affects all the life stages of the mosquito will be vital in improving rearing protocols. Mosquito survival and output for release programmes can be improved as microbial analysis of the laboratory larval environment might provide solutions to rearing challenges such as ammonia build-up, low larval survival, pupae and adult size, fecundity and male competitiveness.

Use of zeolites in *An. gambiae* s.l. insectary

Water management in mosquito insectaries to minimise the presence of ammonia and encourage beneficial microorganisms is key to achieving optimal rearing results both for small cultures and for mass-rearing facilities (HDV, 2017; Mamai et al., 2017, 2016). Zeolites are microporous crystalline aluminosilicates with chemically neutral basic honeycomb-like structures that originate from volcanic rocks (Ghasemi, et al., 2018). The chemical structure of zeolite forms a network of channels and cavities allowing easy penetration of micron-sized molecules which are filtered according to size, polarity and shape, thereby serving as an efficient filter adsorbing various substances that come in contact with it (Abdel-rahim, 2017). Zeolite has been widely used in aquaculture to improve water quality and feed quality as well as reduce the negative environmental impacts of aquaculture and improve the quality of seafood (Skleničková et al., 2020). Water quality is improved when the zeolite is used because it can adsorb ammonia, heavy metals, pesticides, smells, radioactive cations and many other toxins (Inglezakis, 2012). Average ammonia adsorption capacity is 25mg NH_4^+ /g of zeolite (Abdel-Rahim, 2017).

There is scarcity of literature on the application of zeolite's tremendous ammonia absorption value in mosquito rearing. Medium and mass-rearing mosquito facilities may benefit from the tremendous value of zeolite rock to improve mosquito yield and quality.

1.5. Aims and Objectives

In the light of insecticide resistance to pyrethroids used in LLINs and unavailability of non-transient vaccine for the malaria parasite, it is clear that additional tools and approaches are required for vector control to sustain the gains of the past and achieve global malaria eradication. Several vector control tools (such as SIT, gene drive technologies, etc) involving the mass release of mosquitoes are being developed in line with WHO's vector control research goals. To support the efforts in improving mass rearing facilities and protocols, this study investigated the larval ecological preferences of *Anopheles gambiae* s.s and *Anopheles coluzzii* in the insectary, by revealing the reaction norms of the sibling species to ammonia and minerals in their larval habitat. To improve water quality in rearing trays, the use of ammonia absorbing zeolite for larval rearing was evaluated. Further, bacteria communities in larval trays were also characterised to understand how the microbial dynamics in these trays affect mosquito development. The overall aim of this study therefore, was to elicit plastic responses in the sibling species in response to biotic and abiotic factors in their larval habitat and how this can used to improve water quality in rearing trays and thus, overall mosquito yield for mass release programmes.

The specific objectives were to:

1. investigate the effect of different water sources on the development and phenotypic quality of *An. gambiae* s.s and *An. coluzzii*.

2. assess and compare the phenotypic responses (mortality rate, larval development, pupation, and adult emergence) of *An. gambiae* s.s and *An. coluzzii* to different concentrations of ammonia under standard insectary conditions.
3. reveal reaction norms of the sibling species in their preferred larval habitat in a microcosm setting.
4. evaluate the use of zeolite for mosquito larval rearing.
5. characterise the microbial communities associated with larval rearing trays using 16S rRNA gene sequencing.
6. analyse and quantify by qPCR, the candidate bacteria species that are beneficial and detrimental to mosquito development in the insectary.

Chapter 2

General methods

Methods described here were mostly those common to more than one experimental chapters, references were made to this chapter in subsequent chapters where relevant.

2.1. Mosquito strain

The Kisumu strain of *An. gambiae* s.s., colonized over 40 years ago, from the area of Kisumu, Kenya, East Africa; 17-year-old Mopti strain of *An. coluzzii*, colonized in 2003 by the Lanzaro Laboratory (UC Davis) from the village of N'Gabacoro droit near Bamako, Mali, West Africa, and a recently-colonised 2-year-old VK3 *An. coluzzii* strain from Vallee du Khou in Burkina Faso, West Africa (supplied by IRSS, Bobo Dioulasso), were used for the experiments. The strains were maintained by the Tripet group in dedicated insectaries of the Centre of Applied Entomology and Parasitology (CAEP), Keele University, UK.

2.2. Mosquito rearing

The insectary

An insectary can be briefly defined as a room where living insects are kept and reared in a manner that yields insects of standard phenotypes that can be utilised for experimentation. At CAEP, the typical insectary was an enclosed, temperature and a humidity-controlled room used for rearing mosquito colonies. Mosquitoes were maintained at 25 ± 2 °C, relative humidity of 70 ± 5%, with a 12-h light/dark photocycle. High hygienic standards were maintained in the insectary. Bleach was used to sterilise contaminated equipment, clean the insectary weekly, and sterilise the humidifier

fortnightly to prevent the build-up of micro-organisms that may be pathological to the colonies.

Adults

Approximately 600 – 800 adults were held in 5L cages covered with netting held in place with masking tape. Sugar was provided via a paper towel soaked in 10% glucose solution, and water *via* a soaked cotton pad in an upturned bowl placed on the cage netting. Seven to eight days post-emergence, female adult mosquitoes were fed with defibrinated horse blood using an artificial feeding membrane (Hemotek feeding membrane system, Discovery workshops, Blackburn, UK). Styrofoam cups (egg cups) containing filter paper and water were placed in the cages four days post blood-feeding, to collect eggs. Following the removal of the egg cups, the cages were washed thoroughly and sterilised with bleach. Mouth aspirators were used to transfer adults from one container to another when necessary.

Figure 2.1: Mosquito cages with emerged adults and egg cup

Figure 2.2: Blood feeding using a hemotek unit and parafilm membrane

The egg cups were removed three days after they were placed in the cages and hatched larvae were transferred to clean larval rearing trays using a pipette. Unhatched eggs were immersed in water, covered and allowed to hatch.

Larvae

First instar larvae were transferred from egg cups into clean white trays with 500ml of deionized water, additional 500ml of deionised water was added on the fifth day. Larvae were fed an optimized diet of ground fish food (Tetramin, Tetra, Melle, Germany) at a rearing density of 200 larvae/litre by manual counting.

Figure 2.3: *An. gambiae* s.l. larval tray set-up in the insectary.

Pupae

Eight days following the transfer of first instar larvae to larval trays, pupae were transferred to 5l plastic cages (*c.*20.5 cm height × 20 cm diameter), covered with netting for adult emergence. The trays are washed thoroughly with only water following the completion of pupation of the population in the tray. Cages had sleeved opening for easy management of mosquitoes and accessories.

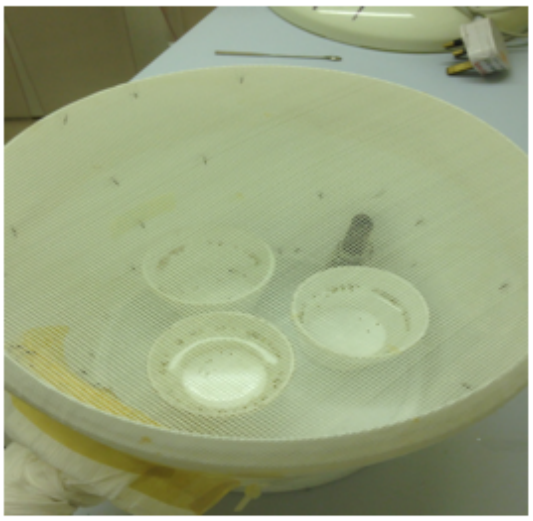

Figure 2.4: Pupae in styrofoam cups with water for adult emergence.

2.3. Mosquito DNA extraction

Adult mosquitoes to be identified were preserved for DNA extraction in 75% ethanol in collection tubes. DNA extraction was done using protocols standardised in Tripet's laboratory as follows: Laboratory bench was washed with soap and water and wiped with 75% ethanol to avoid contamination. Individual mosquitoes were placed in 1.5ml centrifuge tube, 100µl of DNAzol was pipetted into the tube and mosquitoes were ground with a pestle until no recognizable body parts were left. The mixture was centrifuged for 10 minutes at 10'000g to form pellets, following which the supernatant was transferred to a new tube. 50µl of 100% ethanol was added to the supernatant to precipitate DNA and mixed by gently shaking/inverting 5-8 times, after which it was left to incubate for 3 minutes at room temperature. The supernatant was then centrifuged for 7 minutes at 7'000g, after which the liquid was gently decanted. Further short spins may be necessary to remove excess ethanol using a pipette. 200µl of TE buffer was pipetted into the tube and set aside (in -20^0C if not to be used immediately) for PCR species identification. Latex gloves and a laboratory coat were worn throughout the procedure. DNA quantification was carried out using Nanodrop 1000 spectrophotometer to verify the amount of DNA present in the samples.

2.4. Polymerase chain reaction (PCR)

PCR is an indispensable molecular biology tool with wide applications in medicine, medical and biological research (Bartlett & Stirling, 2003). It was first described and experimentally applied by the Nobel Laurette, Kary Mullis in 1985. PCR is an amplification technique that generates millions of copies of a specific segment of DNA (amplicon) from a small quantity of starting amount (DNA template), using primers, DNA polymerase, nucleotides, and specific ions (Kralik & Ricchi, 2017).

PCR for mosquito species identification was done using SINE200 cycle (Santolamazza et al., 2008). Master mix for the reaction consisted of 20.38µl of deionized water, 2.5µl of 10x buffer, 0.5µl of DNTPs (10mM), 0.25µl of primer S200X6-1F (10pM), 0.25µl of S200X6-1R (10pM), 0.13µl of dreamtaq, giving a final volume of 24µl for one reaction. The volume prepared was proportionate to the number of samples to be identified. The master mix reagents, test samples were placed in an ice box throughout the process. 24µl of master mix and 1µl of DNA solution (sample) was placed in a 5µl PCR well (Starlab) and labelled accordingly using a PCR well chart, before running the PCR. The amplifications were carried out using a 'DNA-Engine Peltier Thermal Cycler' (BioRad). Other PCR conducted in this study followed similar protocol described above, with specific primers to amplify target regions. PCR cycles used are referenced in relevant chapters.

2.5. Gel electrophoresis

Following PCR amplification, the amplicons were visualised via gel electrophoresis. Agarose gel was prepared using 1.8g of agarose powder per 100ml of 1 x TBE buffer in a bottle. The solution was heated in a microwave until the powder dissolved. 1µl of gel red was added per 10ml agarose solution. The gel was left to cool before pouring gently into the plexiglass gel cast. Following gel electrophoresis at 70 volts for approximately 1 hour, the gel was placed in a GenSys unit to read off the bands. The PCR was said to be successful if bands of predicted size were visible on the gel (Figure 2.5).

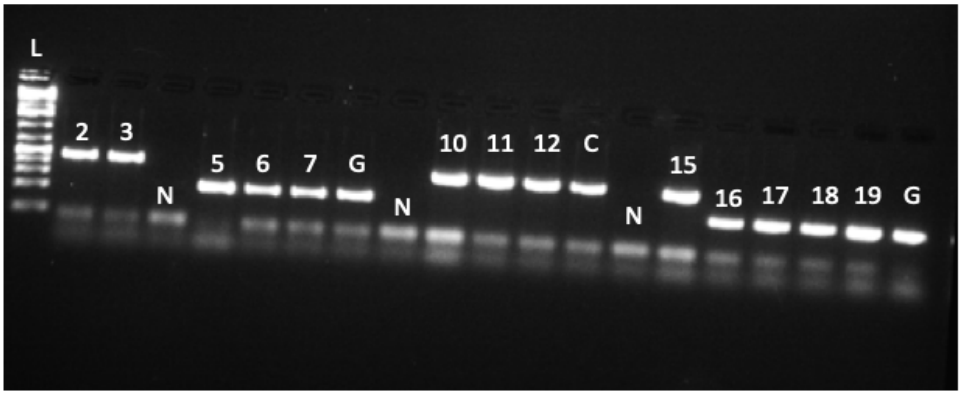

Figure 2.5: Species identification of culture samples on agarose gel (1.8%). L= 100bp DNA weight marker, lanes 2,3, 10-12, 15 = *An. coluzzii;* N= No template control; lanes 5-7, 16-19 = *An. gambiae* s.s.; G= positive control for *An. gambiae* s.s.; C= positive control for *An. coluzzii.*

2.6. Real-time polymerase chain reaction (qPCR)

Monitoring DNA amplification in real time through the tracking of fluorescence (quantitative PCR), is a substantial improvement on the utilization of PCR. It allows for the measurement of fluorescence after each PCR cycle, the magnitude of the fluorescent signal mirror the momentary amount of DNA amplicons in the sample at a definite time (Kralik & Ricchi, 2017; Kubista et al., 2006). The number of DNA ideally doubles after each PCR cycle resulting in 100% efficiency (Johnson et al., 2013; Kralik & Ricchi, 2017). At the initial cycles in a qPCR reaction, fluorescence is too low to be detected from the background, but as fluorescence intensity increases above detectable levels, it reaches a point where it corresponds to the number of template DNA molecules in the sample (Kubista et al., 2006). This point is called the quantification cycle (*Cq*) and its value allows for the determination of the absolute quantity of target DNA in a given sample in relation to a standard calibration curve derived from serially diluted samples of known concentration or copy numbers. Ideally, an amplification reaction should follow this equation: $N_n = N_0 \times (1 + E)^n$, where N_n is the number of PCR amplicons after n cycles, N_0 is the initial number of template copies in the sample, E is the PCR efficiency that can assume values from 0-1 (0-100%) and n is the number of cycles

(Kralik & Ricchi, 2017). The equation can be further simplified to $N_n = 2^n$ where one copy of the template is used in the reaction. The value of *n* is then 3.322 where PCR efficiency is 100%. In practice though, amplification efficiency varies between 90 and 100%.

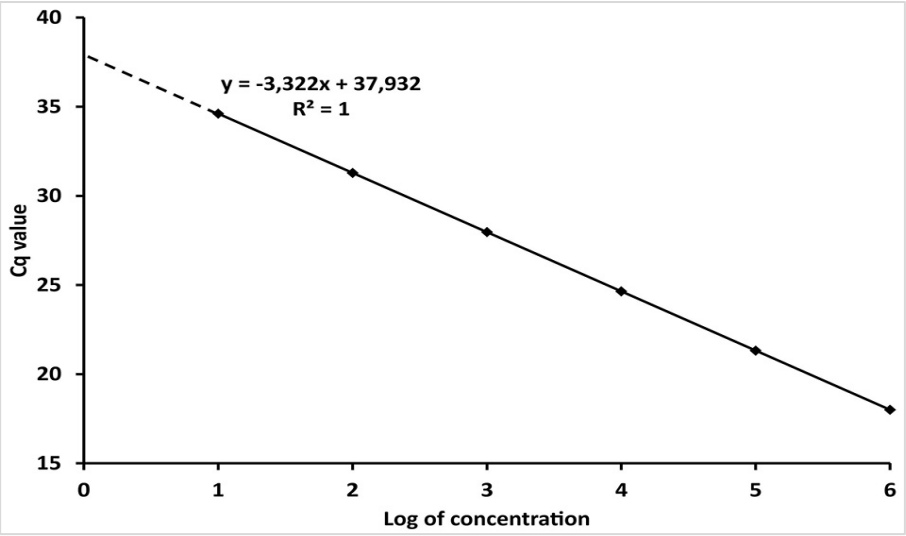

Figure 2.6: Model calibration curve with regression equation (characterised by the slope and intercept) and regression coefficient. (Kralik & Ricchi, 2017)

The Log_{10} of the concentration or copy number of each standard is then plotted against its *Cq* value to derive this correlation equation: $y = kx + C$ (where *x* is the concentration/amount of target, *y* is the *Cq* value of the target, *K* is the regression coefficient or slope and *C* is the intercept; which is subsequently used to quantify unknown samples (Figure 2.6) (Johnson et al., 2013; Kralik & Ricchi, 2017).

Two main methods for the visualisation of amplified DNA fragments in real-time PCR include, non-specific fluorescent DNA dyes and fluorescently labelled DNA probes (Kralik & Ricchi, 2017). The fluorescence dye used in this study is SYBR Green dye (KAPA biosystems, Massachusetts, United States), both for library quantification of bacteria 16S gene for sequencing and quantification of candidate bacteria species in

subsequent chapters. The KAPA SYBR FAST qPCR Master Mix (2×) kit contains a novel DNA polymerase which is an engineered version of *Taq* DNA polymerase designed specifically for real-time PCR using SYBR Green I chemistry. This DNA polymerase is not active at room temperature, preventing the formation of mis-primed products and primer-dimers during preparation stages before the first denaturation step, resulting in accurate quantification and high DNA specificity (KAPA BIOSYSTEMS, 2016). SYBR Green I bind all double-stranded DNA molecules , emitting a fluorescence signal on binding. The kit also uses a reference dye (ROX) to provide a stable baseline against which PCR-related fluorescent signals are normalized, thus, compensating for non-PCR related variations in fluorescence detection, such as slight variations in volumes and well position. It does not interfere with the reaction and has an emission spectrum different from that of SYBR Green I. (Appendix J)

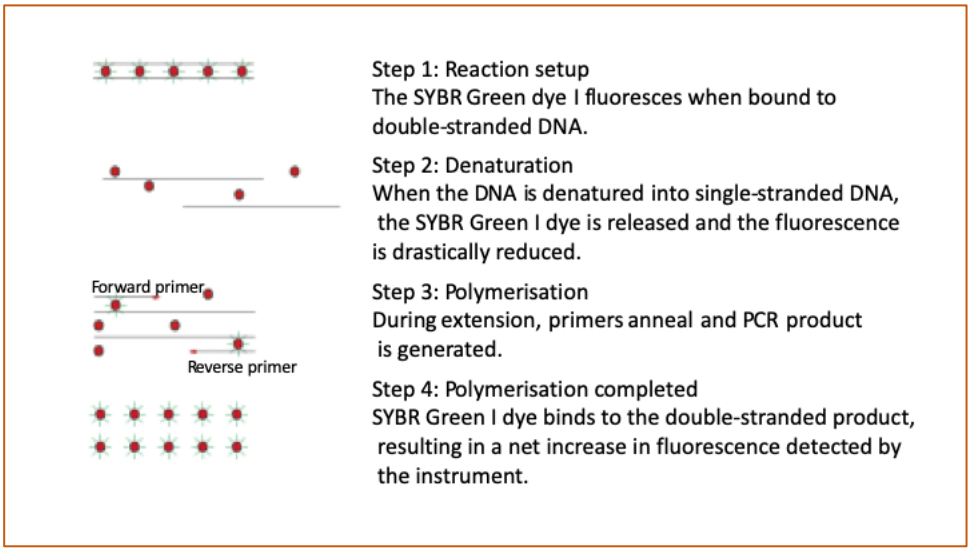

Figure 2.7: SYBR Green I qPCR process (modified from KAPA BIOSYSTEMS, 2016)

qPCR provides the following advantages for use in microbial quantification. Following the geometric rise in the availability of sequencing data, it is possible to design specific primers for almost every microorganism of interest. qPCR provides fast and high-

throughput detection and quantification of target DNA sequences in different samples (Klein, 2002; Kralik & Ricchi, 2017). The simultaneous amplification and visualisation of newly formed amplicons offered by qPCR lowers the time spent in the laboratory (Kubista et al., 2006). In terms of preventing cross-contamination, qPCR offers a safer option as no further manipulations of samples are required after amplification. Finally, qPCR has a wide range from quantification (7-8 Log_{10}) and also allows for multiplexing, for the amplification of several targets into a single reaction (Yang & Rothman, 2004). The downside of the use of qPCR for microbial quantification is that the method cannot distinguish among viable and dead cells when DNA is used as the template, but the use of RNA can mitigate this shortfall if the need arises (Kralik & Ricchi, 2017).

2.7. 16S ribosomal RNA gene sequencing for bacteria community analysis

The traditional methods for microbial identification are arduous and time-consuming, requiring the recognition of differences in morphology, growth, enzymatic activity and metabolism (Petti, et al., 2005). Further, these phenotypic profiles generated are compared against algorithms and databases based on characteristics observed in known and reference strains with predictable biochemical and physical properties under optimal growth conditions. These characteristics however in sampled bacteria species are not static and can change with stress and evolution (Ochman, et al., 2005). Human bias, inexperience handlers, outdated databases and the presence of uncommon phenotypes in common microorganisms, can result in misidentification of bacteria species (Edgar, 2018; Petti et al., 2005). In this thesis, where we investigated environmental samples from *Anopheles* larval trays, the traditional method will have proven to be herculean and ineffective as the list of possible species to be cultured would have been endless.

Next-generation sequencing of the prokaryotic 16S ribosomal RNA (rRNA) gene is the current gold standard for the study of microbial communities in environmental samples (Cho & Blaser, 2012; Hartmann et al., 2014; Moran, 2015; Pflughoeft & Versalovic, 2012). Although not perfect, the emergence of genotypic identification of microbial communities by 16S rRNA gene sequencing has revolutionised the study of microorganisms in environmental samples (Bosshard, et al., 2004; Edgar, 2018). It is a more objective, accurate and reliable method for bacterial identification with the added advantage of defining taxonomical relationships among bacteria (Petti, et al., 2005). 16S rRNA gene sequencing allows for the exploration of unculturable biodiversity and ecological characteristics of whole microbial communities and individual microbial taxa (Caporaso et al., 2011; Klindworth et al., 2013). These 16S rRNA studies are able to reveal which microbial taxa are present in a sample because the 16S rRNA is an excellent phylogenetic marker (Winand et al., 2020). The 16S rRNA gene is present in all bacteria, it is approximately 1500bp long, containing nine variable regions that are interspersed between conserved regions (Hermans, et al., 2017; Kirchman, et al., 2010). The Illumina sequencing platform is the preferred sequencing technique employed for microbial characterisation compared to the earlier Sanger and 454 sequencing methods (Caporaso et al., 2011; Klindworth et al., 2013). It is a high-throughput sequencing technology with advanced computational tools that can exploit metadata (sample description) to relate hundreds of samples to one another in a way that reveal clear biological patterns (Caporaso et al., 2011). It provides a cost effective alternative for the identification of microbial phylotypes present in environmental samples without the need for laborious cultures (Caporaso et al., 2011). Standard protocols for illumina MiSeq sequencing of the 16S rRNA gene include, amplicon primer design, library preparation, sequencing on MiSeq and secondary data analysis on BaseSpace or CLC genomics workbench (Full

methodology are described in Chapter 7 and protocol links are available in Appendices K, L, M, N, and O).

2.8. Mosquito wing measurement

One wing of all emerged adults was measured from the distal end of the allula to the apical margin (radius veins), excluding the fringe scale using a binocular microscope A stage micrometer of 1mm ruler length (Graticules Ltd, Kent, UK) was used for calibration on 2.5 magnification on a scale of 1 microscope unit = 0.04 mm). (Araújo & Gil, 2012).

2.9. Statistical analysis

All data collected were analysed using the software JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA). All data were checked for deviations from normality and heterogeneity, and analyses were conducted using parametric and non-parametric methods as appropriate. Data from all replicates were used for analysis, replicate effects were tested but are only reported when significant. Interactions between independent variables were tested using step-wise models and only those significant were retained in the final models.

Chapter 3

Effect of water source and feed regimes on development and phenotypic quality in *Anopheles gambiae* (*s.l*).

3.1. Introduction

Despite the steady inflow in malaria funding, this persistent, multifaceted disease was still responsible for 405,000 deaths in 2018, with 93% of these cases occurring in the African region (WHO, 2019). Since 2016, there has been a stall in progress towards reduction in malaria morbidity, incidence and mortality rates, and in some cases, a standstill. More worrisome is the rise in malaria cases across some high-burden countries in Africa (WHO, 2019). This illustrates how fragile are the gains made in our quest to control, and ultimately eradicate the disease. In the absence of effective vaccines and the evolution of resistance to available drugs by *Plasmodium* malaria parasites, vector control continues to be the most cost-effective line of defence as it interrupts the disease cycle by preventing the transfer of malaria-causing parasites to humans (Lees, et al., 2015).

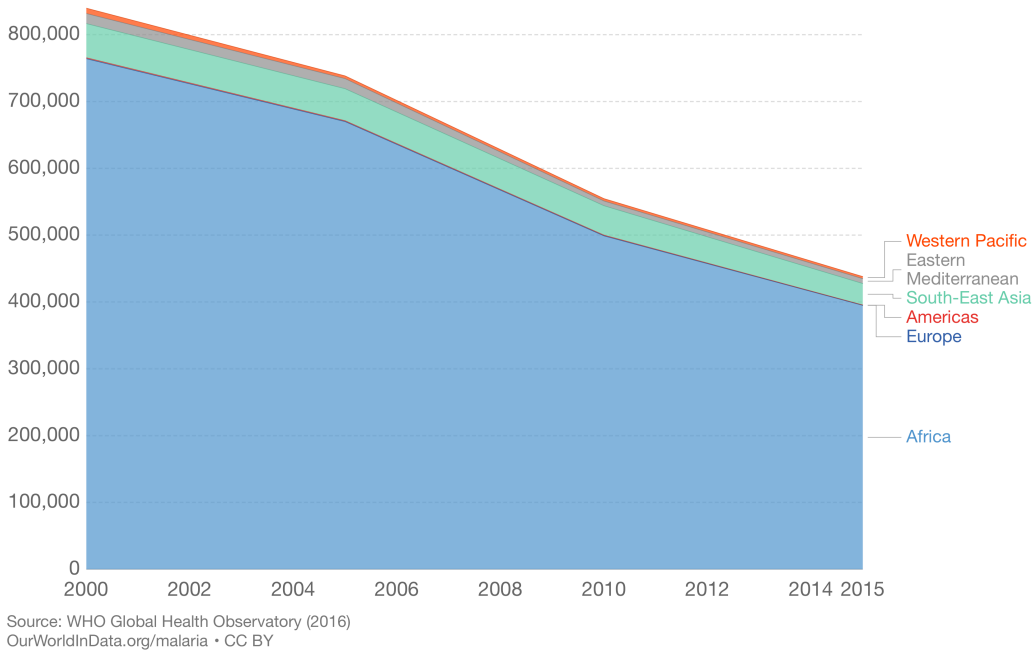

Figure 3.1: Global malaria deaths by world region.

In sub-Saharan Africa, malaria is primarily transmitted by mosquitoes of the *Anopheles gambiae* complex with *Anopheles gambiae* s.s. and *Anopheles coluzzii* being the most abundant and widespread (della Torre et al., 2002). These morphologically indistinguishable sibling species co-occur over large areas of sub-Saharan Africa and do not exhibit intrinsic post-mating barriers to reproduction (Aboagye-Antwi et al., 2015). *Anopheles gambiae* s.s. is widespread throughout the region, extending across the continent all the way from West Africa, through Central and East Africa and into Madagascar. *Anopheles coluzzii* has a westerly distribution which spans from Northern Senegal, West-central Africa and Angola (della Torre et al., 2005). In many of these regions, the two species are found in sympatry and are separated genetically by strong assortative mating, hence low hybridization rates (Diabaté et al., 2006; Tripet et al., 2001). The first exception to this rule is some sympatric populations from far-West of Africa in Guinea-Bissau and The Gambia where hybrid frequencies as high as 22.9% have been recorded (Caputo et al., 2011). The second exception is populations in which the kdr-resistance allele of *An. gambiae* recently selectively introgressed into *An. coluzzii* which resulted in a temporary increase in hybrid-like genotypes (Lee et al., 2013; Norris et al., 2015)

The current toolbox for controlling these prevalent vectors includes the use of insecticide-treated nets (ITN), indoor residual spraying (IRS) and integrated vector management (IVM) (WHO, 2017). Although these methods have been very effective in the reduction of mortality and morbidity over the past decade, 50% of the countries with ongoing malaria transmission which were on track towards critical targets for reduction in mortality and morbidity, have recorded a stall in progress (WHO, 2017). Insecticide resistance amongst other factors stands out as a major driver for this change in trajectory. This current trend brings to the forefront the research for new vector control methods to

complement the existing IVM techniques. The release of sterile or genetically-modified mosquitoes for the replacement suppression of mosquito populations is one such promising tool (Burt, 2014; Diabate & Tripet, 2015; Klassen, 2009). These approaches bear similarities with 1950s and 1960s sterile male releases, in that they involve the production of large number of males which by mating with wild females will cause either the decline in the target population over a short period (Lees, et al., 2015), or its replacement with a population refractory to the malaria parasite (Burt, 2014). Successful implementation of these techniques is based amongst other factors, on rearing protocols designed specifically for *An. gambiae* (s.l.), a species for which no large-scale release programme has ever been conducted. Since the species in this complex are particularly demanding in terms of water cleanness compared to culicid species (Subra, 1981; Tene Fossog et al., 2013), the need for efficient water management whilst providing enough larval food for production is crucial. Equally important is the need for the resulting sterile or genetically-modified male mosquitoes to be of sufficient phenotypic quality to ensure optimal survival and mating competitiveness after releases (Lees, et al., 2015).

In nature, the sibling species, *An. gambiae* and *An. coluzzii* differ in their preferred larval breeding sites (Kamdem et al., 2012). Although larvae of both species can be found in the same habitat, *An. coluzzii* prefers more permanent breeding sites resulting from human activities such as irrigated rice fields, reservoirs, abandoned mines and quarries, deforestation, and drainage ditches (Diabaté et al., 2009). In contrast, *An. gambiae* s.s, whose populations usually peak during the rainy season, thrives in habitats that are more ephemeral and rain-dependent (della Torre et al., 2005; Edillo et al., 2006; Kamdem et al., 2012; Lehmann & Diabate, 2008). Larval habitat divergence has repeatedly been cited as a possible driver of ecological speciation between these species and larval transplant experiments have shown that *An. coluzzii* avoids the aquatic predators associated with

more permanent habitats more effectively (Coetzee et al., 2013; Gimonneau et al., 2010). Whether *An. gambiae* s.s. develops better in the water with low mineral content such as rain filled pools is currently unknown. However, there is some evidence that *An. coluzzii* may tolerate water with a higher mineral content at least in some areas of Africa (Mattah et al., 2017)

There is a dearth of information on the effect of water source (in relation to mineral content or hardness) on the development and phenotypic quality of *An. gambiae* (s.l.) in the laboratory. Deionised water is commonly used in mosquito rearing in the laboratory regardless of strain. Although, it has been demonstrated that different feed regimes affect mosquito phenotypic quality and development time (Faeza, et al., 2012; Yahouédo et al., 2017), these studies focused on the effect of feed in isolation and not in relation to different water sources. Some of these studies have linked food quality to development of larger mosquitoes which have higher fecundity and longevity especially in females (Faeza et al., 2012; Takken et al., 1998)

In this study, we investigated the impact of different water sources (in relation to hardness and minerals) and feed regimes on the development and phenotypic quality of two well-established strains and one recently colonized strain of *An. gambiae* s.l. in the laboratory. First-instar larvae were reared in three water types with different levels of hardness and data were collected on larval survival, pupal survival and mortality, adult emergence, development time, wing-length and sex. Larvae were fed with food delivered as floating flakes, referred to as 'powder feed', or as liquid solution or 'solution feed'. The results obtained show an improvement in mosquito phenotypic quality when mineral water is used, and this could be beneficial for mosquito rearing programmes small or large. These findings should lead to better protocols for mass-rearing of sterile or genetically-modified male anopheline mosquitoes towards releases for vector control.

3.2. Materials and methods

3.2.1. Mosquito maintenance

All experiments were conducted in dedicated insectaries of the CAEP, Keele University, UK. The Kisumu strain of *An. gambiae*, colonized over 40 years ago, from the area of Kisumu, Kenya, East Africa; 17-year-old Mopti strain of *An. coluzzii*, colonized in 2003 by the Lanzaro Laboratory (UC Davis) from the village of N'Gabacoro droit near Bamako, Mali, West Africa, and a recently-colonised 2-year-old VK3 *An. coluzzii* strain from Vallee du Khou in Burkina Faso, West Africa (supplied by IRSS, Bobo Dioulasso), were used for the experiments

3.2.2. Experimental design

Ten first-instar larvae were placed in styrofoam cups containing 150 ml of water at 5 cm depth. Mosquitoes were reared in three water types with different levels of hardness: deionised water, mineral water, and a 50:50 mix of both water types: (i) deionised water which was sourced from a reverse osmosis unit (PURELAB Prima, Wycombe, United Kingdom) installed in the laboratory. The water quality specifications of treated deionised water were: Total organic carbon < 0.1ppm, bacteria < 5 CFU/ml, 98% rejection of inorganics, > 99% rejection of organics, > 99% rejection of particles. (ii) mineral water: bottled water containing minerals which are natural compounds formed through geological processes, sourced from a local shop with the following typical nutrient values/litre: calcium (11 mg), magnesium (3.5 mg), potassium (2.5 mg), sodium (10 mg), bicarbonate (25 mg), sulphate (11 mg), nitrate (15 mg), chloride (14 mg), dry residue at 180 °C (85 mg) and pH (6.2); and (iii) mix water: a 50:50 mix of deionised and mineral water.

Although water hardness is usually defined as the total concentration of calcium and magnesium in water in mg/l, it is caused by a variety of dissolved polyvalent metallic ions, mainly calcium and magnesium and other ions such as aluminium, barium, iron, manganese, strontium and zinc (Rubenowitz-Lundin & Hiscock, 2013). To determine the water hardness/nutrient content of the water treatments used, 42 readings of conductivity (µS), total dissolved solids (mg/l), and salinity (ppm) per treatment were taken at 3 points during the experiment, using an EXTECH conductivity/TDS/salinity/Temperature hand-held meter (FLIR Commercial Systems, Inc., Nashua, USA). Mean values for TDS (Total Dissolved Solids), salinity and conductivity of the three water types at 3 points during the study are shown in Table 3.1. Larvae were fed with two standardised feeding regimes (solution and powder feed). Powder feeding regime consist of daily rations of ground fish food, using a spatula to spread on the water surface: 0.1µl of Liquifry liquid fish food (Interpret Ltd, Surrey, UK) on day 1, 2mg on days 2–3, 4mg on day 4, and 10mg on day 5 until pupation.

Table 3.1 Mean (95% CI) of total dissolved solids, salinity and conductivity: measure of water hardness

Water type	TDS (mg/l)	Salinity (ppm)	Conductivity (µS)
Deionized	27.55 (25.93 – 29.18)	18.48 (17.38 –19.58)	39.54 (37.24 – 41.84)
Mix	70.54 (68.97–72.12)	47.47 (46.38 – 48.55)	100.85 (98.58 –103.12)
Mineral	112.21 (110.53 – 113.89)	75.78 (74.65 – 76.91)	160.4 (157.99 – 162.80)

Notes: Ninety-five percent confidence intervals are in parentheses. Sample size is 42

Solution feeding regime consist of the same food quantity dissolved in deionized water (0.1µl of Liquifry on day 1, 0.1ml of 1g/50ml of TetraMin Baby on days 2–3, 0.2ml of 1g/50ml of TetraMin Baby on day 4, and 0.5ml of 1g/50ml of TetraMin Baby on day 5 until pupation) and injected into the larval tray using a pipette. The resulting balanced experimental design consisted of 3 strains × 3 water types × 2 feeding patterns × 10

replicates × 10 larvae per pot, for a total sample size of 1800 larvae (Figure 3.2). Larvae from each experimental group were transferred to fresh water (same water source as the original set-up) containers daily.

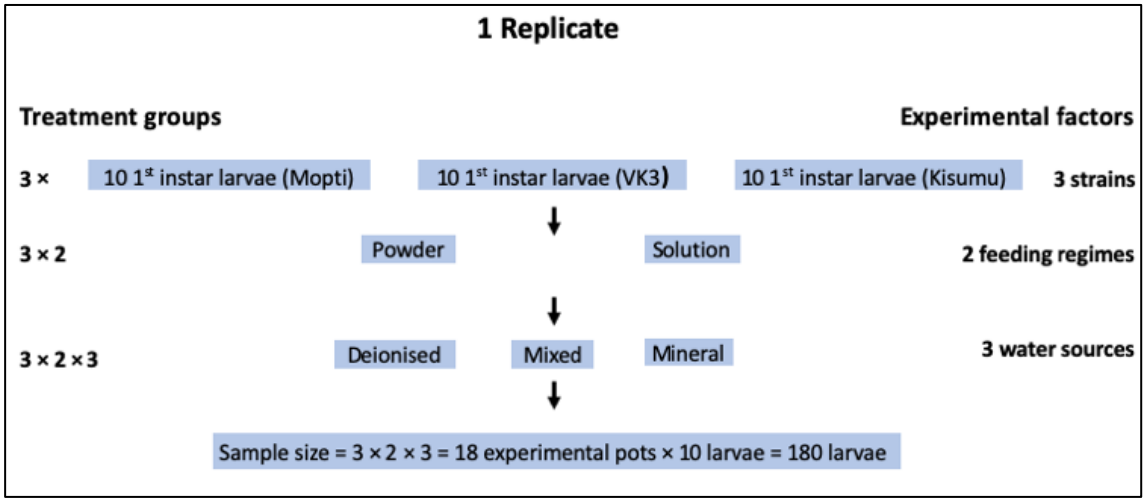

Figure 3.2. Experimental design showing experimental factors in one replicate.

3.2.3. Collection of data at life-cycle stages

Depending on the life-cycle stage of the mosquitoes the following data were observed and recorded: (i) larval survival: determined as the percentage of larvae that developed into pupae from the total number of larvae for each treatment; (ii) pupal survival: determined as the percentage of mosquitoes that emerged as adults from those that pupated in each treatment; (iii) pupal mortality: determined as the percentage of mosquitoes that died at the pupal stage from the total number of mosquitoes per treatment; (iv) adult emergence: determined as the percentage of mosquitoes that emerged as adults from the total number of larvae in each treatment; (v) development time: determined as the number of days from placement of first instar larvae in treatment cups until adult emergence; and (vi) wing-length: following emergence, adult mosquitoes were sexed and stored in 75% ethanol and wing-length was subsequently measured as described in Chapter 2.

3.2.4. Statistical analysis

All data collected were analysed using the software JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA). All data were checked for deviations from normality and heterogeneity, and analyses were conducted using parametric and non-parametric methods as appropriate. Data from all replicates were used for analysis, replicate effects were tested but were only reported when significant. Interactions between independent variables were tested using step-wise models and only those significant were retained in the final models. For analyses of proportion of larvae, pupae and adults, likelihood odds ratios were used for *post-hoc* pairwise group comparisons following logistic regressions. Body size was analysed through general linear models followed by Tukey's HSD *post-hoc* pairwise comparisons. Finally, developmental times (day of emergence) were analysed by Cox Proportional-Hazard models with likelihood odds ratios for *post-hoc* pairwise comparisons.

3.3. Results

3.3.1. Effect of water types and feed regimes on larval survival

Across all experiments *An. gambiae* (Kisumu) larvae survived significantly (93%) better than *An. coluzzii* (Mopti: 82%; VK3: 77%) (Table 3.3, 3.4, Figure 3.3). A full logistic regression model showed that water source had a small but significant positive effect on larval survival across all strains ($P = 0.0405$) but that its impact differed between strains ($P = 0.0117$) (Tables 3.2). The same analyses performed within strains showed that water source significantly impacted larval survival for Kisumu and VK3 strains but not Mopti (Figure 3.3,Table 3.5). *Post-hoc* pairwise comparisons (Odds-ratio tests) revealed that mineral water significantly improved larval survival compared to deionised water ($P = 0.0186$), other water type comparisons were non-significant (Table 3.4).

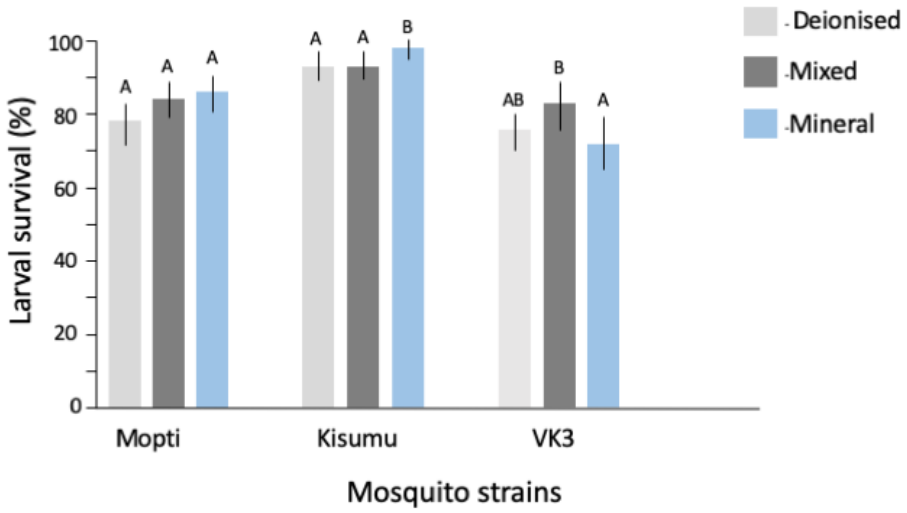

Figure 3.3: Effect of water source on larval survival. The percentage larval survival in deionised (light grey), mixed (dark grey) and mineral (blue) is shown across three strains of mosquitoes (Mopti, Kisumu, VK3). Whiskers represent 95% confidence intervals. Within strains, bar plots sharing a letter are not significantly different, those with different letters are significantly different.

Within the Kisumu strain there was 98% larval survival in mineral water compared to 93% for both mix and deionised water. VK3 strain conversely had the highest larval survival in mix water (83%) followed by deionised water (76%) with

mineral water having the lowest larval survival of 72% (Figure 3.3). Although overall feed type was not significant for larval survival, there was a significant interaction between feed and strain (Table 3.2). For Kisumu strain, solution feed type resulted in significantly higher larval survival (Table 3.5).

Table 3.2: Logistic regressions of the overall effect of water types and feed regime on development

Parameter	Source	df	Likelihood ratio	P-value
Larval survival	Strain	2	86.74	<0.0001***
	Water type	2	6.41	0.0405*
	Feed	1	1.88	0.1708^{ns}
	Feed*Strain	2	7.32	0.0258*
	Water type*Strain	4	12.92	0.0117*
Pupal mortality	Strain	2	23.09	<0.0001***
	Water type	2	0.02	0.9898^{ns}
	Feed	1	1.22	0.2690^{ns}
	Feed*Strain	2	5.62	0.0601 ^{ns}
Adult emergence	Strain	2	25.61	<0.0001***
	Water type	2	4.11	0.1283^{ns}
	Feed	1	2.51	0.1129^{ns}
	Feed*Strain	2	13.36	0.0013*
	Water type*Strain	2	9.75	0.0448*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

3.3.2. Pupal mortality in relation to water types and feed regimes

Pupal mortality was significantly different between strains ($P < 0.0001$), with 10% mortality in Kisumu strain, 4% and 3% in Mopti and VK3 respectively (Tables 3.2, 3.3). *Post-hoc* comparisons revealed significant difference in pupal mortality between *An. gambiae* and both strains of *An. coluzzii* (Odds ratio tests: $P < 0.0014$ in both cases) (Table 3.4). There were no significant effects of water source or feed regimes on pupal mortality (Table 3.2).

Table 3.3: Effect of water types and feed regimes on life history stages

Strain	Water type	Feed regime	% Larval survival	% Pupal survival	% Pupal mortality	% Adult emergence
Mopti (*An. coluzzii*)	Total	Total	82 (79–85) 600		4(3–7) 600	78 (74–81) 600
	Deionized	Solution	77 (68–84) 100	96 (89–99) 77	3 (1–8) 100	74 (65–82) 100
		Powder	79 (70–86) 100	97 (91–99) 79	2 (1–7) 100	77 (68–84) 100
	Mixed	Solution	82 (73–88) 100	93 (85–97) 82	6 (3–12) 100	76 (67–83) 100
		Powder	85 (77–91) 100	94 (87–97) 85	5 (2–11) 100	80 (71–87) 100
	Mineral	Solution	84 (76–90) 100	90 (82–95) 84	8 (4–15) 100	76 (67–83) 100
		Powder	87 (79–92) 100	95 (89–98) 87	4 (2–10) 100	83 (74–89) 100
	Total	Total	94 (92–96) 600		10 (8–12) 600	84 (81–87) 600
Kisumu (*An. gambiae*)	Deionized	Solution	96 (90–98) 100	89 (81–93) 96	11 (6–19) 100	85 (77–91) 100
		Powder	89 (81–94) 100	85 (77–91) 89	13 (8–21) 100	76 (67–83) 100
	Mixed	Solution	96 (90–98) 100	97 (91–99) 96	3 (1–8) 100	93 (86–97) 100
		Powder	90 (83–94) 100	83 (74–90) 90	15 (9–23) 100	75 (66–82) 100
	Mineral	Solution	98 (93–99) 100	93 (86–96) 98	7 (3–14) 100	91 (84–95) 100
		Powder	97 (92–99) 100	90 (82–94) 97	10 (6–17) 100	87 (79–92) 100
	Total	Total	77 (73–80) 600		3 (2–5) 600	74 (70–77) 600
VK3 (*An. coluzzii*)	Deionized	Solution	69 (59–77) 100	97 (90–99) 69	2 (1–7) 100	67 (57–75) 100
		Powder	82 (73–88) 100	95 (88– 98) 82	4 (2–10) 100	78 (69–85) 100
	Mixed	Solution	87 (79–92) 100	98 (92–99) 87	2 (1–7) 100	85 (77–91) 100
		Powder	79 (70–86) 100	95 (88–98) 79	4 (2–10) 100	74 (64–82) 100
	Mineral	Solution	72 (63–80) 100	96 (88–99) 72	3 (1–8) 100	69 (59–77) 100
		Powder	72 (63–80) 100	94 (87–98) 72	4 (2–10) 100	68 (58–76) 100

Notes: Ninety-five percent confidence intervals are in parentheses and sample sizes are italicized. Larval survival, pupal mortality and emergence rates are calculated out of an initial number of 100 larvae (per treatment) and 600 larvae in total, and pupal survival is calculated out of a variable number of surviving larvae at pupation.

Table 3.4: Odds ratios for pairwise group comparisons of the effect of water types and feed on life cycle stages

Parameter	Source	Level	Odds ratio	P-value
Larval survival	Strain	Kisumu vs Mopti	0.23	<0.0001***
		VK3 vs Mopti	1.40	0.0214*
		VK3 vs Kisumu	6.03	<0.0001***
	Water type	Mix vs Deionized	0.74	0.0912^{ns}
		Mineral vs Deionized	0.61	0.0186*
		Mineral vs Mix	0.82	0.3572 ^{ns}
	Feed	Powder vs Solution	1.24	0.1768 ^{ns}
Pupal mortality	Strain	Kisumu vs Mopti	0.46	0.0014*
		VK3 vs Mopti	1.52	0.1829 ^{ns}
		VK3 vs Kisumu	2.69	<0.0001***
	Water type	Mix vs Deionized	1.00	1.0000 ^{ns}
		Mineral vs Deionized	0.97	0.9016 ^{ns}
		Mineral vs Mix	0.97	0.9016 ^{ns}
	Feed	Powder vs Solution	0.77	0.2708 ^{ns}
Adult emergence	Strain	Kisumu vs Mopti	1.69	0.0008**
		VK3 vs Mopti	0.80	0.1058 ^{ns}
		VK3 vs Kisumu	0.47	<0.0001***
	Water type	Mix vs Deionized	1.29	0.0707 ^{ns}
		Mineral vs Deionized	1.27	0.0971 ^{ns}
		Mineral vs Mix	0.98	0.9153 ^{ns}
	Feed	Powder vs Solution	0.83	0.1141 ^{ns}

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

3.3.3. Effect of water types and feed regimes on adult emergence

Overall, adult emergence significantly differed among strains ($P < 0.0001$) and was also affected by the interaction of strain with feed type and water type ($P < 0.05$ in both cases) (Table 3.2). *Post-hoc* analysis showed that the Kisumu strain of *An. gambiae* had significantly higher adult emergence (84%) than Mopti (78%) and VK3 (74%) (Tables 3.3, 3.5, Figures 3.3, 3.4).

Table 3.5: Logistic regressions of the effect of water types and feed regime on life stages within strains

Parameter	Strain	Source	DF	Likelihood ratio	P-value
Larval survival	Mopti	Water type	2	4.08	0.1300^{ns}
		Feed	1	0.74	0.3900^{ns}
	Kisumu	Water type	2	6.52	0.0384*
		Feed	1	6.34	0.0118*
	VK3	Water type	2	7.61	0.0222*
		Feed	1	0.05	0.8159^{ns}
		Feed*Water type	2	6.66	0.0358*
Adult emergence	Mopti	Water type	2	0.94	0.6248^{ns}
		Feed	1	1.89	0.1693^{ns}
	Kisumu	Water type	2	5.83	0.0543^{ns}
		Feed	1	12.55	0.0004**
	VK3	Water type	2	7.04	0.0296*
		Feed	1	0.10	0.7560^{ns}
		Feed*Water type	2	6.81	0.0331*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

Within strains, water type significantly impacted adult emergence in the VK3 strain with mineral water having the lowest emergence (69%), followed by deionised water (73%), and mix (80%) (Table 3.2;Figure 3.4,) but there were no effects on Mopti and Kisumu (Table 3.5). In Kisumu, solution feed yielded 10% ($P < 0.001$) more adults compared to powder feed (Table 3.3,3.5 Figure 3.5).

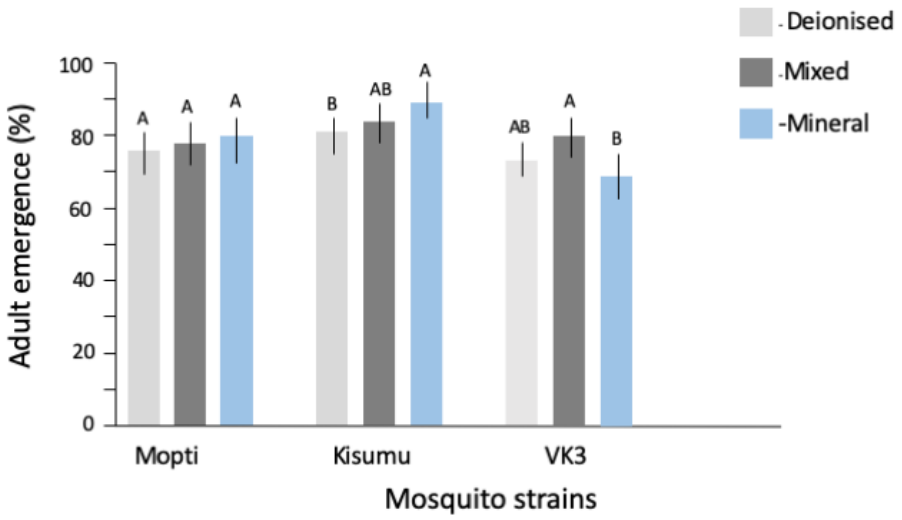

Figure 3.4: Effect of water source on adult emergence. The percentage adult emergence across three water types, deionised (light grey), mixed (dark grey) and mineral (blue) for mosquito strains, Mopti, Kisumu and VK3). Whiskers represent 95% confidence intervals. Within strains, bar plots sharing a letter are not significantly different, those with different letters are significantly different.

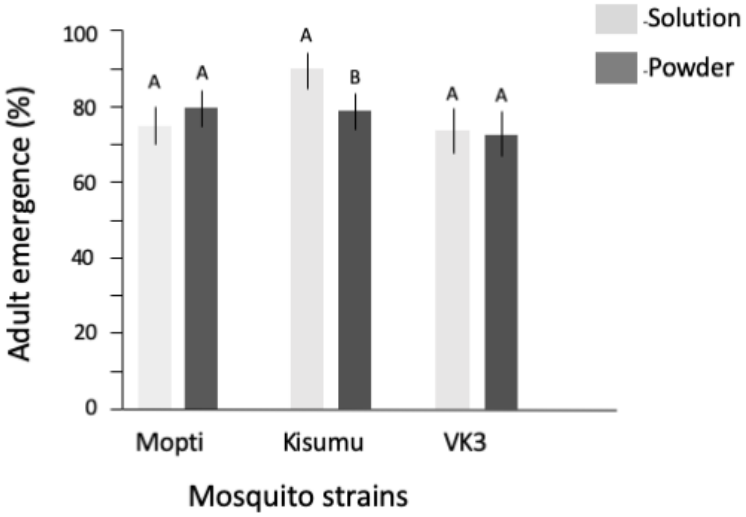

Figure 3.5: Effect of feed regimes on adult emergence. The percentage adult emergence for solution (light grey), and powder (dark grey) feed regimes across three mosquito strains (Mopti, Kisumu and VK3). Whiskers represent 95% confidence intervals. Within strains, bar plots sharing a letter are not significantly different, those with different letters are significantly different.

3.3.4. Wing length of emerged adults in relation to water types and feed regimes

Water source and feed regime significantly ($P < 0.0001$) impacted wing length of both sexes of adult mosquitoes of all strains. There were significant interactions between feed by strain and sex by water type (Tables 3.6; 3.8). Generally, females had longer wing-

length than males. However, females from deionised water were significantly (P < 0.0001) smaller than those from mineral and mix water (Table 3.6). Similarly, males from deionised water had significantly shorter wing length compared to those from mineral water (Table 3.6). *Post-hoc* pairwise comparisons (Tukey's tests) revealed mineral water yielded the largest adults, followed by mix, with deionised water producing the smallest mosquitoes (Table 3.7, Figure 3.6).

Table 3.6: General linear model of the effect of water types and feed regime on wing length

Parameter	Source	df	F-ratio	P-value
Wing length	Strain	2	60.08	<0.0001***
	Water type	2	26.07	<0.0001***
	Feed	1	7.17	0.0075*
	Sex	1	146.50	<0.0001***
	Feed * Strain	2	8.60	0.0002**
	Sex * Water type	2	4.23	0.0147*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

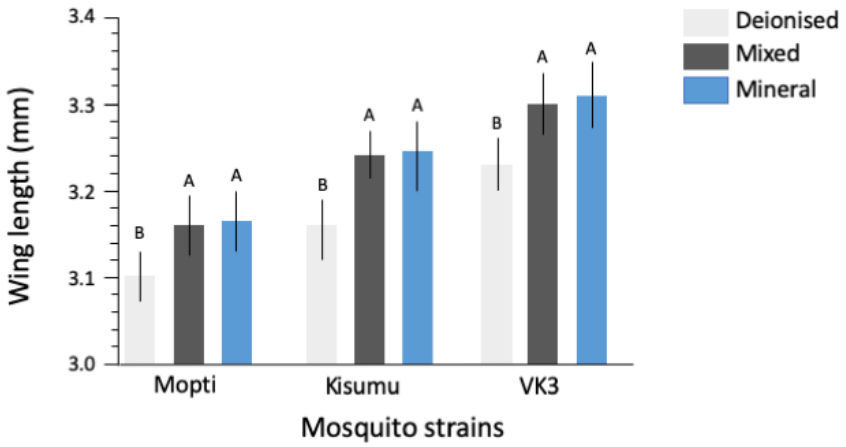

Figure 3.6: Effect of water source on wing-length. The mean wing-length for deionized (light grey), mixed (dark grey) and mineral water (blue) types for Mopti, Kisumu and VK3. Whiskers represent 95% confidence intervals. Within strains, bar plots sharing a letter are not significantly different, those with different letters are significantly different.

Adults that emerged from powder feed were significantly larger than those from solution feed (Figure 3.7, Table 5). Amongst strains, VK3 adults were significantly ($P < 0.0001$) the largest, then Kisumu, lastly Mopti (Figure 3.7, Table 3.7). Powder feed impacted positively on adult size for VK3 and Kisumu strain but was not significant for Mopti (Figure 3.7, Table 3.7).

Table 3.7: Post-hoc following general linear model ,Turkey's pairwise differences on wing length

Source	Level	t-ratio	P-value
Strain	Mopti vs Kisumu	-6.66	<0.0001***
	Mopti vs VK3	-10.88	<0.0001***
	Kisumu vs VK3	-4.51	<0.0001***
Water type	Deionized vs Mix	-6.44	<0.0001***
	Deionized vs Mineral	-6.41	<0.0001***
	Mix vs Mineral	-0.36	0.9294^{ns}
Sex * Water type	Female-Deionized vs Female-Mix	-6.21	<0.0001***
	Female-Deionized vs Female-Mineral	-5.72	<0.0001***
	Male-Deionized vs Male-Mineral	-3.32	0.0117*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

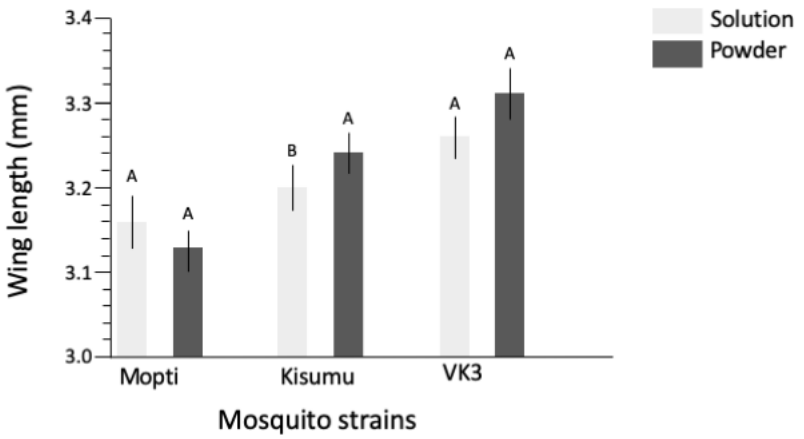

Figure 3.7: Effect of feed regimes on wing-length. The mean wing-length for solution (light grey), and powder feed (dark grey) across three mosquito strains, Mopti, Kisumu and VK3. Whiskers represent 95% confidence intervals. Within strains, bar plots sharing a letter are not significantly different, those with different letters are significantly different.

3.3.5. Developmental time in different water types and feed regimes

Overall, the duration of development from first instar larvae to adults was significantly impacted for all strains by water source (Cox Proportional Hazard: $P < 0.0001$) (Tables 3.8; 3.9). Mosquito development was significantly longer in deionised water compared to mix and mineral water ($P < 0.05$) (Table 3.10, Figure 3.8). Developmental time was not significantly different between mix and mineral water (Table 3.10, Figure 3.8). Across all water types, development time was significantly ($P < 0.0001$) longer in Kisumu strain compared to Mopti and VK3 (Table 3.9, Figure 3.8). Feed regime did not significantly impact development time (Figure 3.9).

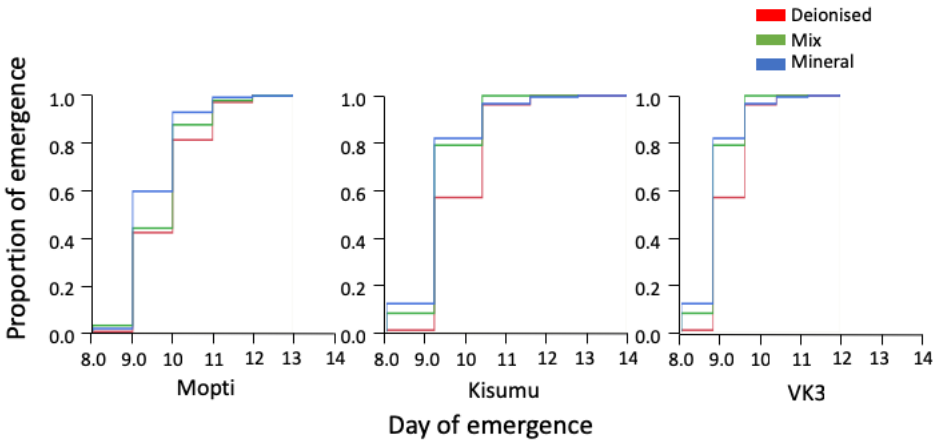

Figure 3.8: Effect of water source on developmental time. The mean developmental time for deionized (red), mixed (green) and mineral (blue) water types is shown for three mosquito strains, Mopti, Kisumu and VK3.

Table 3.8: Effect of water types and feed on mosquito body size (wing length) and day of emergence

Strain	Water type	Feed regime	Mean wing length (mm)	Days till emergence
Mopti (*An. coluzzii*)	Deionized	Solution	3.13 (3.08–3.17) *74*	9.70 (9.54–9.87) *74*
		Powder	3.07 (3.03–3.11) *77*	9.91 (9.70–10.11) *77*
	Mixed	Solution	3.19 (3.14–3.25) *76*	9.72 (9.54–9.90) *76*
		Powder	3.13 (3.09–3.18) *80*	9.68 (9.49–9.86) *80*
	Mineral	Solution	3.16 (3.11–3.22) *76*	9.50 (9.32–9.68) *76*
		Powder	3.17 (3.13–3.21) *83*	9.48 (9.34–9.63) *83*
Kisumu (*An. gambiae*)	Deionized	Solution	3.13 (3.08–3.17) *85*	10.52 (10.39–10.65) *85*
		Powder	3.18 (3.14–3.23) *76*	10.45 (10.29–10.60) *76*
	Mixed	Solution	3.23 (3.19–3.28) *93*	10.26 (10.13–10.38) *93*
		Powder	3.26 (3.21–3.29) *75*	10.01 (9.90–10.12) *75*
	Mineral	Solution	3.24 (3.18–3.27) *91*	10.32 (10.16–10.48) *91*
		Powder	3.28 (3.23–3.32) *87*	9.92 (9.79 – 10.05) *87*
VK3 (*An. coluzzii*)	Deionized	Solution	3.21 (3.16–3.27) *67*	9.60 (9.42– 9.78) *67*
		Powder	3.25 (3.21–3.28) *78*	9.69 (9.52 – 9.87) *78*
	Mixed	Solution	3.27 (3.23–3.31) *85*	9.58 (9.44– 9.74) *85*
		Powder	3.34 (3.28–3.40) *75*	9.40 (9.22– 9.58) *75*
	Mineral	Solution	3.28 (3.23–3.32) *69*	9.59 (9.41– 9.78) *69*
		Powder	3.35 (3.30–3.40) *68*	9.22 (9.03– 9.41) *68*

Notes: Ninety-five percent confidence intervals are in parentheses and the samples sizes, the number of surviving individuals out of an initial number of 100 larvae are italicized.

Table 3.9: Cox Proportional-Hazard analyses on development time

Parameter	Source	df	Likelihood ratio	P-value
Day of emergence	Strain	2	79.61	<0.0001***
	Water type	2	15.26	0.0005**
	Feed	1	2.25	0.1337^{ns}

P- value: *** < 0.0001, ** < 0.001, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom.

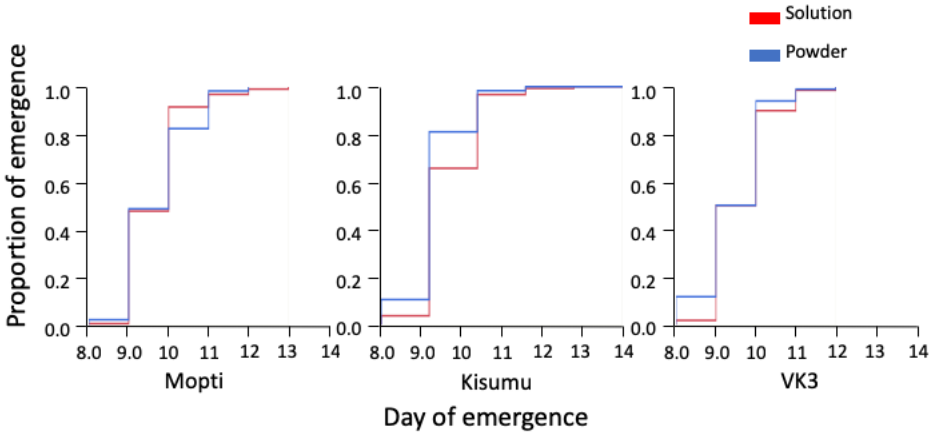

Figure 3.9: Effect of feed regimes on developmental time. The mean developmental time for solution (red) and powder (blue) feed is shown for Mopti, Kisumu and VK3.

Table 3.10: Post-hoc analysis following proportional-hazards fit for development time

Source	Level	Risk ratio	P-value
Strain	Kisumu vs Mopti	0.65	<0.0001***
	VK3 vs Mopti	1.13	0.0713*
	VK3 vs Kisumu	1.73	<0.0001***
Water type	Mix vs Deionised	1.17	0.0196*
	Mineral vs Deionised	1.29	0.0001**
	Mineral vs Mix	1.11	0.1128 ^{ns}
Feed	Powder vs Solution	1.08	0.1337 ^{ns}

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

3.4. Discussion

The results from this study show that mineral water and mixed water produced significantly larger mosquitoes compared to deionised water in the laboratory. This was true for all three strains, from the very old Kisumu strain to the recently-colonized VK3 colony. In mosquitoes, environmental conditions at the larval stage determine the size of the imagos (Aboagye-Antwi & Tripet, 2010), and larger body size is usually synonymous with higher phenotypic quality (Kivuyo et al., 2014). In *An. gambiae* (s.l.), optimal larval nutrition has been linked to female body size, fecundity and increased vectorial capacity (Takken et al., 2013). In males, body size has been directly linked to successful mating and longevity in the wild and in the laboratory (Diabate & Tripet, 2015). Standardization of mosquito adult size and phenotypic quality during rearing is essential to ensure the reproducibility and efficiency of vector control programmes relying on mosquito releases (Valerio, et al., 2016). Therefore, the results of this study suggest that the use of mineral water in mosquito culture laboratories can play an important part in achieving adequate standards of mosquito phenotypic quality. This is relevant to small-scale rearing in mosquito insectaries and may prove crucial for mass rearing protocols for larger mosquito release control programmes.

Quality and delivery of larval diet has been shown to impact on mosquito size and development (Linenberg, et al., 2016). In this study, the powder diet resulted in improved mosquito size, hence phenotypic quality, in two of the three strains studied, the Kisumu and VK3 strains. No significant effect of larval diet in adult size was observed for the Mopti strain. Both the Mopti and VK3 strains are of the *An. coluzzii* sibling species which is thought to be more prone to bottom feeding to avoid predators (Gimonneau et al., 2010; Mattah et al., 2017). Therefore, the contrasted responses to liquid feed of these two strains remains to be explained. Kisumu strain is a strain of *An. gambiae* s.s., which is thought

to prefer surface filter feeding (Gimonneau et al., 2010). In addition, it is a far older strain which may have become better adapted to powder food because it is widely used in insectaries.

The duration of the developmental cycle is another important parameter for mosquito rearing that is dependent on an optimal combination of feed quantity and quality, water type and ambient conditions (Araújo & Gil, 2012). An ideal culture timeline should be the shortest possible time required to produce good sized, long-lasting and viable mosquitoes that are able to compete with those in the wild (Araújo & Gil, 2012). Mineral water led to faster development, longer wing length, therefore, it stands out as the best option for improved mosquito culture. Deionised water on the other hand, had the longest developmental time and resulted in smaller mosquitoes. Small size females have been reported to have higher mortality especially after a blood feed or do not produce viable eggs (Araújo & Gil, 2012; Tchigossou et al., 2018).

Although mineral water positively impacted larval survival for Kisumu strain and had no effect on the Mopti strain, it seemed to negatively impact larval survival in the newly colonised VK3 strain. This effect might be a due to the fact that this strain is not yet adapted to the confinement conditions in the insectary or other factors. Of more importance, wing-length and development time was positively impacted by mineral water even for VK3 strain. Mixed water and mineral water had similar impact on larval survival, adult emergence, wing length and development time despite the former having 50% less mineral content. Given that mineral water represents a significant cost, using mixed water is a cost-effective and sustainable option for mosquito rearing, especially in areas of water scarcity and with limited financial resources.

Ad-hoc tests revealed strong differences between the three species in how they responded to water source and feed type. Overall, *An. gambiae* (Kisumu) larvae survived

significantly better than both *An. coluzzii* strains (Mopti and VK3). This higher larval survival of the *An. gambiae* Kisumu strain may be the result of 40 years of selection for insectary rearing compared to the Mopti strain of intermediate age and the young VK3 strain. The contrasted age of insectary maintenance of the colonised strains, whilst relevant for understanding adaptations to the laboratory, limited our ability to distinguish species-specific differences such as hardness tolerance and generally prevented inferences with regards to processes that associated to larval ecological speciation between the sibling species (della Torre et al., 2005; Diabaté et al., 2009; Edillo et al., 2006; Kamdem et al., 2012; Lehmann & Diabate, 2008; Mattah et al., 2017)

Based on the results of this study, the use of mineral or mixed water resulting in hardness (TDS 70.5–112.2 mg/l, salinity 47.5–75.8 ppm, conductivity 100.6–160.4 µS) is recommended for rearing *An. coluzzii* and *An. gambiae* s.s. to ensure optimal qualitative yield. Powder feed is also recommended. Although the cost of mineral water may be an economic challenge in some settings, the results obtained show that a mix of mineral and deionised water produces a similar result as mineral water. Further research is needed to investigate if these gains are directly linked to mosquito longevity and fecundity as well as male competitiveness.

Chapter 4

Contrasted adaptive responses in *Anopheles gambiae* s.s. and *Anopheles coluzzii*

4.1. Introduction

The exploitation of a new environment by an organism can lead to a new set of environmental pressures that promote divergence of a novel sub-population and, eventually, sub-species from the ancestral species (Price, et al., 2003). Phenotypic variations shaped by genotype-by-environment (G×E) interactions which usually result in an improvement in growth, survival, or reproduction are collectively called phenotypic plasticity (Li, et al., 2018; Price et al., 2003). Plasticity could be adaptive; occurring when individuals showing a plastic response have higher fitness than those who do not. Evolution of life-history traits and their plasticity determine the population dynamics of interacting species (Stearns, 1992). Phenotypic plasticity has two vital roles in evolution; first, it modifies the relationship among given phenotypic traits and their fitness and therefore changes the selection pressures on traits across environments. Secondly, by regulating the expression of genetic variation (single traits) and genetic covariation (pairs of traits), it changes the genetic response to selection across environments (Stearns, 1992).

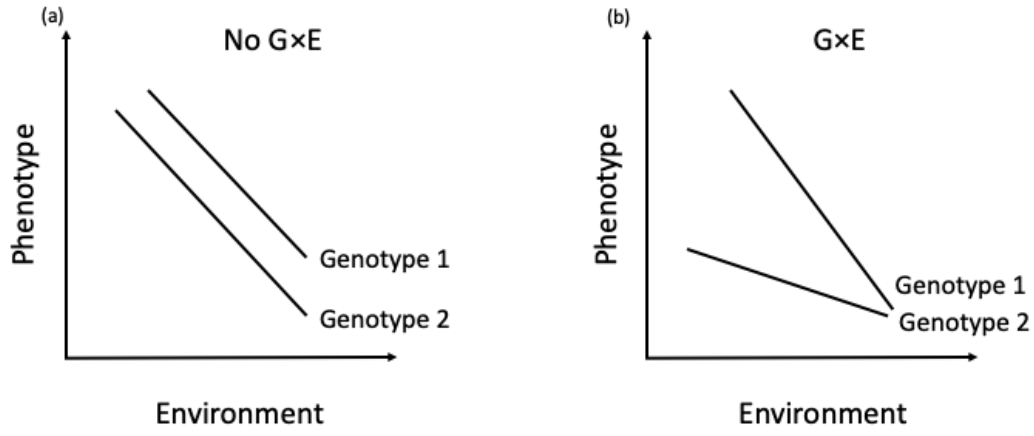

Figure 4.1: (a) Reaction norms of the genotypes present run parallel to each other and there is no genotype × environment interactions. (b) Reaction norms with different slopes represent genotype × environment interactions. (modified from Stearns, 1992).

These two processes highlight the fact that ecological speciation is often driven via contrasted selection regime acting on phenotypically plastic traits. Understanding reaction norms of traits associated with ecological speciation is thus key to appreciating their process of divergence (Stearns, 1992).

Sub-Saharan Africa is malaria-endemic, with 93% worldwide incidence and 85% of malaria mortality occurring in this region in 2018 (WHO, 2019). *Anopheles gambiae* s.s and *Anopheles coluzzii* are the major and efficient vectors of malaria in this region because of their close association with human dwellings with larval habitats resulting from anthropogenic sources; as well as their endophilic and endophagic behaviour (Lehmann & Diabate, 2008). Both species are morphologically identical and share similar resources, they are however uniquely divergent, owing to strong assortative mating with occasional hybrids and resultant low levels of genetic introgression leading to speciation (Aboagye-Antwi et al., 2015; Niang et al., 2015; Reidenbach et al., 2012). Genome-wide comparisons have shown that they are genetically-differentiated only over 3% of their genome; commonly known as 'speciation islands' (Santolamazza et al., 2015; White et al., 2010). In addition to assortative mating, larval ecological adaptations (drought resistance, larval predator tolerance, pollution tolerance, oviposition site preference, interspecific larval competition) have also been highlighted as drivers of eco-speciation (ecological speciation) in these species. (Dao et al., 2014; Kamdem et al., 2012; Roux, et al., 2013, 2014). The persistence of sympatric populations of *An. coluzzii* and *An. gambiae* s.s. occurring at the same time despite imperfect premating barriers suggests that other factors possibly ecological, contribute to their isolation (Roux et al., 2014; Tene Fossog et al., 2015).

Prior to 2013, *An. gambiae* s.s and *An. coluzzii* were considered a single species having two molecular forms known as *An. gambiae* s.s -M and S molecular forms

(Coetzee et al., 2013). This earlier classification into molecular forms was based on an effort to describe assortative mating populations of the former *An. gambiae* s.s populations which were distinguished by form-specific SNPs (single nucleotide polymorphism) in the rDNA intergenic spacer region (della Torre et al., 2001). The study highlighted the lack of association between their chromosomal constitution, suggesting ecotypic adaptation and speciation (della Torre et al., 2001). The M and S molecular forms displayed a significant lack of gene flow evidenced by the absence or rarity of hybrids and DNA genotypes, despite sharing similar chromosomal inversions and chromosome-2 karyotype (Fanello, et al., 2002; Favia et al., 2007; Tripet et al., 2001). Restricted gene flow between the molecular forms has been attributed to premating mechanisms of reproductive isolation, selection against hybrids and ecologically-driven divergent larval selection (Diabaté et al., 2009; Pennetier et al., 2010). Despite isolated cases of hybridisation in stable hybridization zones with secondary contact, further analysis of 400,000 SNPs across genomes of paired population samples of M and S from Mali, Burkina Faso and Cameroon led to the conclusion that the two taxa are evolving collectively on independent evolutionary trajectories (Caputo et al., 2011; Marsden et al., 2011; Oliveira et al., 2008; Reidenbach et al., 2012; Weetman et al., 2012). Based on these genomic evidences, the M and S molecular forms of the former *An. gambiae* s.s. were recognised as cohesive and exclusive taxonomic groups across their shared range, leading to their elevation to species status (Coetzee et al., 2013).

In West Africa, the main ecological distinction between the presumed ancestral *An. gambiae* s.s. and the derived *An. coluzzii* is their larval habitat. *An. coluzzii* preferably breeds in irrigated rice fields and its process of speciation may be strongly linked with that of rice domestication in Africa 3000- 3,500 years ago (Bambaradeniya & Amarasinghe, 2003; Chang, 1976; Li Zhi-Ming et al., 2011; Reidenbach et al., 2012). *An.*

gambiae s.s. on the other hand, prefers rain-fed larval habitats that are temporary (Diabate et al., 2005). The rice field ecosystem has facilitated changes in the bionomics of *An. coluzzii* arising from irrigation development and increased rice cultivation (Bambaradeniya & Amarasinghe, 2003; Reidenbach et al., 2012). In sub-Saharan Africa, rice fields serve as larval sites for *An. gambiae* s.l. throughout the rice planting season until harvest (Mwangangi et al., 2010). These observations have been made in Kenya (Mwangangi et al., 2006), Burkina Faso (Epopa et al., 2017; Sawadogo et al., 2017), The Gambia (Lindsay et al., 1991), Madagascar (Marrama et al., 2004), Senegal (Faye et al., 1995), and Mali (Dolo et al., 2004). This ability to exploit more permanent, predator-rich, freshwater habitats gives *An. coluzzii* a vectorial capacity advantage over *An. gambiae* s.s., since it can to breed and transmit *Plasmodium* all year-round compared to its seasonal sibling (Roux et al., 2014).

Pre-irrigational agriculture, it was assumed that populations of *An. coluzzii* (then *An. gambiae* s.l.) reduce their reproduction during the dry season and possibly aestivate at low densities to survive harsh environmental conditions allowing them to persist in arid areas (Adamou et al., 2011). However, intensive rice cultivation facilitated the persistence of larval breeding sites that better suit *An. coluzzii* larvae and promote its strong dominance throughout the year (Diabaté et al., 2009). According to a WHO review in 1954, attention to the presence of members of the *Anopheles* g*ambiae* complex in rice fields has been made since 1918 by Legendre and corroborated by Couvy in 1925 (Holstein, 1954) who concluded from his work in Madagascar that rice fields were a major cause of illness. In 1937, Monier established that cultivated rice field was a principal factor in the rapid breeding of *An. gambiae* s.l. (Holstein, 1954). Similar reports were made by Granger in Kisumu, Kenya, De Meillon (Holstein, 1954) in Mozambique, Bruce-Chwatt in Gambia amongst several corroborating reports leading to the conclusion

by that " wherever rice growing has been undertaken, gambiae had developed"(Holstein, 1954). This association, however, is not typical to rice fields as *An. gambiae* s.l. has been known to occur wherever there was plant cultivation, with resultant stagnant pools. Construction projects such as dams, wells and borrow pits have also serve as pullulation sites for *An. gambiae* s.l. (Holstein, 1954). Larval predation is more prevalent in *An. coluzzii* typical habitat (rice paddy) compared to the temporary, predator-free, shallow larval habitat of *An. gambiae* s.s. and is another major force prompting niche differentiation between the sibling species *An. coluzzii* and *An. gambiae* s.s. (Gimonneau et al., 2012; Munga et al., 2006).

The presence of ammonia in the rice paddies due to biological waste from its inhabitants is an important aspect of rice field ecosystem-chemistry. Water supply in irrigated rice paddies are sourced either from rivers or through rainfall and this influences the floodwater chemistry and composition of aquatic biota. Natural levels of ammonia in rice paddies range from 0.53 ± 0.1 mg/l and this depends on water supply and fluctuate with the changing activities throughout the planting season (Bambaradeniya & Amarasinghe, 2003; Baolan et al., 2012). Ammonia is a by-product of biological waste production in aquatic ecosystems; it is excreted as the end-product of protein metabolism and may be toxic if allowed to accumulate (Hargreaves, 1998). Aquatic organisms, including invertebrates, excrete mostly ammonia which permeates cell membranes easily due to its high solubility in water (Wright, 1995). For every gram of ammonia ingested, aquatic animals require 400ml of water for dilution to concentrations below toxicity (Wright, 1995). Ammonia concentrations between 0.05-1mg/l has been reported to result in reduction in catfish growth in aquatic ponds; with a 50% reduction at 0.5mg/l (Hargreaves, 1998). In a typical rice field (paddy), ammonia is generated as part of the nitrogen (N) cycle, a biogeochemical process in which nitrogen is converted to multiple

chemical forms include; N fixation, ammonification (N mineralisation), nitrification and denitrification (Figure 4.2). Lower levels of oxygen limits nitrification as well as nitrogen immobilisation (conversion of inorganic nitrogen to organic nitrogen by bacteria) resulting in the accumulation of ammonium in the soil/water (Ishii, et al., 2011). Ammonia in its un-ionized form (NH_3) is the most toxic to fish and aquatic invertebrates (Tene Fossog et al., 2013) with a toxicity limit of 0.2mg/l for most aquatic animals (Eddy, 2005; U.S. Environmental Protection Agency, 2013). The toxicity of un-ionised ammonia is a function of pH, temperature, and total ammonia concentration and the added buffering effect of minerals which masks any additional toxicity over pH 8 (Florescu, et al., 2011; Hargreaves, 1998). Toxicity increases at elevated pH and temperature, shifting the ionisation equilibrium to the toxic, un-ionised, gaseous form.

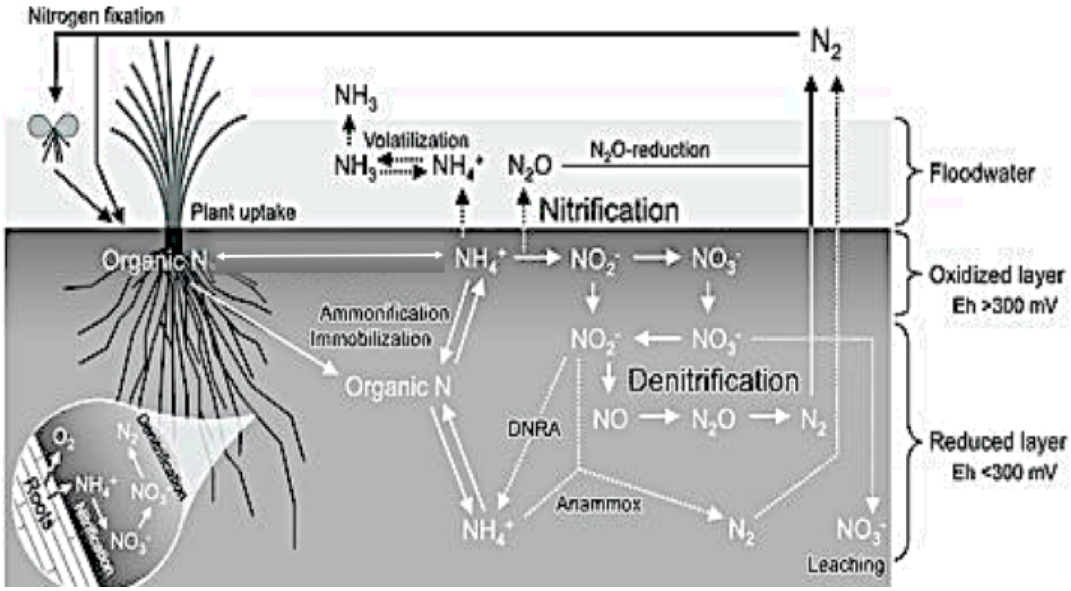

Figure 4.2: Overview of nitrogen cycling in rice paddy soils. Nitrogen fixation can occur in the surface water of rice paddies or rice rhizospheres, by free-living or plant-associated bacteria. When a plant or animal dies or expels waste, bacteria/fungi converts organic nitrogen to ammonium (ammonification/mineralisation). In the oxidized layer, ammonium mineralized from organic matter is oxidized to nitrate via nitrite (nitrification) by bacteria. Nitrate and nitrite can be diffused to the reduced layer where these compounds are reduced stepwise to gaseous end products by denitrification (NO, N_2O, and N_2). Ammonia volatilization may become large in rice paddy soils with high pH (>8.5). (Ishii, et al., 2011).

This risk of pH and temperature elevation is greater in poorly buffered (low alkalinity) ponds at the hottest point in the day (Hargreaves, 1998).

The hypothesis that the larval stages of *An. coluzzii* and *An. gambiae* s.s. exhibit contrasted responses to ammonia had been investigated in an extensive survey of *An. gambiae* s.l populations along gradients of urbanization in Yaounde, Cameroon (Tene Fossog *et al.*, 2013). This study used standardized acute toxicity bioassays to reveal that *An. coluzzii* has higher ammonia tolerance than *An. gambiae* s.s., a difference which may explain the distribution of sibling species in aquatic habitats (Tene Fossog *et al.*, 2013). The concentrations of ammonia used in the study were far higher than levels (0.53 ± 0.1 mg/l) that would be obtained from the pre-fertilization rice field ecosystem (Bambaradeniya & Amarasinghe, 2003; Baolan et al., 2012). Moreover, observations were made within a short period and life-history traits were not accounted for (Tene Fossog et al., 2013). Other studies investigating ammonia tolerance in *An. gambiae* s.l. focused on the relationship between tolerance and insecticide resistance (Tene Fossog et al., 2012).

The aim of this study was to investigate the effect of chronic exposure of immature stages of *An. gambiae* s.s. and *An. coluzzii* to ammonia on larval development into adulthood. The hypothesis was that there will be a significant difference in the plastic responses between *An. coluzzii* and *An. gambiae* s.s. to increasing levels of ammonia similar to those in rice paddies. First instar mosquito larvae were exposed to increasing ammonia concentrations until adult emergence. A second experiment was conducted to tease apart the potential carry-over effects of ammonia exposure at larval stage to adults on pupal mortality. Evidence of distinct phenotypic plastic response in long-colonised strains of the sibling species support the idea that ancestral contrasted norms of reaction to rice field like levels of ammonia may have played a role in their ecological speciation.

4.2. Materials and Methods

4.2.1. Mosquito strain

The Kisumu strain of *An. gambiae* s.s, colonized over 40 years ago, from the area of Kisumu, Kenya, East Africa and the 17-year-old Mopti strain of *An. coluzzii*, colonized in 2003 by the Lanzaro Laboratory (UC Davis) from the village of N'Gabacoro droit near Bamako, Mali, West Africa, were used for the experiments. The strains were maintained by the Tripet group in dedicated insectaries of the Centre of Applied Entomology and Parasitology (CAEP), Keele University, UK, as described in Chapter 2.

4.2.2 Effect of ammonia on *An. gambiae* s.s. and *An. coluzzii* development.

Experimental factor 1: Using a 3ml plastic pipette, ten first instar larvae of *An. coluzzii* and then, *An. gambiae* s.s., were placed in white styrofoam cups containing 300ml of water with variable test concentrations comprising of three distinct factors as described below:

Experimental factor 2: Mosquitoes were reared in two water types with different levels of hardness (i) deionised water which was sourced from a reverse osmosis unit (PURELAB Prima, Wycombe, United Kingdom) installed in the laboratory. The water quality specifications of treated deionised water were: Total organic carbon < 0.1ppm, bacteria < 5 CFU/ml, 98% rejection of inorganics, > 99% rejection of organics, > 99% rejection of particles. (ii) mineral water: bottled water containing minerals which are natural compounds formed through geological processes, sourced from a local shop with the following typical nutrient values/litre: calcium (11 mg), magnesium (3.5 mg), potassium (2.5 mg), sodium (10 mg), bicarbonate (25 mg), sulphate (11 mg), nitrate (15 mg), chloride (14 mg), dry residue at 180 °C (85 mg) and pH (6.2).

Experimental factor 3: For each water type and feeding regimes, larval cups were reared in 7 ammonia (NH_3) concentrations: 0mg/l (control); 0.6mg/l; 1.3mg/l; 2.5mg/l; 12.5mg/l, 25mg/l and 62.5mg/l, informed by published reports of natural levels of ammonia in rice field flood water equivalent or higher than 0.5mg/l and toxicity level of unionised ammonia to freshwater aquatic organism at concentration above 0.2mg/l (Hargreaves, 1998; Eddy F B, 2005; Florescu *et al.*, 2011; Baolan *et al.*, 2012; U.S. Environmental Protection Agency, 2013). A stock solution of ammonium hydroxide (Sigma-Aldrich, Missouri, USA) equivalent to ~ 28-30% NH_3 at 14.8M was diluted to 1% (~2.5g/l) by adding the proportionate volume of water (using a fume hood and gloves). Final NH_3 concentration of 1% working solution was calculated using the molecular weight of NH_3 = 17.034 (14.01- Nitrogen, 1.008 x 3- Hydrogen):

$1M\ of\ NH_3 = 17.034\ g/l \therefore 14.8M = (14.8 \times 17.034)\ g/l = 252.10\ g/l \therefore$

$1\% \sim 2.5 g/l$

Further dilutions were made to achieve the 7 test concentrations of ammonia using the following dilution formula in mg/l: $C1V1 = C2V2$, where $C1$ (initial concentration) = 2.5g/l; $V1$ (initial volume of working solution) = 0µl(control), 250µl, 500µl, 1000µl, 5ml, 10ml, and 25ml respectively; $C2$ = desired concentration of NH_3 and $V2$ (final volume) = 1000ml.

Experimental factor 4: Larvae were fed with two standardised feeding regimes (solution and powder feed). Powder feeding regime consists of daily rations of ground-fish food, using a spatula to spread on the water surface: 0.1 µl of Liquifry liquid fish food (Interpret Ltd, Surrey, UK) on day 1, 2 mg on days 2–3, 4 mg on day 4, and 10 mg on day 5 until pupation. Solution feeding regime consist of the same food quantity dissolved in deionized water (0.1 µl of Liquifry on day 1, 0.1 ml of 1 g/50 ml of TetraMin Baby on

days 2–3, 0.2 ml of 1 g/50 ml of TetraMin Baby on day 4, and 0.5 ml of 1 g/50 ml of TetraMin Baby on day 5 until pupation) and injected into the larval pot using a pipette. Individual pipettes were used for the strains/treatment group and also for the controls (0.0mg/l) to avoid contamination. The experimental pots were completely randomized to ensure there was no bias towards lighting, ventilation, humidity or other factors in the insectary that variates with space. Observations were done “blindly” by using codes instead of the name of the strains for labelling. This was to minimize bias from the researcher tilting results in favour of known facts. A replicate consisted of the two species exposed to the three experimental factors (2 species x 7 NH_3 concentrations x 2 water types x 2 feeding pattern = 56 treatment pots × 10 larvae = 560 larvae). A total of 1680 (560 × 3) larvae were sampled in 3 replicates. Larvae from each experimental group were transferred to freshwater (same water source as the original set-up) containers daily until pupation. At pupation, pupae were transferred to a sister netted cup containing 100ml of "rearing water"(same water source as the original set-up).

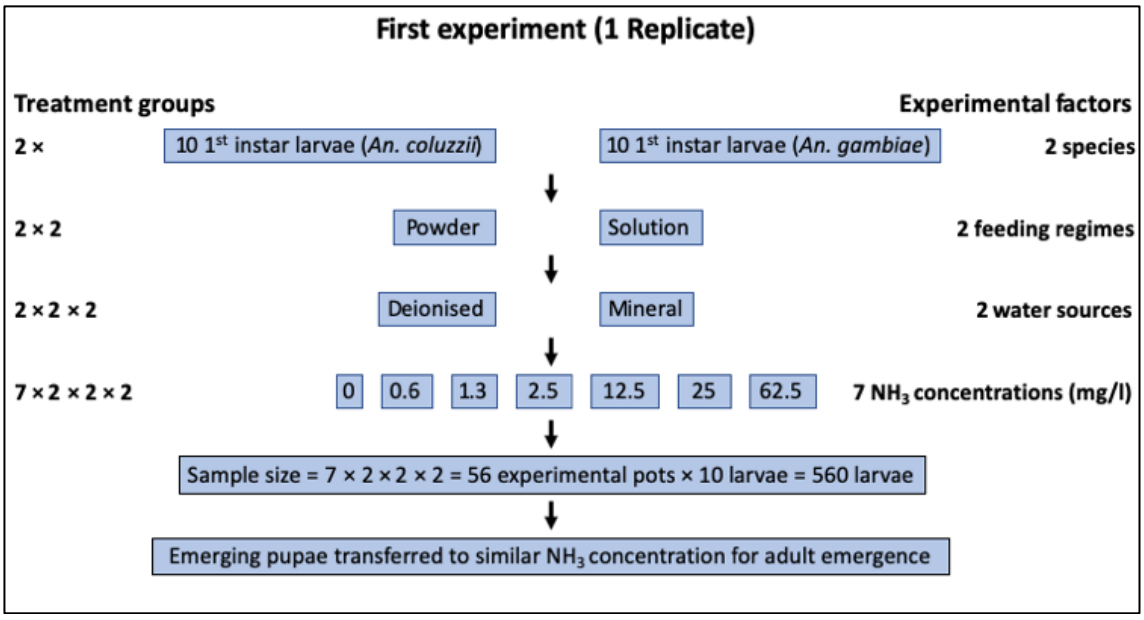

Figure 4.3: Experimental set-up for the effect of NH_3 concentrations on *An. coluzzii* and *An. gambiae* s.s. development.

Collection of data at life-cycle stages

Depending on the life-cycle stage of the mosquitoes, the following data were observed and recorded: (i) larval survival: determined as the percentage of larvae that developed into pupae from the total number of larvae for each treatment; (ii) pupal survival: determined as the percentage of mosquitoes that emerged as adults from those that pupated in each treatment. (iii) pupal mortality: determined as the percentage of mosquitoes that died at the pupal stage from the total number of mosquitoes per treatment; (iv) adult emergence: determined as the percentage of mosquitoes that emerged as adults from the total number of larvae in each treatment; (v) development time: determined as the number of days from placement of first instar larvae in treatment cups until adult emergence; and (vi) wing-length: following emergence, adult mosquitoes were sexed and stored in 75% ethanol and wing-length was subsequently measured as described in Chapter 2.

4.2.3 Carry-over effect of ammonia on *An. coluzzii* adults

Due to the deviation from the expected result in the first experiment (*An. coluzzii* had significantly higher larval mortality, consequently lower adult emergence but with higher pupal survival compared to *An. gambiae* s.s.), a second experiment to investigate any carryover effect of ammonia exposure at the larval stage on *An. coluzzii* adults was designed, focusing on the effect of ammonia and water types on pupation. The experimental set-up was similar to that described in section 4.2.2. using three NH_3 concentrations (2.5mg/l, 12.5mg/l and 25mg/l) for larval rearing. An experimental set-up of 1 species × 2 feeding regimes × 2 water sources × 3 NH_3 concentrations = 120 larvae × 4 replicates = 480 larvae (Figure 4.4). However, at pupation, pupae were split into two groups, one set placed in “rearing water”, the other set placed in the same water type as

before, but without ammonia. Data were collected at all life stages and adult body size determined as before.

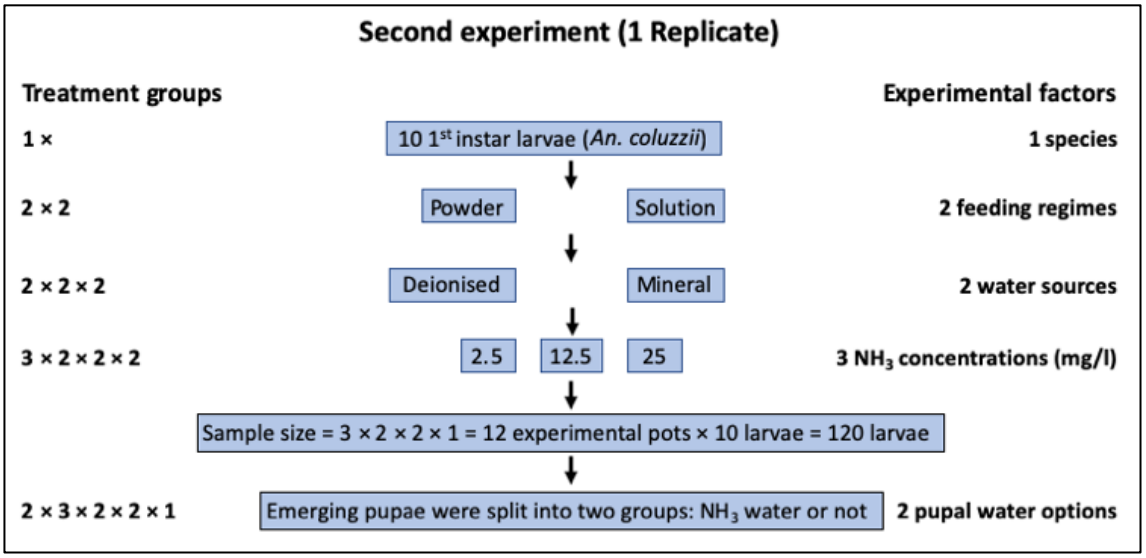

Figure 4.4: Experimental set-up for the carry-over effect of ammonia concentrations on *An. coluzzii* adults.

4.2.4. Water quality analysis

Physicochemical water parameters; nitrate, ammonia, pH, general hardness and carbonate hardness were measured using API aquarium test kits (Aquarium Pharmaceuticals, Mars Fishcare, North America, 50E Hamilton St., Chalfont, PA,18914, USA). Change in pH was indicated by colour change with precise readings to 7.6 after which increasing darkening colour changes were indicated with ordinal symbols (+) showing increasing pH. Readings were taken 10 days after the experiment set-up (Table 4.1).

4.2.5. Statistical analysis

All data collected were analysed using the software JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA). All data were checked for deviations from normality and heterogeneity, and analyses were conducted using parametric and non-parametric

methods as appropriate. Data from all replicates were used for analysis, replicate effects were tested but were only reported when significant. Interactions between independent variables were tested using step-wise models and only those significant were retained in the final models. For analyses of proportion of larvae, pupae and adults, likelihood odds ratios were used for *post-hoc* pairwise group comparisons following logistic regressions. Body size was analysed through general linear models followed by Tukey's HSD *post-hoc* pairwise comparisons. Finally, developmental times (day of emergence) were analysed by Cox Proportional-Hazard models with likelihood odds ratios for *post-hoc* pairwise comparisons.

Table 4.1: Mean of nitrate, General hardness, Carbonate hardness, pH and Ammonia

Water type	NH_3	Nitrate (mg/l)	Ammonia (mg/l)	General hardness (mg/l)	Carbonate hardness (mg/l)	pH
Deionised	0	5	1	17.9	17.9	7.05
	0.6	5	3	17.9	17.9	7.05
	1.3	5	8	17.9	17.9	7.05
	2.5	5	-	17.9	35.8	7.2
	12.5	5	-	17.9	89.5	7.6
	25	-	-	-	-	-
Mineral	0	5	1.5	53.7	35.8	7.4
	0.6	5	2	53.7	35.8	7.6
	1.3	5	8	53.7	35.8	7.6+
	2.5	5	-	53.7	53.7	7.6 ++
	12.5	10	-	53.7	89.5	7.6+++
	25	10	-	35.8	125.3	7.6++++

Notes: Sample size=12. Beyond pH 7.6, + show increasing darkening in colour change and pH of the solution without precise measurement of concentration.

4.3. Results

4.3.1. Plastic response of *An. gambiae* s.s. and *An. coluzzii* in response to ammonia exposure.

Overall, a decline in larval survival was inversely proportional to the increase in NH_3 concentration (Figure 4.5; Table 4.2). Significant decreases in larval survival was observed from 1.3mg/l to 25mg/l NH_3, with no larvae surviving at 62.5mg/l. There was a significant difference ($P < 0.0001$) for the percentage of larval survival between *An. gambiae* and *An. coluzzii* with the former having 14% higher larval survival across NH_3 concentrations and in both water types, deionised and mineral water (Figure 4.6; Table 4.3).

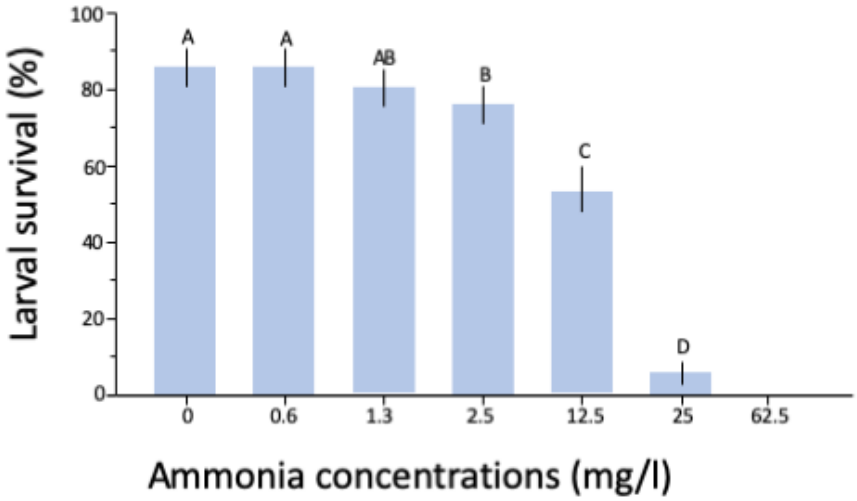

Figure 4.5: Effect of NH_3 on larval survival. Bar plots show the percentage of larval survival across seven NH_3 concentrations. Bar plots sharing the same letter are not significantly different. Whiskers represent 95% confidence intervals.

Logistic regression model did not reveal an effect of feed regime on larval survival (Table 4.3). Larval survival in mineral water was 12% higher ($P < 0.0001$) than in deionised water for both species and across NH_3 concentrations (Figure 4.5, Table 4.3). Interactions between water types and NH_3 significantly ($P < 0.0001$) impacted larval survival at 2.5mg/l and 12.5 mg/l for *An. coluzzii* and at 12.5mg/l for *An. gambiae*. There was significantly higher larval survival in mineral water at these concentrations (Figure

4.6). *An. gambiae* larvae reared at 25mg/l NH_3 only survived in mineral water; all larvae died in deionised water for this concentration (Figure 4.6).

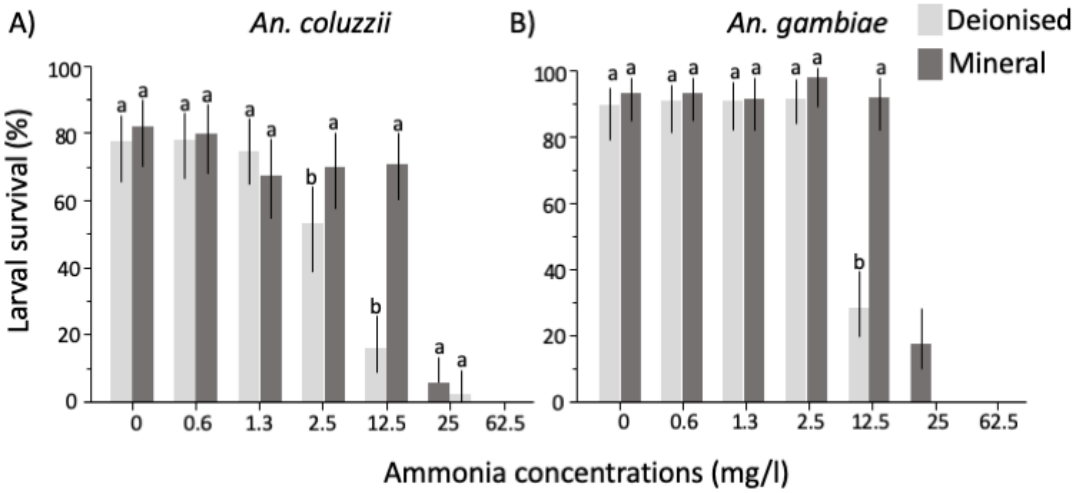

Figure 4.6: Effect of NH_3 and water types on larval survival. The percentage larval survival for *An. coluzzii* and *An. gambiae* s.s. for both deionised water (light grey) and mineral water (dark grey). Bar plots sharing the same letter are not significantly different for each concentration of NH_3. Whiskers represent 95% confidence intervals.

Pupal mortality was significantly impacted by NH_3 ($P < 0.0001$) concentrations (during larval stage) with an increase in pupal mortality with increasing NH_3 concentrations (Table 4.2;4.3; Figure 4.7). Overall, pupal mortality was 4% higher in *An. gambiae* s.s. compared to *An. coluzzii* and this was significant ($P = 0.0052$) across NH_3 concentrations (Tables 4.2; 4.3). Pupal mortality was 1% ($P = 0.0363$) higher in deionised water at higher NH_3 concentrations (Figure 4.7; Tables 4.2 4.3). From 2.5mg/l upwards, there was a significant increase in pupal mortality for both species (Table 4.2, 4.3; Figure 4.7). To investigate these interactions further, a second experiment was designed to ascertain their effect on pupal mortality (section 4.3.2).

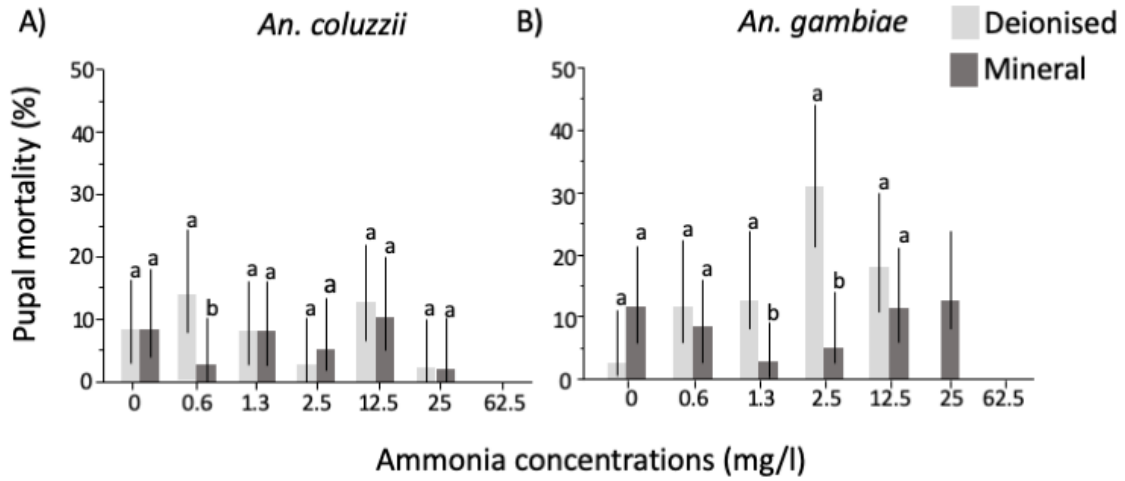

Figure 4.7: Effect of NH_3 and water types on larval pupal mortality. The percentage pupal mortality for *An. coluzzii* and *An. gambiae* for both deionised water (light grey) and mineral water (dark grey). Bar plots sharing the same letter are not significantly different for each concentration of NH_3. Whiskers represent 95% confidence intervals.

Table 4.2: Effect of NH_3, water types and feed regimes on life history stages.

Species	Water type	Feed regime	NH_3 (mg/l)	%Larval survival	%Pupal mortality	%Adult emergence
An. coluzzii	Deionised	Solution	0	77 (59-88)	10 (3-26)	67 (49-81)
			0.6	73 (56-86)	0	73 (56-86)
			1.3	70 (52-83)	13 (5-30)	57 (39-73)
			2.5	57 (39-73)	3 (1-17)	53 (36-70)
			12.5	33 (19-51)	23 (12-41)	10 (3-26)
			25	3 (0-17)	3 (0-17)	0
			62.5	0	0	0
		Powder	0	77 (59-88)	3 (1-17)	73 (56-86)
			0.6	83 (66-93)	27 (14-44)	57 (39-73)
			1.3	80 (63-90)	0	80 (63-90)
			2.5	50 (33-67)	3 (1-17)	47 (30-64)
			12.5	0	0	0
			25	0	0	0
			62.5	0	0	0
	Mineral	Solution	0	80 (63-90)	7 (2-21)	73 (56-86)
			0.6	80 (63-90)	3 (1-17)	77 (59-88)
			1.3	73 (56-86)	10 (3-26)	63 (46-78)
			2.5	67 (49-81)	3 (1-17)	63 (46-78)
			12.5	73 (56-86)	3 (1-17)	70 (52-83)
			25	10 (3-26)	3 (1-17)	7 (2-21)
			62.5	0	0	0
		Powder	0	83 (66-93)	10 (3-26)	73 (56-86)
			0.6	80 (63-90)	3 (1-17)	77 (59-88)
			1.3	60 (42-75)	3 (1-17)	57 (39-73)

Species	Water type	Feed regime	NH_3 (mg/l)	%Larval survival	%Pupal mortality	%Adult emergence
			2.5	73 (56-86)	7 (2-21)	67 (49-81)
			12.5	70 (52-83)	17 (7-34)	53 (36-70)
			25	0	0	0
			62.5	0	0	0
An. gambiae	Deionised	Solution	0	86 (70-95)	7 (2-21)	80 (63-90)
			0.6	97 (83-99)	10 (3-26)	87 (70-95)
			1.3	90 (74-97)	0	90 (74-97)
			2.5	87 (70-95)	17 (13-34)	70 (52-83)
			12.5	43 (27-61)	23 (12-41)	20 (10-37)
			25	0	0	0
			62.5	0	0	0
		Powder	0	93 (79-98)		93 (79-98)
			0.6	87 (70-95)	13 (5-30)	73 (56-86)
			1.3	93 (79-98)	27 (14-44)	67 (49-81)
			2.5	100(89-100)	47 (30-64)	53 (36-70)
			12.5	13 (5-30)	13 (5-30)	0
			25	0	0	0
			62.5	0	0	0
	Mineral	Solution	0	93 (79-98)	17 (7-34)	77 (59-88)
			0.6	97 (83-99) 30	10 (3-26) 30	87 (70-95) 30
			1.3	87 (70-95)	3 (1-17)	83 (66-93)
			2.5	100(89-100)	0	100(89-100)
			12.5	93 (79-98)	10 (3-26)	83 (66-93)
			25	13 (5-30)	7 (2-21)	7 (2-21)
			62.5	0	0	0
		Powder	0	93 (79-98)	7 (2-21)	87 (70-95)
			0.6	90 (74-97)	3 (1-7)	87 (70-95)
			1.3	97 (83-99)	0	97 (83-99)
			2.5	93 (79-98)	10 (3-26)	83 (66-93)
			12.5	90 (74-97)	13 (5-30)	77 (59-88)
			25	20 (10-37)	20 (10-37)	0
			62.5	0	0	0

Notes: Ninety-five percent confidence intervals are in parentheses. Larval survival, pupal mortality and emergence rates were calculated out of an initial sample size of 30 larvae (per treatment).

As a reflection of the effect on the overall mosquito survival, regression models on adult emergence revealed significant ($P < 0.0001$) decline in survival with increasing NH_3 concentration (Table 4.2, 4.3; Figure 4.8). At 1.3mg/l, 12.5mg/l and 25mg/l, adult emergence reached significant levels of reduction compared to lower concentrations of NH_3 (Figure 4.8). The percentage of adult emergence in *An. gambiae* s.s. (11% higher)

were significantly ($P < 0.0001$) higher than that of *An. coluzzii* for both water types and across NH_3 concentrations (Table 4.3; Figure 4.9).

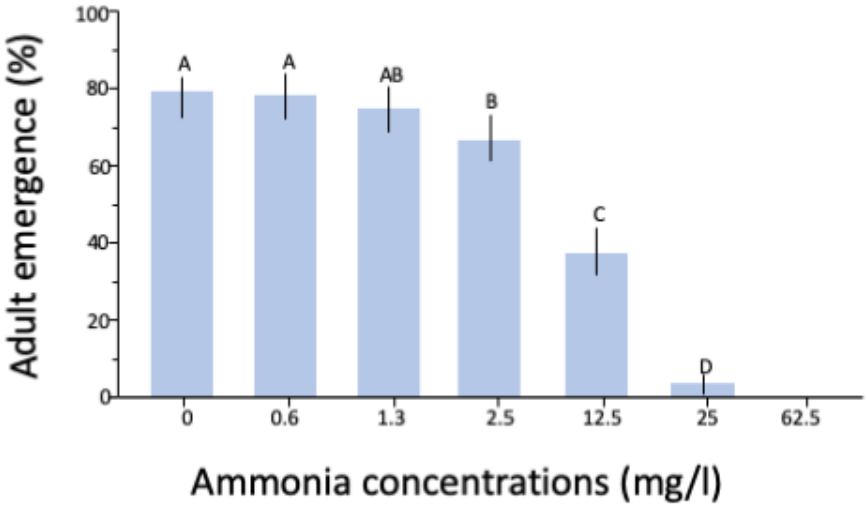

Figure 4.8: Effect of NH_3 on adult emergence. Bar plots show the percentage adult emergence across six NH_3 concentrations. Bar plots sharing the same letter are not significantly different. Whiskers represent 95% confidence intervals.

Significant interactions ($P < 0.0001$) between water type and NH_3 impacted on adult emergence at 2.5mg/l and 12.5mg/l. Overall, adult emergence was significantly higher (23% and 63% higher respectively) in mineral water at these concentrations compared to emergence in deionised water. At 25mg/l, adults only emerged from the mineral water set-up but not from the deionised water (Figure 4.9, Table 4.2). At 2.5mg/l, solution feed yielded significantly ($P = 0.0141$) higher adults compared to powder feed for *An. gambiae* (Figure 4.10, Table 4.3). At 25mg/l, adults-only emerged from solution feed but not from powder feed.

There was significant ($P < 0.0001$) decrease in adult body size (represented by wing-length) with an increase in NH_3 concentration (Tables 4.4, 4.5). Adults emerging from mineral water were significantly ($P < 0.0001$) bigger than those from deionised water (Table 4.5, Figure 4.11) for both species and across NH_3 concentrations.

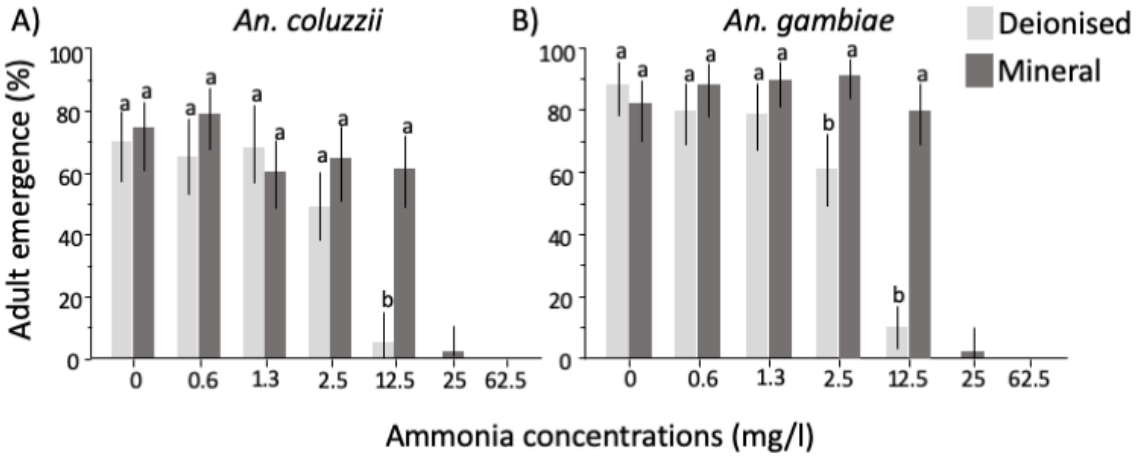

Figure 4.9: Effect of NH_3 and water types on adult emergence. The percentage adult emergence for *An. coluzzii* and *An. gambiae* s.s. for both deionised water (light grey) and mineral water (dark grey). Bar plots sharing the same letter are not significantly different for each concentration of NH_3. Whiskers represent 95% confidence intervals.

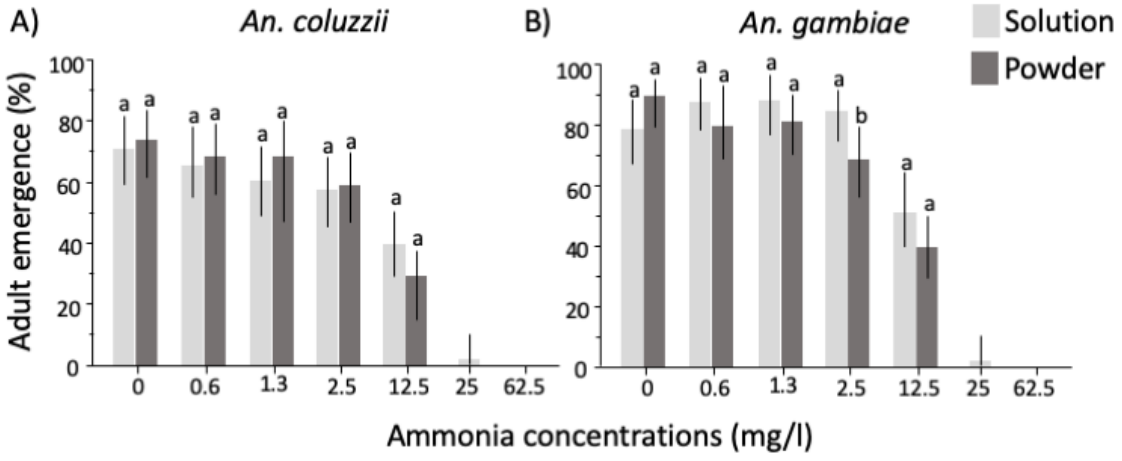

Figure 4.10: Effect of NH_3 and feed regimes on adult emergence. The percentage adult emergence for *An. coluzzii* and *An. gambiae* s.s. for both solution feed (light grey) and powder feed (dark grey). Bar plots sharing the same letter are not significantly different for each concentration of NH_3. Whiskers represent 95% confidence intervals.

Table 4.3: Logistic regressions of the effect of NH_3, water types and feed regimes on life history stages.

Parameter	Source	DF	Likelihood ratio	P-value
Larval survival	Species	1	20.614	<0.0001***
	Water type	1	78.060	<0.0001***
	Feed	1	1.565	0.2109^{ns}
	NH_3 at larval stage	1	1072.061	<0.0001***
	NH_3 * Water type	1	31.720	<0.0001***
	NH_3 * Species	1	7.325	0.0068**
Pupal mortality	Species	1	8.062	0.0045**
	Water type	1	4.328	0.0375*
	Feed	1	1.286	0.2567^{ns}
	NH_3 at larval stage	1	32.472	<0.0001***
Adult emergence	Species	1	37.496	<0.0001***
	Water type	1	92.267	<0.0001***
	Feed	1	3.872	0.0491*
	NH_3 at larval stage	1	941.705	<0.0001***
	NH_3 * Water type	1	43.053	<0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

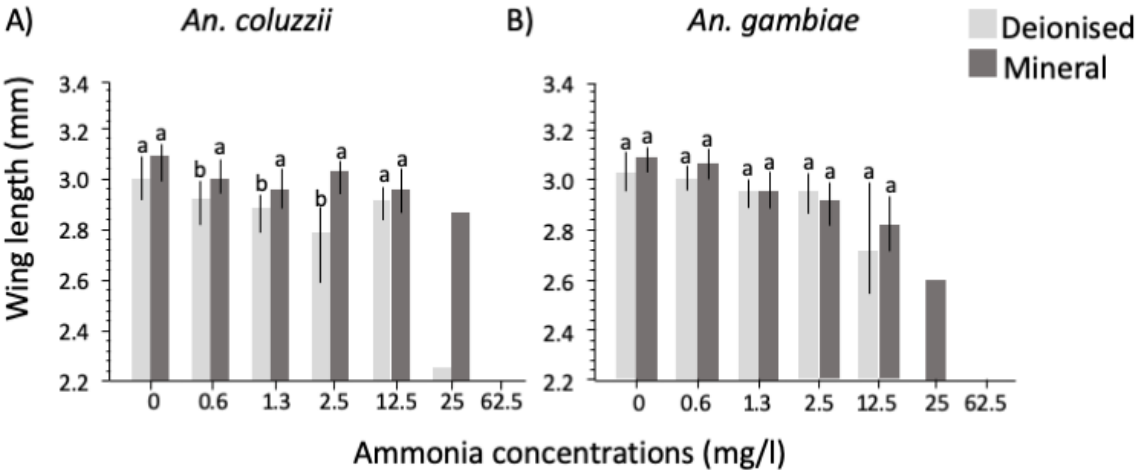

Figure 4.11: Effect of NH_3 wing-length. The mean wing-length for *An. coluzzii* (light grey) and *An. gambiae* s.s. (dark grey) shown for two water types (A and B). Whiskers represent 95% confidence intervals.

Significant interactions (P = 0.0398) between species and water type impacted on wing-length from 2.5mg/l upwards. In mineral water, *An. coluzzii* wing-length was significantly longer than *An. gambiae* s.s. and vice versa in deionised water (Figure 4.11; Tables 4.4; 4.6). Feed regime had no significant impact on wing-length and overall, the sibling species were not significantly different in adult body size (Table 4.5).

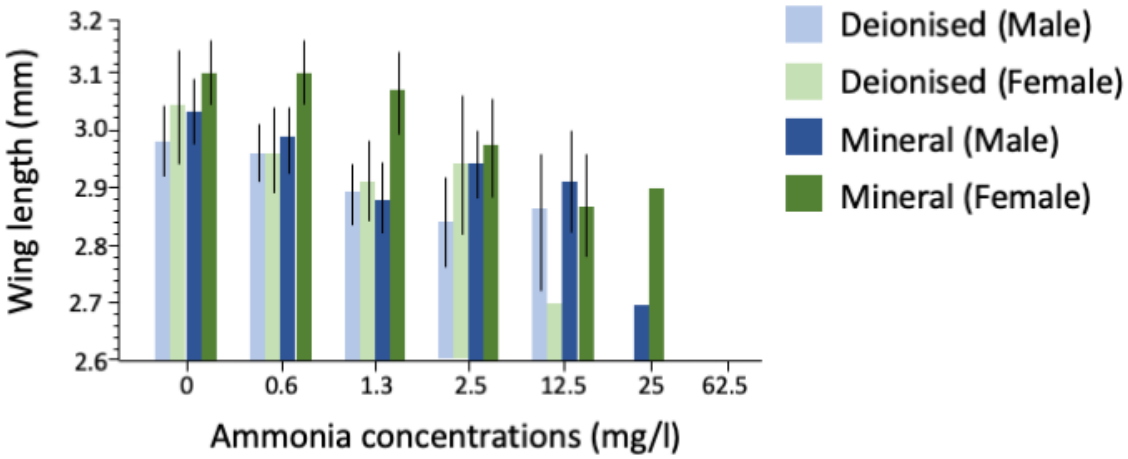

Figure 4.12: Effect of NH_3 on mosquito adult body size by gender. The mean wing-length of adult males in deionised water (light blue), mineral water (dark blue) and females in deionised water (light green), mineral water (dark green). Whiskers represent 95% confidence intervals.

Overall, females were significantly bigger than males, whereas, females reared in mineral water were significantly bigger than other groups (Figure 4.12; Table 4.5). Interactions between water type and species (P = 0.0087), NH_3 and species, sex and NH_3, significantly impacted on the sex ratio of emerged adults (Table 4.5). Post-hoc tests (Tukey HSD pairwise comparisons) revealed significant differences in wing-length in adults emerging from *An. coluzzii*/deionised and *An. gambiae*/deionised, *An. coluzzii*/deionised and *An. coluzzii*/mineral, lastly between, *An. coluzzii*/deionised and *An. gambiae*/mineral (Table 4.6).

Table 4.4. Effect of NH_3, water types and feed regimes on body size (wing length) and day of emergence.

Species	Water type	Feed regime	NH_3 (mg/l)	Mean wing length (mm)	Days till emergence
An. coluzzii	Deionised	Solution	0	3.00 (2.86–3.13) *20*	9.85 (9.41–10.29) 20
			0.6	2.95 (2.86-3.04) *22*	9.77 (9.44-10.11) *22*
			1.3	2.81 (2.68-2.93) *17*	10.24 (9.67-10.80) *17*
			2.5	2.81 (2.66-2.95) *16*	9.75 (9.25-10.25) *16*
			12.5	2.9 (2.24-3.56) *3*	11.33 (9.89-12.77) *3*
			25	0	0
			62.5	0	0
		Powder	0	3.00 (2.87–3.11) *22*	9.82 (9.52–10.11) *22*
			0.6	2.85 (2.74-2.97) *17*	9.76 (9.38-10.15) *17*
			1.3	2.88 (2.77-2.98) *24*	10.42 (10.14-10.69) *24*
			2.5	2.76 (2.58-2.94) *14*	10.29 (9.81-10.76) *14*
			12.5	0	0
			25	0	0
			62.5	0	0
	Mineral	Solution	0	3.07 (2.98–3.16) *22*	9.64 (9.31–9.96) *22*
			0.6	2.98 (2.89-3.07) *23*	9.74 (9.44-10.04) *23*
			1.3	2.92 (2.80-3.04) *19*	9.37 (9.13-9.61) *19*
			2.5	3.04 (2.94-3.13) *19*	9.68 (9.36-10.01) *19*
			12.5	2.97 (2.88-3.07) *21*	9.67 (9.40-9.93) *21*
			25	2.90 (2.90-2.90) *2*	10.5 (-8.56-29.56) *2*
			62.5	0	0
		Powder	0	3.03 (2.93–3.13) *22*	9.86 (9.40–10.32) *22*
			0.6	3.04 (2.95-3.14) *23*	9.43 (9.14-9.72) *23*
			1.3	3.01 (2.86-3.16) *17*	9.06 (8.77-9.34) *17*
			2.5	2.98 (2.92-3.04) *20*	9.50 (9.22-9.78) *20*
			12.5	2.93 (2.75-3.10) *16*	9.63 (9.30-9.95) *16*
			25	0	0
			62.5	0	0
An. gambiae	Deionised	Solution	0	2.97 (2.86–3.08) 24	10.38 (10.10–10.65) 24
			0.6	3.02 (2.95-3.08) 26	10.15 (9.91-10.40) 26
			1.3	2.97 (2.89-3.04) 27	10.41 (10.16-10.66) 27
			2.5	2.95 (2.83-3.07) 21	10.29 (10.08-10.50) 21
			12.5	2.77 (2.55-2.98) 6	12.67 (11.23-14.10) 6
			25	0	0
			62.5	0	0
		Powder	0	3.07 (2.96–3.18) *28*	10.36 (10.00–10.71) *28*
			0.6	2.99 (2.91-3.08) *22*	10.23 (9.96-10.50) *22*
			1.3	2.92 (2.84-2.99) *20*	10.35 (10.12-10.58) *20*
			2.5	2.93 (2.81-3.05) *16*	10.75 (10.51-10.99) *16*
			12.5	0	0
			25	0	0
			62.5	0	0
	Mineral	Solution	0	3.06 (2.98 –3.14) *23*	9.43 (9.22–9.65) *23*
			0.6	3.05 (2.98-3.13) *26*	9.85 (9.60-10.09) *26*
			1.3	2.98 (2.90-3.06) *25*	10.08 (9.88-10.28) *25*
			2.5	2.82 (2.72-2.93) 30	9.60 (9.39-9.81) 30
			12.5	2.88 (2.76-2.99) *25*	10.76 (10.51-11.01) *25*
			25	2.6 (-2.48-7.68) *2*	11.50 (5.15-17.85) *2*
			62.5	0	0

Species	Water type	Feed regime	NH_3 (mg/l)	Mean wing length (mm)	Days till emergence
		Powder	0	3.08 (3.00–3.17) *26*	9.50 (9.29 – 9.71) *26*
			0.6	3.06 (2.98-3.15) *26*	9.54 (9.28-9.80) *26*
			1.3	2.95 (2.84-3.05) *29*	9.79 (9.61-9.98) *29*
			2.5	3.02 (2.90-3.14) *25*	9.48 (9.27-9.69) *25*
			12.5	2.81 (2.67-2.94) *23*	10.35 (10.14-10.56) *23*
			25	0	0
			62.5	0	0

Notes: Ninety-five percent confidence intervals are in parentheses and the samples sizes, the number of surviving individuals out of an initial number of 30 larvae are italicized.

Table 4.5: General linear model of the effect of NH_3, water types and feed regimes on wing length

Parameter	Source	df	F-ratio	P-value
Wing-length	Species	1	3.1706	0.0754^{ns}
	Feed	1	0.4523	0.5014^{ns}
	NH_3	1	30.6251	<0.0001***
	Water type	1	15.8197	<0.0001***
	Sex	1	11.3657	0.0008**
	Sex*NH_3	1	5.2245	0.0225*
	NH_3*Species	1	6.6604	0.0100*
	Water type*Species	1	6.9135	0.0087*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

Table 4.6: Tukey's HSD pairwise comparison of the effect of NH_3, water types and feed regimes on wing -length

Source	Standard Error	P-value
An. coluzzii/deionised vs *An. gambiae*/deionised	0.027	0.0191*
An. coluzzii/deionised vs *An. coluzzii*/mineral	0.027	<0.0001***
An. coluzzii/deionised vs *An. gambiae*/mineral	0.025	0.0003**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

The development time of emerged adults was significantly impacted by water type and increasing NH_3 concentrations (Table 4.7). The duration from first instar larvae to adult increased with increasing NH_3 concentrations (Figure 4.13).

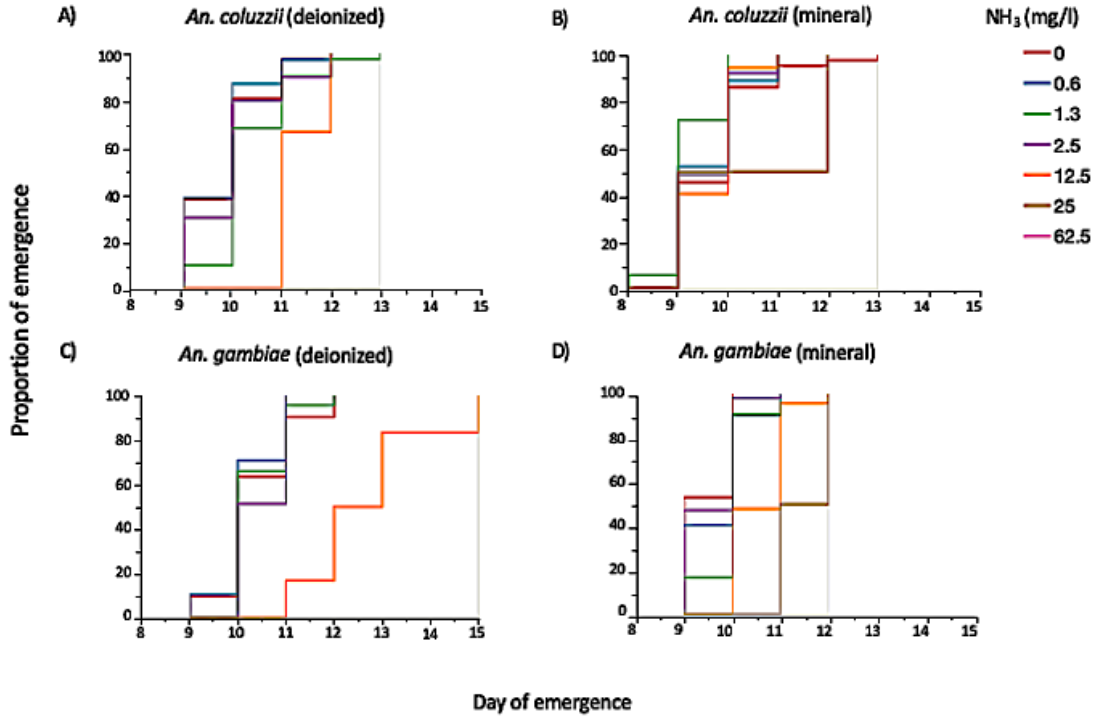

Figure 4.13: Effect of NH_3 on development time. The mean development time for *An. coluzzii* **(A,B)** and *An. gambiae* **(C,D)** reared in deionised and mineral water across 7 concentrations of NH_3.

Table 4.7: Cox Proportional-Hazard analyses of development time

Parameter	Source	df	Wald Chi Square	P-value
Day of emergence	Species	1	9.272	0.0023**
	Water type	1	48.369	<0.0001***
	Feed	1	0.607	0.4361^{ns}
	NH_3	1	19.552	<0.0001***
	NH_3*Species	1	5.74302292	0.0166*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

Mosquitoes reared in deionised water took longer to complete their development cycle (Figure 4.11). At higher concentrations (from 12.5mg/l), development time was markedly longer in deionised water compared to mineral water. There was also a significant interaction between the effect of species and NH_3 concentrations on development time.

In deionised water, at 12.5mg/l NH_3, *An. coluzzii* emerged two days earlier than *An. gambiae* s.s. (Figure 4.13;Table 4.7).

4.3.2. Carry-over effect of NH_3 exposure on *An. coluzzii.*

The second experiment on the carry-over effect of NH_3, water types and feed regimes revealed a similar pattern as before on mosquito development. Larval survival was higher in mineral water, significantly so at higher NH_3 concentrations (12.5mg/l) compared to survival in deionised water at the same NH_3 concentration. There were no significant differences in larval survival, pupal mortality and adult emergence among the two water types for mosquito larval stage at 2.5mg/l NH_3 concentration. At 12.5mg/l NH_3 (larval stage) concentration, there was 100% pupal mortality for all mosquitoes reared in deionised water, hence no adult emergence from that water type at that concentration. Inversely, adult emergence was observed in mineral water at 12.5mg/l NH_3 (larval stage) concentration (Figure 4.14; Tables 4.8; 4.9).

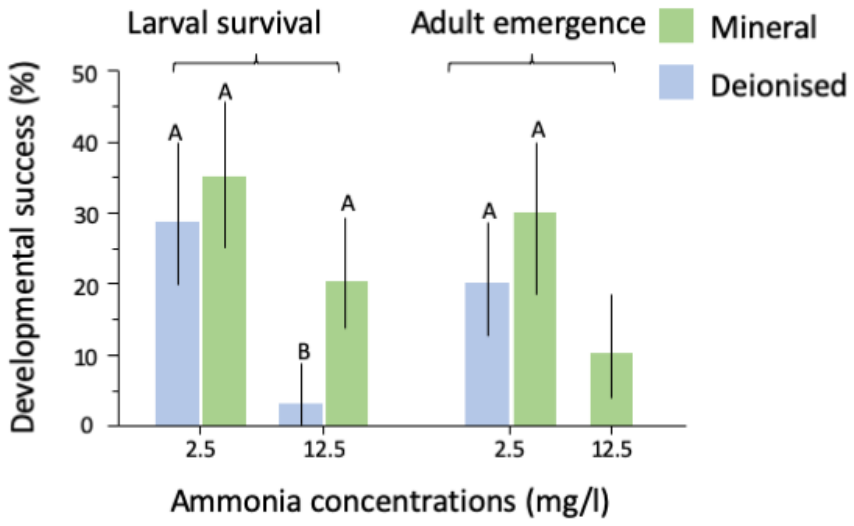

Figure 4.14: Effect of NH_3 on mosquito life-history stages. Percentage larval survival, pupal mortality and adult emergence are shown for mineral (blue) and deionised (green) water types. Whiskers represent 95% confidence intervals. Bar plots sharing the same letter are not significantly different for each concentration of NH_3.

At 25mg/l there was 100% mortality in all experimental groups for both species (Table 4.8). Although NH_3 concentrations at the larval stage were significant for pupal mortality, there was no significant effect of the presence/absence of NH_3 in the water in which the pupae emerged to adult (Table 4.8; 4.9). Pupation in NH_3 or not, did not significantly impact adult emergence in this carry-over effect experiment, therefore the impact of NH_3 was carried over from the larval stage to adult emergence (Table 4.9). Overall, solution feed was significantly more favourable for larval survival compared to powder feed (Table 4.8;4.9, Figure 4.15)

Table 4.8: Effect of NH_3, water types and feed on mosquito development

Species	Water type	Feed regime	NH_3 (mg//)	%Larval survival	%Pupal mortality	%Adult Emergence
An. coluzzii	Deionised	Solution	2.5	40 (26-55)	8 (3-20)	32 (20-48)
			12.5	5 (1-17)	5 (1-17)	0
			25	0	0	0
		Powder	2.5	20 (11-35)	13 (5-26)	7 (3-20)
			12.5	0	0	0
			25	0	0	0
	Mineral	Solution	2.5	43 (29-58)	10 (4-23)	33 (20-50)
			12.5	25 (14-40)	10 (4-23)	15 (7-29)
			25	0	0	0
		Powder	2.5	28 (16-43)	3 (0-13)	25 (14-40)
			12.5	18 (9-32)	10 (4-23)	8 (3-20)
			25	0	0	0

Notes: Ninety-five percent confidence intervals are in parentheses. Larval survival, pupal mortality and emergence rates were calculated out of an initial number of 40 larvae (per treatment).

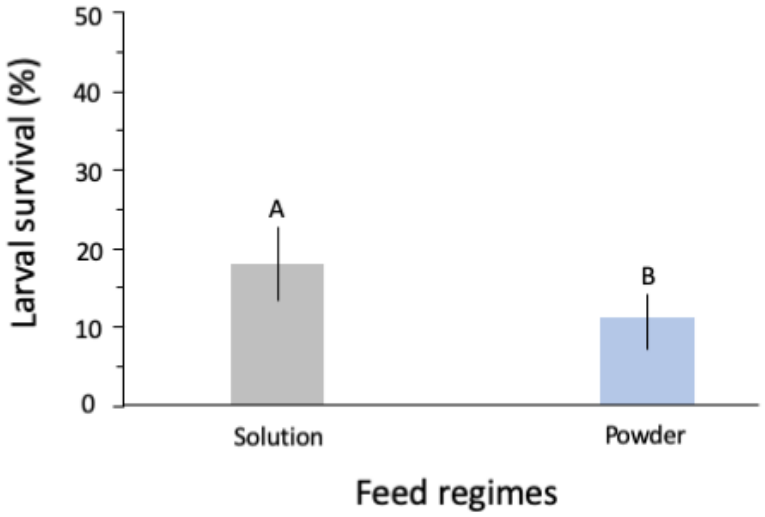

Figure 4.15: Effect of feed regimes on larval survival. Percentage larval survival shown for solution (grey) and powder (blue) feed regimes. Bar plots sharing the same letter are not significantly different. Whiskers represent 95% confidence intervals.

Table 4.9: Logistic regressions of the effect of NH_3, water types and feed on mosquito development.

Parameter	Source	df	Likelihood ratio	P-value
Larval survival	Water Type	1	0.74137177	0.3892^{ns}
	Feed	1	6.54913484	0.0105*
	NH_3 at larval stage	2	85.9388699	<0.0001***
	NH_3*Water Type	2	8.02483615	0.0181*
Pupal mortality	Water Type	1	3.61184053	0.0574 ^{ns}
	Feed	1	0.96877351	0.3250 ^{ns}
	NH_3 at larval stage	1	6.97265644	0.0083*
	Pupation in NH_3	1	0.04236003	0.8369
Adult emergence	Water Type	1	3.61184053	0.0574 ^{ns}
	Feed	1	0.96877351	0.3250 ^{ns}
	NH_3 at larval stage	1	6.97265644	0.0083*
	Pupation in NH_3	1	0.04236003	0.8369

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

4.4. Discussion

The main findings of this study highlights for the first time, contrasted strain specific plastic responses to an environmental toxicant (ammonia) (Hargreaves, 1998; Ishii, et al., 2011; Wright, 1995) that is characteristic of more eutrophic habitats in *An. gambiae* s.s. and *An. coluzzii*, under insectary conditions. The higher phenotypic quality (measured here as adult body size) of emerged adult *An. coluzzii* in this study supports the assumption that the species should cope better with levels of ammonia that are characteristic of its preferred putative ancestral ecological speciation niche (Dao et al., 2014; Gimonneau et al., 2012; Kamdem et al., 2012; Roux et al., 2014).

Overall, mosquito survival decreased with increasing ammonia concentrations revealing significant genotype × environment interactions for *An. coluzzii* and *An. gambiae* s.l. (Xin Li et al., 2018; Price et al., 2003). What is particularly interesting, is that we find that a strain of *An. coluzzii* that is comparatively younger (by ~ 23 years), outperforms in phenotypic quality, the well laboratory-adapted *An. gambiae* s.s. strain in conditions that are closer to more eutrophic habitats and/or like rice fields. Adult body size (wing-length) of *An. gambiae* s.s. was found to be negatively affected with increasing NH_3 concentrations. Despite having higher survival rates across water types, the adult body size of *An. gambiae* s.s. was more severely impacted at high NH_3 concentrations than *An. coluzzii*. Adult mosquito size is an important phenotypic trait in *Anopheles* mosquitoes. Larger body size in *An. gambiae* s.l. has been demonstrated to be reflective of larval growth conditions (environment) (Takken et al., 2013; Valerio et al., 2016). Larger females exhibit optimal lifetime fitness with increased ability to withstand stress as adults (Aboagye-Antwi & Tripet, 2010; Takken et al., 2013). In males, body size correlates with mating attractiveness and lifespan as smaller males usually die a few days after emergence as virgins (Abdoulaye Diabate & Tripet, 2015). This reaction norm in

An. coluzzii adult phenotypic quality in response to larval environment is a classic example of phenotypic plasticity which commonly emerges in stressful or adverse habitats, revealing pre-existing genetic and developmental of organismal homeostasis (Badyaev, 2005; Roux, et al., 2014). In the case of *An. coluzzii*, this reaction norm is adaptive and might be as a result of pre-existing traits developed due to selection pressures created by rice domestication in Africa (Mwangangi et al., 2010; Reidenbach et al., 2012).

Although this study did not show clear reaction norms in mosquito survival among the sibling species to ammonia as we had expected, it did reveal significant effects of the interaction of ammonia and water mineralisation on the developmental success and body size of *An. gambiae* s.s. and *An. coluzzii*. Larval survival, pupal survival and adult emergence were significantly higher in mineral water compared to deionised water. This supports published evidence that the ammonia toxicity risk of elevated pH and temperature can be greatly reduced with alkaline buffer above pH 8 (Florescu et al., 2011; Hargreaves, 1998). Mineral water used in the study had a mean general hardness of 53.7mg/l compared to the 17.9mg/l of deionised water, resulting in higher pH values above the minimum threshold (pH 8) at which the buffering effect which masks additional ammonia toxicity was activated (Florescu et al., 2011). The results of this study clearly show that the presence of minerals in the water constituted a buffer that resulted in the reduction of the impact of ammonia toxicity, especially at higher NH_3 concentrations. Additionally, adults emerging from mineral water were significantly bigger than those from deionised water. Although development time increased with increasing NH_3 concentration, both species developed faster in mineral water compared to deionised water. The duration from first instar larvae to adult,. *An. gambiae* however, had significantly longer development time in deionised water compared to *An. coluzzii*.

These results further strengthen the argument for the buffering capacity of mineral water to reduce the effect of ammonia on aquatic organisms, resulting in the higher percentage survival and better adult quality of *An. gambiae* and *An. coluzzii* (Akpodiete et al., 2019; Florescu et al., 2011; Hargreaves, 1998).

Below NH_3 concentrations that trigger toxicity, *An. gambiae* s.s. is expected to outperform *An. coluzzii* and this has been demonstrated elsewhere (Akpodiete et al., 2019; Tene Fossog et al., 2013). However, the higher survival rates of *An. gambiae* s.s. at higher NH_3 concentrations was unexpected as *An. coluzzii*, the more recently derived taxon, is known to prefer, permanent, nutrient-rich habitat and so should be better adapted to mineralisation and hence survive better in eutrophic conditions (Diabate et al., 2005; Tene Fossog et al., 2013). This situation may be attributed to the longer adaptation of *An. gambiae* s.s. (~ 40 years of laboratory maintenance compared to the ~ 17-year old *An. coluzzii* strain) to insectary conditions (Baeshen et al., 2014). Testing of such conditions (abiotic stressors) with additional strains from different locations could help improve the understanding of whether this disparity in adaptive response to NH_3 is species-specific. However, current field populations of the sibling species are under heavy selection pressures from chemical control, agricultural chemical residue and other pollutants (Cassone et al., 2014; Kamdem et al., 2012; Tene Fossog et al., 2013). The sibling species have also recently introgressed with one another, resulting in the possible selective introgression of important pesticide resistance loci and possibly ammonia detoxification ones (Cassone et al., 2014). Therefore, in this study, we favoured older strains with as little evolutionary history of such recent anthropomorphic selection pressures and associated introgression (Caputo et al., 2011; Mancini et al., 2015).

The second experiment which focused on the carry-over effect of NH_3 reveal that the effect of NH_3 exposure at larval stage was carried over to adult emergence. Although

NH_3 exposure at larval stage significantly impacted pupal mortality, there was no significant difference in pupal mortality and adult emergence between mosquito pupae transferred to NH_3 water for emergence and those transferred to non-NH_3 water. The pupal stage in mosquitoes is a short, non-feeding stage, this limits the expression of any phenotypic response to the larval stage that is relatively longer (~1-10 days) with active feeding (White, 1977). The result also supports studies that have shown that larval environmental conditions (temperature, nutrition, toxicants, microbial communities, predators), shape the outcomes for *Anopheles* adults, impacting on body size, vectorial capacity, distribution, prevalence, stress-tolerance and epidemiology (Aboagye-Antwi & Tripet, 2010; Chobu, et al., 2015; Christiansen-Jucht, et al., 2014; Diabate et al., 2005; Gimonneau et al., 2012).

In summation, the findings of this study are important both as supporting evidence of larval ecological divergence amongst the sibling species resulting in eco-speciation, sympatric occurrence of the sibling species without outcompeting each other, and for direct application for vector control. Firstly, the contrasted adaptive response to ammonia in rice field proposed here as a key ecological process driving speciation was found to be still present in these so-called laboratory-adapted strains as evidenced in the higher phenotypic quality of adult *An. coluzzii* (Li et al., 2018; Price et al., 2003; Tene Fossog et al., 2015). Earlier studies have demonstrated that these differences are related to their contrasted abilities to detoxify ammonia (Dias, et al., 2019; Tene Fossog et al., 2013). The *An. coluzzii* strain (Mopti from Bamako Mali) used for this study is comparatively 'younger' and might have been colonized from a strain that already had some adaptations to pesticide exposures of recent evolutionary times, thus presenting a possible confounding factor for the interpretation of the results. For this study however, it was difficult to replicate these ammonia plastic responses with additional older colonized

strains because available *An. coluzzii* insectary populations do not pre-date the M and S diagnostic (della Torre et al., 2001). Although the metabolic insecticide resistance pathways in mosquitoes have been demonstrated to evolve from allelochemicals and xenobiotic detoxification pathways, the ammonia detoxification pathway in mosquitoes has been described as a distinct pathway with no available literature linking these pathways (David et al., 2013; Kirchman et al., 2010; Scaraffia, et al., 2010). Metabolic insecticide resistance primarily involves the increased biodegradation of insecticides through the overproduction of detoxification enzymes such as P450s, glutathione S-transferases (GSTs) and carboxy/cholinesterases (CCE) (David et al., 2013; Hemingway, et al., 2004; Hemingway & Ranson, 2000). In contrast, ammonia is mainly removed by mosquitoes through the synthesis of glutamine and proline, via glutamine synthetase (GS), glutamate synthase (GltS), glutamate dehydrogenase (GH) and alanine transferase (AT) pathways and by the excretion of uric acid, allantoin, allantoic acid and urea (Scaraffia et al., 2010). Even in the unlikely event of an overlap between insecticide and ammonia detoxification pathways, it has been demonstrated that target site resistance by kdr mutation was not present in the *An. coluzzii* strains used for this study (Tripet et al., 2007). The possibility of metabolic resistance in this *An. coluzzii* population is highly unlikely as a study that investigated insecticide resistance in Mali, sampling *An. coluzzii* populations for the 25 year period before 2005 did not detect CYP9K1 (a P45O gene closely related to P450 genes that have been proven to metabolise pyrethroid insecticides in vitro) (Main et al., 2015). These studies in Mali were based on the background of the dramatic increase in insecticide-treated bed-net usage in 2005 (Ng et al., 2017) which likely altered the fitness landscape of *An. gambiae* s.l. and promoted adequate introgression of kdr (knockdown resistance) from *An. gambiae* s.s into *An. coluzzii* (Norris et al., 2015).

The second significance of this study is that the contrasted slopes of reaction norms to ammonia exemplifies why and how sympatric sibling species can be found in the same region despite imperfect premating barriers and considerable overlap in larval habitats use, yet without one outcompeting another over time (Roux et al., 2014; Tene Fossog et al., 2015). Current demographic explosion in malaria-endemic regions of the world and the attending demand for food security has led to increased rice domestication, often implemented via irrigation (Sikirou et al., 2015). Irrigational agriculture often involves the use of fertilizers, to improve crop yield, thus introducing sub-lethal doses of ammonia in these aquatic ecosystems (Tene Fossog et al., 2015). Field based acute toxicity assays in Yaounde, Cameroon, found *An. coluzzii* (M form) to have high tolerance for organic pollution associated with high levels of ammonia ions, and alkaline water collections (Tene Fossog et al., 2013). Earlier studies had demonstrated that irrigational agriculture though not impacting malaria transmission in areas of stable transmission (Ijumba & Lindsay, 2001) can alter malaria transmission patterns from seasonal to perennial in semi-arid zones of Africa (Dolo et al., 2004). Planned and unplanned urbanization characterised by environmental pollution, are also typical of these malaria endemic regions leading to the onset of urban malaria (Kamdem et al., 2012; Tene Fossog et al., 2015). These human interventions such as irrigate rice fields, open quarry mines, unplanned urbanization, will serve to further expand the niche of *An. coluzzii* vectors, bringing these virulent vectors in closer proximity with their human host and expand disease transmission all year round (Kamdem et al., 2012).

Of direct translational impact, the third significance of this study is that the results can inform modifications in *An. gambiae* s.l. rearing protocols to include mineral water instead of the commonly used deionised water. Ongoing efforts towards mass release mosquitoes modified by gene drive technology, SIT (sterile insect techniques)

implementation and other vector control strategies that rely on large scale production of mosquitoes could benefit from the introduction of mineral water in mass rearing protocols to improve mosquito yield and adult phenotypic quality. As a result of this study, water mineralisation, ammonia, and water depth were highlighted as major factors impacting larval mortality and phenotypic quality in the sibling species. We believed that these factors needed to interact together in a microcosm to reveal divergent reaction norms in mosquito survival as well as adult phenotypic quality, thus, informing the experimental design of Chapter 5.

In conclusion, the overall epidemiological consequence of these reaction norms by *An. coluzzii* to ammonia resulting in larval niche expansion might be a contributing factor to the stall in the progress towards malaria control and possible eradication (WHO, 2018). Larvicidal and adulticidal based vector control methods will need to be revised, to account for the niche expansion and distribution of these vectors resulting from ammonia tolerance in *An. coluzzii.*

Chapter 5

Contrasted microcosms experiment and developmental success reaction norms in *Anopheles gambiae* s.s. and *Anopheles coluzzii.*

5.1. Introduction

The Cambridge dictionary defines microcosm as " a small place, society or situation that has the same characteristics as something much larger" (Cambiridge Dictionary, 2020). In the context of scientific research, microcosms are simplified, ecosystems, that are used to simulate and predict the behaviours of natural ecosystems under controlled conditions (Roeselers, et al., 2006). Microcosms provide an experimental area for ecological research, with substantial and valuable information on the structure of ecosystems, natural processes, and the functioning of biotic communities within these ecosystems (Draggan, 1976; Roeselers et al., 2006). As models of natural ecosystems, the characteristics of microcosms are defined by those of the larger systems they represent (Draggan, 1976). They are conceptually similar in function to, but may differ in origin and structure from the natural system they mimic (Altermatt et al., 2015). A microcosm can either be a subset of a natural ecosystem (such an intact soil section) or a laboratory fabrication (such as a soil column), and this factor is important in the conceptualisation of microcosms as models of real-world situations (Draggan, 1976). Another equally important factor in conceptualizing of a microcosm is the hypotheses posed by the investigator of the expected behaviour of organisms in a given microcosm (Altermatt et al., 2015).

These miniaturised ecosystems (microcosms) can be very useful to study the effects of disturbance or determine the ecological role of a key species (Roeselers, et al., 2006). They serve as a good compromise between field experiments, which are often challenging due to environmental variability, high cost and difficult logistics, and

laboratory-based experiments, which are scarcely representative of the natural systems (Grenni, et al., 2012). Other advantages of laboratory microcosm studies include the possibility of multiple replications due to the compact size of these systems and the ability to vary experimental conditions individually to establish a cause-effect relationship between variables and the test organism (Grenni, et al., 2012). Results from microcosm studies sometimes do not directly translate to the real world, but they are a great starting point for ecosystem research (Altermatt et al., 2015).

Phenotypic plasticity has been defined as the change in the expressed phenotype of a genotype in response to the environment (Forsman, 2015). It involves the ability of a single gene to produce more than one alternate form of morphology, physiological state and/or behaviour in response to environmental conditions (Forsman, 2015). Phenotypic plasticity commonly emerges in stressful or adverse habitats, revealing pre-existing genetic and development of organismal homeostasis (Badyaev, 2005; Li et al., 2018). An organism's ability to tolerate stress might be a result of its complexity and accumulation of unexpressed variation by genetic and phenotypic developmental systems facilitating evolutionary changes under extreme conditions (Badyaev, 2005). An increase in variation in individual organismal systems and their subsequent reorganisation is thought to enable the formation of novel adaptations, promoting its persistence under adverse conditions and establishment in novel environments before genuine genetic adaptations evolve (Badyaev, 2005; Gulisija et al., 2016). Phenotypic plasticity may incur a fitness cost such as the development and maintenance of structures and systems involved in osmo- or thermoregulation (Gulisija, et al., 2016). Where the cost of plasticity exceeds the benefit, for instance, when environmental conditions improve or become favourable, plasticity is selected against (Gulisija, et al., 2016). These observed phenotypic variations are shaped by genomes, environment and their interactions (Li et al., 2018). Genotype-environment

(G × E) interactions are widespread in nature, such as inbreeding depression and heterosis in crops, and natural populations manifest average fitness differences magnified in harsh conditions (Kondrashov & Houle, 1997)

The phenotypic profile of a genotype across environments is described by the reaction norm, while phenotypic plasticity measures the variation among these phenotypic values (Li et al., 2018). The reaction norm is the pattern of phenotypes produced by a given genotype under different environmental conditions, thereby mediating the expression of genetic variation (Li et al., 2018; Stearns, 1992). Where a quantitative index is generated for the environments, G × E can be modelled as different performance curves of genotypes (i.e. reaction norms) along with this index (Li et al., 2018). It is onerous to pinpoint critical environmental determinants to establish a quantitative index that is both biologically essential and prognostic for the natural field (Li et al., 2018). In the presence of several genotypes, environments can differ qualitatively in the distribution of genotypes, often leading to the crossing of reaction norms (Stearns, 1992) (Figure 5.1). Crossing reaction norms is a strong form of G × E interaction, that have two critical effects on phenotypic distribution. Firstly, they determine if the variation in the phenotype is heritable. In the region where the norms cross, the genotypic variation is unclear, phenotypes cannot be assigned unambiguously to genotypes near this point. Outside that crossing region, the genotype variation is clear. Secondly, reaction norms influence the ranking of phenotypes. In the environments to the right of the crossing point, the phenotypic ranks of the genotypes are the opposite of those in the environment to the left (Stearns, 1992).

Mosquitoes in the *Anopheles gambiae* s.l. complex consists of the major vectors of human malaria throughout sub-Saharan Africa (Simard et al., 2009).

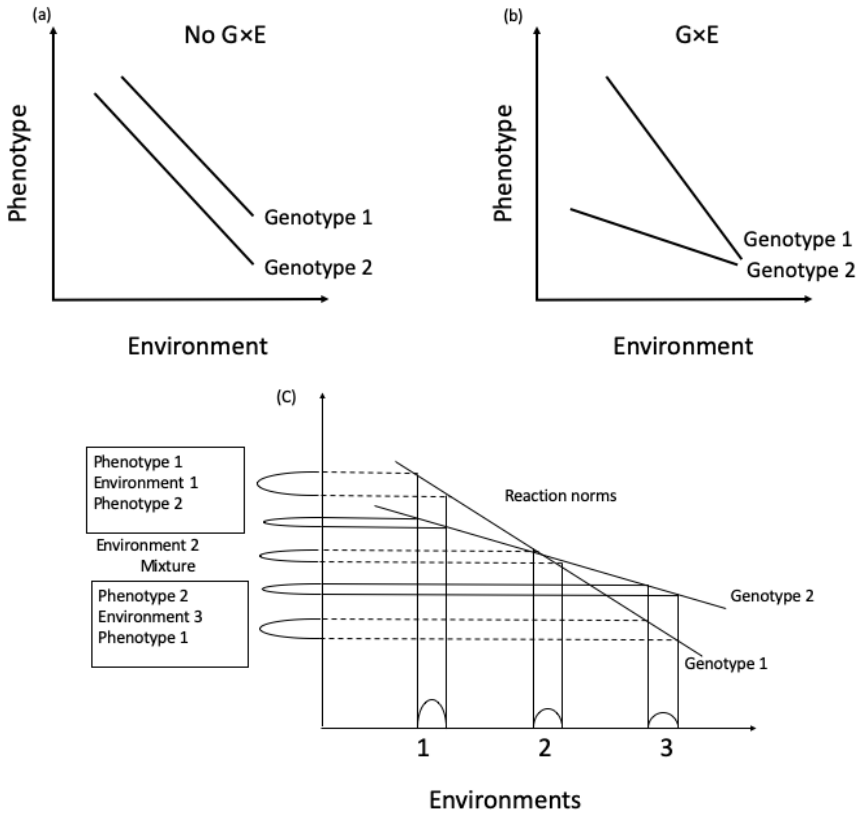

Figure 5.1: (a) Reaction norms of the genotypes present run parallel to each other and there is no genotype × environment interactions. (b) Reaction norms with different slopes represent genotype × environment interactions. (c) Crossing reaction norms convert three similar environmental distributions. In environments 1 and 3, the genotypes can be distinguished in the phenotypes and perceived genetic variation is significantly different from zero. In environment 2, near the crossing point of the norms, the genotypes are indistinguishable in the phenotypic mixture and perceived genetic variation is nil. Between environments 1 and 3, the phenotypic ranking of the genotype reverses (modified from Stearns, 1992)

Speciation among members of this complex is thought to be encouraged by disruptive selection and ecological divergence dependent on sets of adaptation genes protected from recombination by polymorphic chromosomal inversions (Simard et al., 2009). The recently speciated sibling species, *Anopheles gambiae* s.s. and *Anopheles coluzzii*, members of the *An. gambiae* s.l complex, are model species for eco-speciation studies (Reidenbach et al., 2012). They have a remarkable preference for human blood and are primarily endophilic and endophagic; together with their high longevity, allowing for the sustainable development of *Plasmodium* parasites under a variety of ecological settings, making them the most proficient malaria vectors in the world (Simard et al., 2009). The

ongoing divergence between these species despite incomplete reproductive isolation has been linked to differential ecological adaptations to divergent larval habitats in different eco-geographical settings (Aboagye-Antwi et al., 2015; della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008).

They are virtually present ubiquitously in sub-Saharan Africa, populating the varying environments typically found on the continent and transmitting malaria to humans in remote areas as well as urban municipalities (Simard et al., 2009). In West African savannahs, the main ecological distinction between the presumed ancestral *An. gambiae* s.s. and the derived *An. coluzzii* is their choice of larval habitat (Reidenbach et al., 2012). *An. coluzzii* is often associated with longer-lasting breeding sites that are associated with predators and high amounts of organic matter such as rice fields, reservoirs, abandoned mines and quarries, and drainage ditches (Diabate et al., 2005). Its ability to breed in irrigated rice fields may be strongly linked with rice domestication in Africa (Reidenbach et al., 2012). *An. gambiae*, on the other hand, usually reaches its population peak during the rainy season, and is well adapted to habitats that are more ephemeral and rain-dependent (Aboagye-Antwi et al., 2015; della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008). This larval habitat preference and the resultant divergent adaptations of these species has been cited as a possible cause of speciation (ecological speciation) among both species (Gimonneau et al., 2010).

Rice is a tropical/sub-tropical crop that is cultivated in several agro-ecological zones. In West Africa, African rice (*Oryza glaberrima*) was domesticated from the wild approximately 3500 years ago in the inland delta flood plains of the upper Niger River in Mali (Chang, 1976). There was a subsequent spread, 500 years later, along the Sahelian rivers to two secondary locations, one in the coastal Gambia, Casamance (Senegal), and

Guinea Bissau; the second location in the Guinea forest between Sierra Leone and the western Ivory Coast (Chang, 1976; Li et al., 2011; Reidenbach et al., 2012). Environments, where rice is grown, include tidal wetlands (coastal regions), upland environments, deep water environments, rain-fed lowland environments and irrigated environments. Water is available in irrigated rice fields throughout the growing season with depths of 5cm to 10 cm (Bambaradeniya & Amarasinghe, 2003). Irrigated rice fields are characteristically lentic water bodies viewed by scientists as agronomically managed wetlands (Mwangangi et al., 2010). Farming activities such as tillage, agrochemical application, and weeding creates a disturbance in this ecosystem as well as rainfall and flooding (Bambaradeniya & Amarasinghe, 2003). The chemistry and biotic composition of irrigated rice paddies are influenced by the source of water supply, which is either from naturally occurring rivers or through rainfall (Baolan et al., 2012; Mwangangi et al., 2010). Natural levels of ammonia in rice paddies ranging from 0.53 ± 0.1 mg/l, is a by-product of protein metabolism of aquatic organisms and can be toxic if allowed to accumulate (Bambaradeniya & Amarasinghe, 2003; Baolan et al., 2012; Hargreaves, 1998). Un-ionised ammonia concentrations above 0.2 ± 0.3mg/l has been reported to be toxic to freshwater aquatic organisms (Florescu et al., 2011).

The levels of ammonia in the floodwater are further increased by fertilizers used by farmers to increase agricultural yield (Bambaradeniya and Amarasinghe, 2003). Approximately 78% of the invertebrates in a typical irrigated rice field ecosystem are insects among which the Dipteran order are dominant, specifically in the family Culicidae (Bambaradeniya & Amarasinghe, 2003). Members of the *An. gambiae* s.l. species complex thrive in shallow inundated rice fields during tilling, transplanting, the first six weeks of the rice growing period and after harvest (Klinkenberg, et al., 2003). In West Africa, rice fields have proved to be particularly suited as larval sites for *An. coluzzii,* one

of the main malaria vectors in this region (Dolo et al., 2004; Epopa et al., 2017; Ijumba & Lindsay, 2001; Klinkenberg et al., 2003; Mwangangi et al., 2010). Although irrigated agriculture has little or no impact on malaria transmission in localities where transmission is stable, the year-round availability of mosquito breeding sites provided by rice field cultivation can alter malaria transmission in drier regions; changing transmission pattern from rainfall seasonality to perennial (Ijumba & Lindsay, 2001; Ijumba, et al., 2002). Observed variation in the distribution and abundance of *Anopheles* populations associated with rice fields (Dolo et al., 2004; Epopa et al., 2017; Faye et al., 1995; Lindsay et al., 1991; Marrama et al., 2004; Mwangangi et al., 2006; Sawadogo et al., 2017) around West Africa reflects the oviposition preferences of gravid mosquitoes and the ability of immature stages to tolerate conditions that prevail within their aquatic habitats (Mwangangi et al., 2010).

Abiotic factors such as habitat stability, or the degree of spatial heterogeneity and biotic factors such as predations are known to influence *Anopheles* populations in the rice field aquatic ecosystem (Diabate et al., 2005; Munga et al., 2006). Intraspecific competition has been cited as a factor for the almost exclusive occurrence of *An. coluzzii* in rice fields in the savannah region of West Africa, a situation indicative of competitive segregation (Diabate et al., 2005). Intraspecific competition in *Anopheles gambiae* s.l. has been known to prolong larval development and reduce the size of adults which results in reduced productivity (Munga et al., 2006). Following a field transplantation experiment, where the sibling species were placed in *An. coluzzii*-typical and *An. gambiae* s.s.-typical larval habitats, the sibling species successfully outperformed one another in their preferred habitats thereby lending support to the hypothesis that competition at the larval stage played a significant role in the divergence between both species (Diabate et al., 2005).

Rice paddies (*An. coluzzii* typical larval habitat) are prone to mosquito larval predators when compared with the temporal, predator free, shallow rain puddles preferred *An. gambiae* s.s. and this has been highlighted as a major driver of the larval niche differentiation between the sibling species (Gimonneau et al., 2012; Munga et al., 2006). The sibling species have been modelled in field transplantation experiments as examples of how predation pressure results in disruptive selection that promote ecological divergence (Gimonneau et al., 2010). In the study, larvae of *An. gambiae* s.s a had shorter pre-imaginal development duration in temporary waters with low predators with *An. coluzzii* developing faster in predator rich environments (Gimonneau et al., 2010). Other studies have also established the fact that *An. coluzzii* is better adapted to live in ecologically more complex and stable habitats where predators are more abundant and diverse compared to the ephemeral predator barren larval habitats of *An. gambiae* s.s. (Munga et al., 2006; Roux et al., 2014; Tene Fossog et al., 2015). In the absence of predation however, the sibling species outcompetes each other in their natural habitats (Gimonneau et al., 2012). Whilst *An. gambiae* s.s. behaves as surface feeders, mainly thrashing at the water surface and foraging through floating vegetation, *An. coluzzii* larvae mimics benthic feeders, spending significantly greater amount of time browsing at the bottom of the container and diving more frequently than the former (Gimonneau et al., 2012).This behaviour has been directly linked to its success in both habitats under predator pressure (Diabaté et al., 2008).

The phenotypic responses mediated by the cultivated rice environment in *An. coluzzii* is not fully understood even though various studies agree that significant changes in its bionomics and epidemiology can be traced to rice domestication (Bambaradeniya & Amarasinghe, 2003; Dolo et al., 2004; Epopa et al., 2017; Faye et al., 1995; Lindsay et al., 1991; Marrama et al., 2004; Mwangangi et al., 2006; Sawadogo et al., 2017). In-

depth study is therefore required to understand the G × E interactions of the rice-agroecosytem as a larval habitat of *An. coluzzii*, as well as the factors that affect its abundance and prevalence in this ecosystem and how this has contributed to the ongoing eco-speciation between *An. gambiae* s.s. and *An. coluzzii*.

To fill this gap in knowledge, we had investigated plastic responses of *An. coluzzii* and *An. gambiae* s.s. to increasing concentrations of ammonia, a common toxicant in the rice field ecosystem in the previous chapter of this thesis (Chapter 4). The results revealed reactions norms for *An. coluzzii* that correlate with stress and ammonia tolerance. In this current study we investigated the phenotypic responses underlying larval ecological adaptations between the sibling species, *An. gambiae* s.s and *An. coluzzii* in microcosm-based experiments that are as close to the natural habitat as possible to reveal plastic responses to natural ecosystems under controlled conditions. This was achieved by observing the larval development and adult emergence of these vectors in species-preferred microcosms and also during transplantation to non-preferred microcosms to reveal G × E interactions amongst the sibling species in an arena that were ecologically more relevant and closer to the natural environment compared to the Chapter 4 set-up. *Anopheles coluzzii* was reared in a simulated rice field microcosm and *Anopheles gambiae* s.s. was reared in a simulated rain-fed larval microcosm and both species were also transplanted to non-preferred microcosms. Adult survival, mosquito wing length and developmental time were recorded as indices of plasticity. Results reveal divergent criss-crossing reaction norms in the developmental success of the sibling species in the two contrasted larval environments. These findings lend support to the hypothesis that adaptations to rice-field like conditions, independent of predator presence may be an important driver of ecological speciation in the sibling species.

5.2. Materials and Methods

5.2.1. Mosquito strain

The Kisumu strain of *An. gambiae* s.s, colonized over 40 years ago, from the area of Kisumu, Kenya, East Africa and the 17-year-old Mopti strain of *An. coluzzii*, colonized in 2003 by the Lanzaro Laboratory (UC Davis) from the village of N'Gabacoro droit near Bamako, Mali, West Africa, were used for the experiments. The strains were maintained by the Tripet group in dedicated insectaries of the Centre of Applied Entomology and Parasitology (CAEP), Keele University, UK, as described in Chapter 2.

5.2.2. Simulated *An. gambiae* s.s.-typical larval microcosm (Rain puddle)

Rain puddle-like larval microcosms were simulated with transparent aquarium-like containers of 19.5cm length, 16.5cm height and 12.5cm width, containing deionised water 4cm deep and 400ml of wet inert light brown sand (UNIPAC aqua gravel, the PetCare division of D-PAC Limited, 4 Sketty close, Brackmills Northampton, NN4 7PL, United Kingdom) of 2cm depth (Figure 5.2).

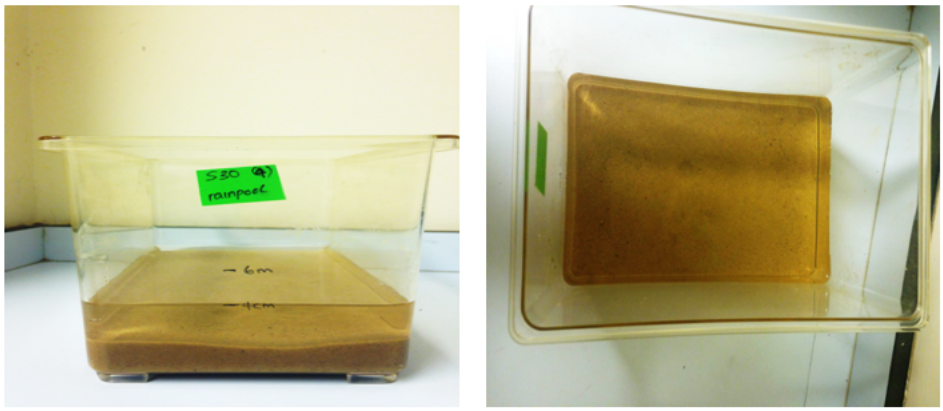

Figure 5.2: Simulated *An. gambiae* s.s. preferred microcosm (Rain puddle).

5.2.3. Simulated *An. coluzzii*-typical larval microcosm (Rice paddy).

An. coluzzii-typical larval microcosm was simulated using similar container (as described in section 5.2.2), containing mineral water at 8cm depth, 1.3mg/l of ammonia, 400ml of inert dark sand 2cm deep (UNIPAC aqua gravel, the PetCare division of D-PAC Limited, 4 Sketty close, Brackmills Northampton, NN4 7PL, United Kingdom), and a patch of intermediate density plastic grass (to imitate the rice field), 11.3cm length × 6.2cm width (patches were 5cm apart) (Figure 5.3).

A: Rice paddy - Side view B: Rice paddy - Top view

Figure 5.3: Simulated *An. coluzzii*-typical larval habitat.

5.2.4. Experimental design

Each replicate consisted of two larval densities (30 and 60 larvae) of first instar larvae of both species placed in the two types of microcosmic larval habitats (2 species × 2 densities × 2 habitats = 8 microcosms), resulting to a total of 720 sampled mosquito larvae for two replicates. The total of 16 microcosms was randomly arranged and rearranged daily from day 5, to avoid confounding effects. To prevent ammonia build-up, on day 5 and afterwards, every other day until the completion of the assay, water was pumped out from the microcosm using a low-pressure pump, down to a minimal level to avoid picking

up larvae and replaced with fresh water as per larval habitat type. Microcosms were covered with mosquito netting material to contain the emergence of mosquito adults and entry of external debris. Coloured tapes were used to differentiate the habitat type and species and the microcosms arrangement in the insectary was completely randomised (Figure 5.5).

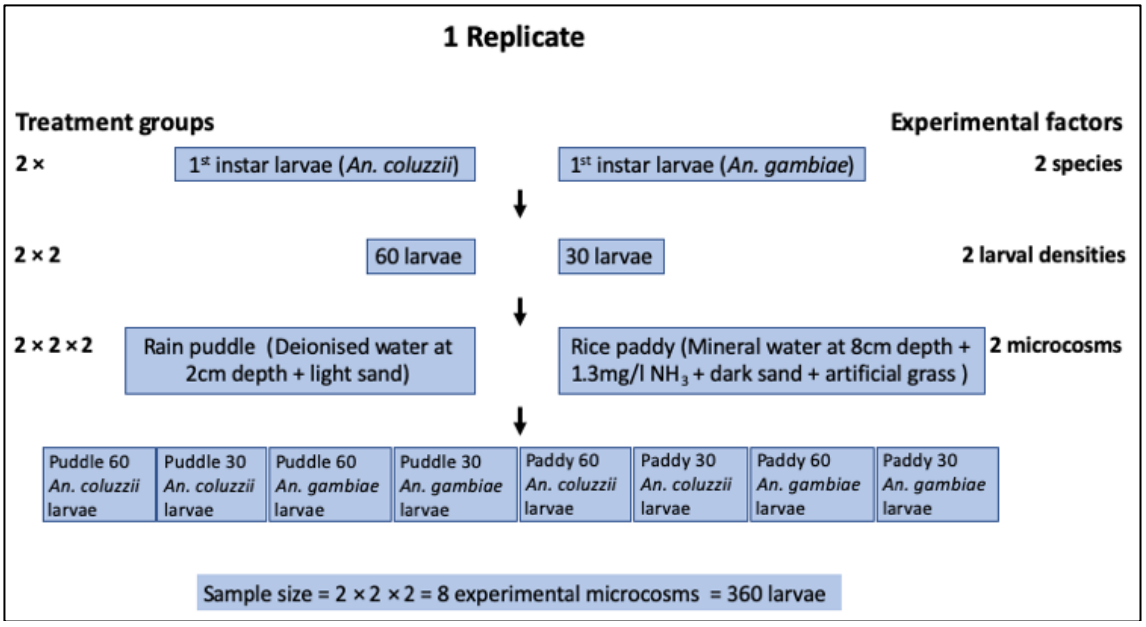

Figure 5.4: Experimental design for the contrasted microcosms experiment.

5.2.5. Larval feeding regime

Larvae were fed daily following a standardised feeding regime. 0.1 µl of Liquifry liquid fish food (Interpret Ltd, Surrey, UK) on day 1. From day 2 until pupation, finely ground Tretramin flakes (Tetra GmbH- Herrentech D-49324, 78 Melle, Germany) was mixed with deionised water and injected into the microcosm in the following proportions: day two to three - 0.25ml of 0.2g/10ml solution of ground Tetramin flakes; day 4 - 0.5ml of 0.16g/10ml solution of ground Tetramin flakes; day 5 until pupation - 1ml of 0.75g/50ml of ground Tetramin flakes.

Figure 5.5: An array of contrasted larval microcosms before randomization (see 5.2.4 for details)

5.2.6. Data collection

Adult emergence was recorded as the number of adults that emerged from the total number of larva placed in a microcosm. Development time was recorded as the duration between day 1 of the experiment when first instar larvae were placed in microcosm and the day of adult emergence. Adults that emerged from the microcosms were collected using a mouth aspirator, sexed and stored in 75% ethanol for subsequent measurement of wing-length as described in Chapter 2.

5.2.7. Water quality analysis

Using API aquarium test kits (Mars Fishcare North America, Inc, 50E Hamilton street, Chalfont, PA, 18914, USA), general hardness, carbonate hardness, pH, nitrates and ammonia readings were taken on day 1 and day 10 following experimental set-up (Table 5.1).

5.2.8. Statistical analysis

All data collected were analysed using the software JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA). All data were checked for deviations from normality and heterogeneity, and analyses were conducted using parametric and non-parametric

methods as appropriate. Data from all replicates were used for analysis, replicate effects were tested but were only reported when significant. Interactions between independent variables were tested using step-wise models and only those significant were retained in the final models. For analyses of proportion of larvae, pupae and adults, likelihood odds ratios were used for *post-hoc* pairwise group comparisons following logistic regressions. Body size was analysed through general linear models followed by Tukey's HSD *post-hoc* pairwise comparisons. Finally, developmental times (day of emergence) were analysed by Cox Proportional-Hazard models with likelihood odds ratios for *post-hoc* pairwise comparisons.

Table 5.1: Mean of general hardness, carbonate hardness, pH, nitrates and ammonia across larval habitats

Treatment	Species	Larval density	Day	Nitrate (mg/l)	Ammonia (mg/l)	General hardness (mg/l)	Carbonate hardness (mg/l)	pH
Rain puddle	*An.coluzzii*	30	1	0.5	0.25	17.9	17.9	6.8
			10	0	8	35.8	53.7	7.2
		60	1	0.5	0.25	17.9	17.9	6.8
			10	40	2.5	44.75	35.8	6.9
	An.gambiae	30	1	0.5	0.25	17.9	17.9	6.8
			10	0	4	35.8	35.8	6.8
		60	1	0.5	0.25	17.9	17.9	6.8
			10	40	0.75	35.8	35.8	6.6
Rice paddy	*An.coluzzii*	30	1	20	0.5	53.7	53.7	7.2
			10	0	4	71.6	53.7	6.7
		60	1	20	0.5	53.7	53.7	7.2
			10	0	8	71.6	53.7	7.2
	An.gambiae	30	1	20	0.5	53.7	53.7	7.2
			10	0	4	71.6	53.7	7
		60	1	20	0.5	53.7	53.7	7.2
			10	0	6	62.7	53.7	7

Notes: Sample size=2.

5.3. Results

5.3.1. Adult emergence across divergent mosquito larval microcosmic habitats

Overall, there was a significant effect ($P < 0.0001$) of larval habitat type on adult emergence (Table 5.2). Adult emergence from rain puddle was 40% higher than that from the rice paddy larval microcosm (Table 5.2; Figure 5.6). Species and density did not significantly impact on the total adult emergence numbers across microcosms (Table 5.2). However, the interaction between species and microcosms (G x E interaction) had a very strong and significant impact on adult emergence (Table 5.2).

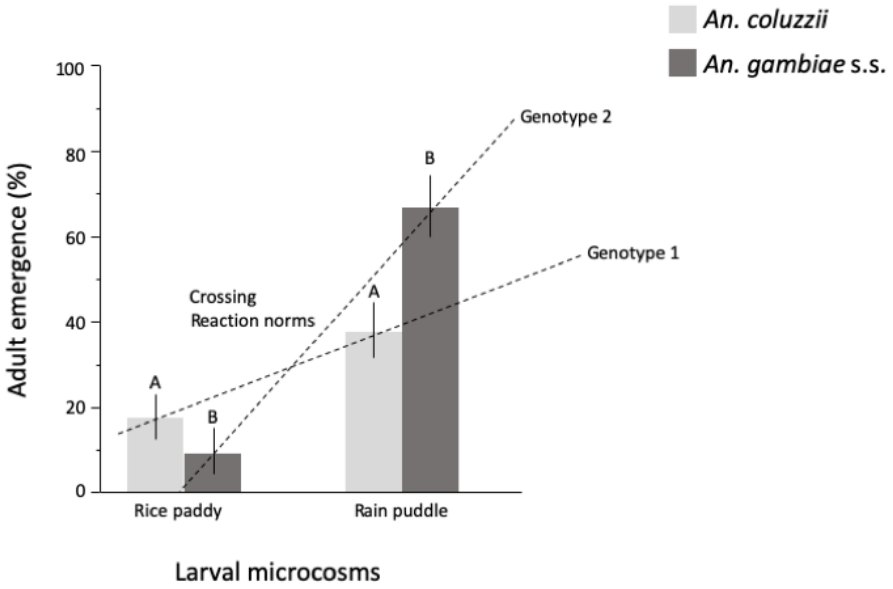

Figure 5.6: Adult emergence of *An. coluzzii* (light grey bars) and *An. gambiae* s.s (dark grey bars) across two larval microcosms. Whiskers represent 95% confidence intervals (CI). Within larval microcosms, significant differences are represented by different letters. The two overlaid dashed lines highlight the crossing G × E reaction norms across two divergent larval environments.

In the rice paddy (*An. coluzzii* typical), adult emergence for *An. coluzzii* (17%) was almost double that of *An. gambiae* s.s. (9%). (Figure 5.6) Inversely, in the rain puddle microcosm (*An. gambiae* typical), *An. gambiae* had significantly higher adult emergence (67%), compared to the 38% emergence in *An. coluzzii*. (Figure 5.6). Further logistic regression fit modelling conducted within microcosms revealed significant differences

among the sibling species within divergent larval microcosmic habitats and for the two larval densities (Table 5.4). As predicted by the significant G × E interaction in the main model, *An. coluzzii* had significantly higher adult emergence in the rice paddy microcosms while *An. gambiae* s.s. had higher adult emergence in the rain puddle microcosms. The effect of density on adult emergence was significant (P = 0.0292) in rice paddy but not in rain puddle, but the interaction between density and species was significant in both microcosms (Table 5.4).

Table 5.2: Logistic regression (Effect Likelihood Ratio Tests) of adult emergence of *An. coluzzii* and *An. gambiae* s.s. in divergent larval microcosms.

Source	DF	Likelihood ratio	P-value
Species	1	0.338	0.1701 ^{ns}
Microcosm	1	60.481	<0.0001***
Density	1	2.646	0.7013^{ns}
Microcosm *Species	1	28.886	<0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

Table 5.3: Adult emergence of *An. gambiae* s.s and *An. coluzzii* across two larval microcosms.

Species	Microcosm	Larval density	% Adult emergence
An. coluzzii	Rice paddy	30	18 (11-30) *60*
		60	16 (10-23) *120*
	Rain puddle	30	32 (21- 44) *60*
		60	42 (33-51) *120*
An. gambiae s.s	Rice paddy	30	2 (0-9) *60*
		60	13 (9-21) *120*
	Rain puddle	30	76 (64-86) *60*
		60	63 (54-71)*120*

Ninety-five percent confidence intervals are in brackets and sample sizes are italicized. Adult emergence was calculated as emerged adults out of the initial sample size.

In the rice paddy at 30 larval density, adult emergence for *An. coluzzii* (18%), significantly higher than the 2% emergence of *An. gambiae* s.s. for the same condition

(Tables 5.3, 5.4; Figure 5.7). There was no significant difference in adult emergence in the rice paddy microcosm at the 60 larval density (Tables 5.3, 5.4; Figure 5.7) for both species. Inversely, in the rain puddle microcosm at 30 larval density, *An. gambiae* had significantly higher adult emergence (76%), compared to the 32% emergence in *An. coluzzii*; and at 60 larval density *An. gambiae* s.s. had 20% higher adult emergence (Tables 5.3, 5.4; Figure 5.6) at both larval densities.

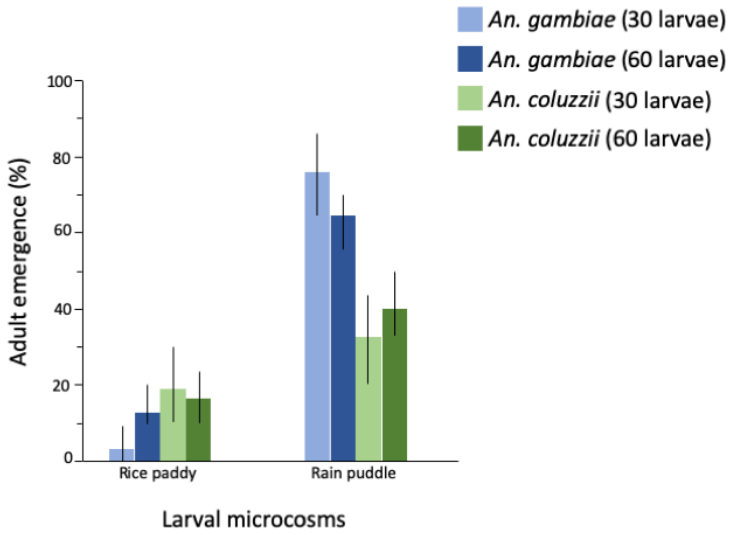

Figure 5.7: Adult mosquito emergence across divergent larval microcosms for two larval densities. Whiskers represent 95% confidence intervals (CI).

Table 5.4: Logistic regressions (Effect Likelihood Ratio Tests) of adult emergence within habitats

Parameter	Habitat	Source	DF	Likelihood ratio	P-value
Adult emergence	Rice paddy	Species	1	10.679	0.0011**
		Density	1	4.756	0.0292*
		Density*Species	1	6.780	0.0092**
	Rain puddle	Species	1	25.409	<0.0001***
		Density	1	0.253	0.6149
		Density*Species	1	5.330	0.0210*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

5.3.2. Development time in divergent larval microcosms

Time of mosquito development from the larval stage to adult emergence was significantly ($P < 0.0001$) impacted by the type of microcosm in which larvae were reared (Table 5.5). Development time was on the average, two days longer in the rice paddy microcosm compared to rain puddle microcosm (Figure 5.8; Table 5.5). There were no significant effects of species and density on the duration of mosquito development from first instar to adult emergence (Table 5.5).

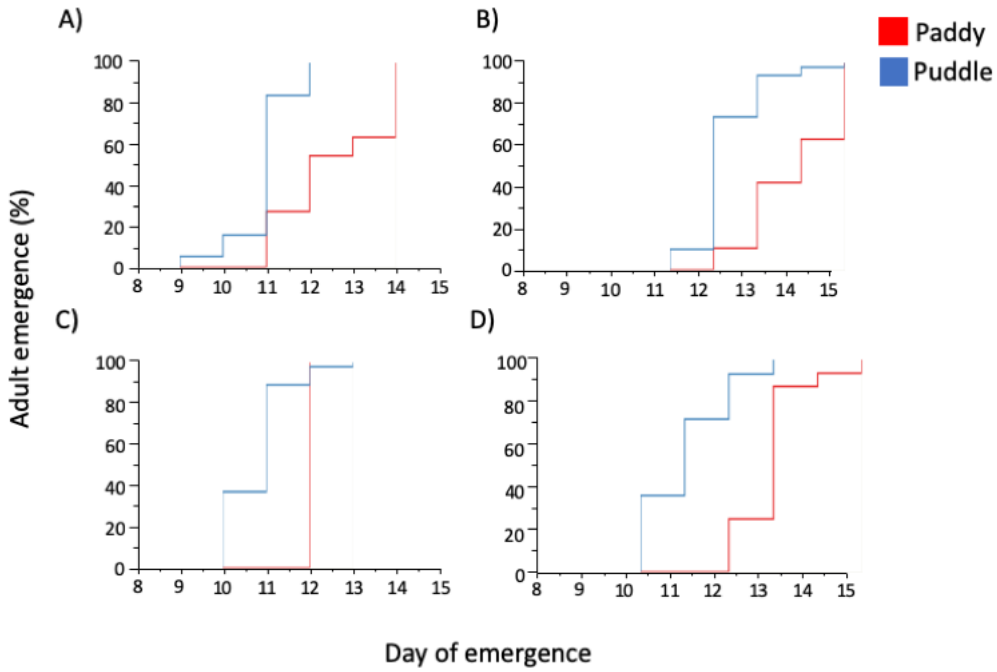

Figure 5.8: Emergence time across larval microcosms for *An. coluzzii* (A-larval density of 30 and B of 60 larval density) and *An. gambiae* (C- 30 larval density and D- 60 larval density).

Table 5.5: Cox Proportional-Hazard analyses of development time

Parameter	Source	df	Likelihood ratio	P-value
Day of emergence	Species	1	1.467	0.2258
	Habitat	1	41.917	<0.0001***
	Density	1	2.759	0.0967

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

5.3.3. Wing-length of emerged adults across larval microcosms.

Across both larval microcosms and rearing densities (30 and 60), *An. coluzzii* adults had significantly (P<0.0001) longer wing-lengths in comparison to *An. gambiae* (Table 5.7; Figure 5.9). Adults emerging from the rain puddle microcosm were significantly (P<0.0001) bigger than those from the rice paddy larval microcosm (Table 5.7; Figure 5.9). Larval rearing density (P = 0.0171), the interaction between density and species (P<0.0001) significantly impacted the wing-length of emerged adults in both divergent larval microcosm (Tables 5.6; 5.7). Larger adults emerged from the 30 larval rearing density compared to the 60 rearing density for *An. coluzzii* across both habitats.

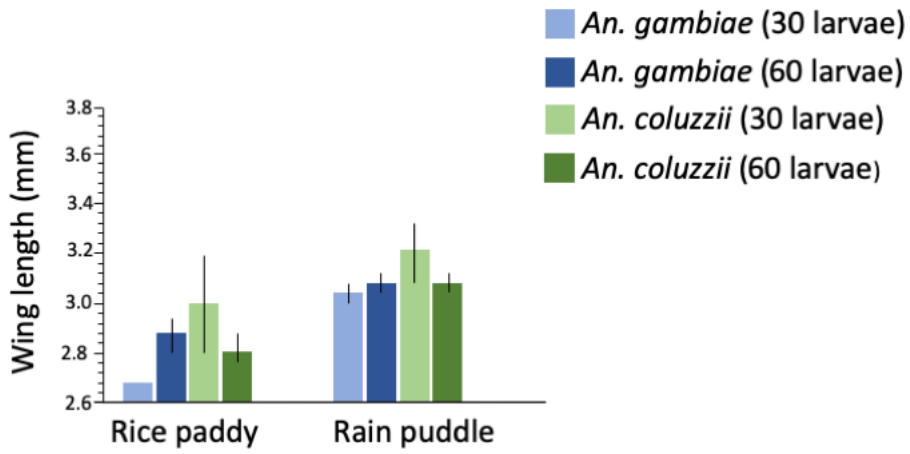

Figure 5.9: Wing-length of *An. gambiae* (light blue-30 larval density, dark blue- 60 larval density) and *An. coluzzii* (light green-30 larval density, dark green- 60 larval density) across microcosms. Whiskers represent 95% confidence intervals.

This effect was only observed in the rice paddy for *An. gambiae*, as there was no significant difference in adult size for this species in rain puddle habitat in relation to rearing density (Figure 5.9; Tables 5.6,5.7). Emerged females for both species were significantly bigger in size than the males, across microcosms and rearing densities (Figure 5.10, Tables 5.7, 5.8). *An. coluzzii* females significantly differed by density

(Table 5.7) with females reared at 30 larval density larger than those at 60 in both larval microcosms.

Table 5.6: Mean wing-length and development time of *An. gambiae* s.s. and *An. coluzzii* in divergent larval microcosms.

Species	Microcosm	Larval density	Mean wing length (mm)	Days till emergence
An. coluzzii	Rice paddy	30	3.00 (2.81–3.18) 11	12.55 (11.68-13.41) 11
		60	2.82 (2.75–2.89) 19	12.84 (12.33-13.36) 19
	Rain	30	3.22 (3.12–3.31) 19	10.95 (10.61-11.29) 19
	puddle	60	3.09 (3.04–3.13) 50	11.24 (11.02-11.46) 50
An. gambiae	Rice paddy	30	2.68 (0–0) 1	12 (0-0) 1
		60	2.88 (2.81–2.94) 16	12.94 (12.53-13.35) 16
	Rain	30	3.04 (2.99 –3.08) 46	10.76 (10.55-10.97) 46
	puddle	60	3.07 (3.04–3.09) 75	10.99 (10.77-11.20) 75

Notes: Ninety-five percent confidence intervals are in parentheses and the samples sizes, the number of surviving individuals out of an initial number of 60 or 120 larvae are italicized.

This effect was absent for *An. gambiae* in the rain puddle, as no significant difference was observed in females of this species by density in that microcosm. In the rice paddy microcosm, however, no female *An. gambiae* emerged from the 30 larval rearing density (Figure 5.10). Within microcosms, males emerging from the rain puddle were larger than those from the rice paddy microcosm.

Table 5.7: General linear model of wing-length across larval microcosms

Parameter	Source	df	F-ratio	P-value
Wing length	Species	1	28.707	<0.0001***
	Microcosm	1	81.735	<0.0001***
	Density	1	5.766	0.0171*
	Sex	1	30.593	<0.0001***
	Density*Form	1	21.548	<0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

No significant differences were observed in males for the species within the rain puddle microcosm. In the rice paddy, *An. coluzzii* males were significantly larger than *An. gambiae* males. Males emerging from the 30 larval rearing density were significantly bigger for *An. coluzzii* compared to those from 60 rearing density (Figure 5.10, Table 5.8).

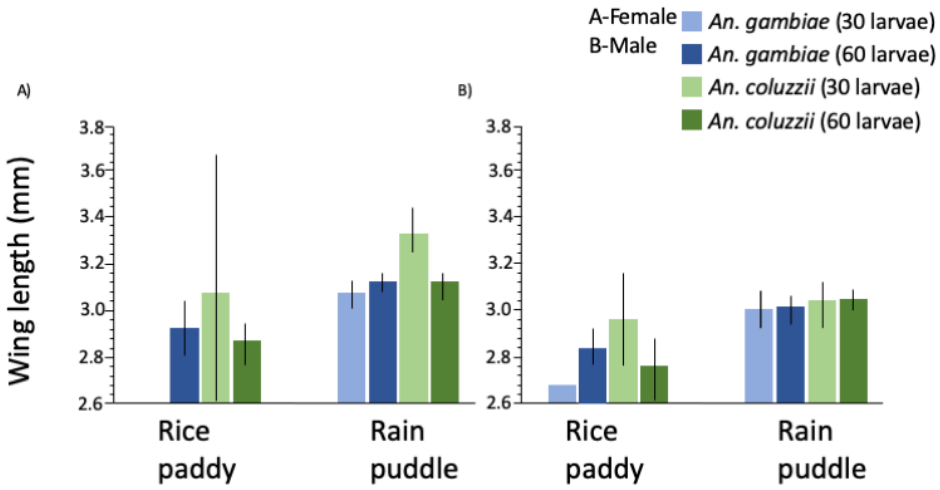

Figure 5.10: Wing-length by sex comparison of *An. gambiae* s.s. (light blue-30 larval density, dark blue- 60 larval density) and *An. coluzzii* (light green-30 larval density, dark green- 60 larval density) across microcosms. Whiskers represent 95% confidence intervals.

Table 5.8: General linear models of wing-length by sex.

Parameter	Sex	Source	df	F-ratio	P-value
Wing length	Female	Species	1	26.573	<0.0001***
		Microcosm	1	39.858	<0.0001***
		Density	1	5.973	0.0160*
		Density*Form	1	20.869	<0.0001***
	Male	Species	1	1.772	0.1860
		Microcosm	1	34.769	<0.0001***
		Density	1	0.208	0.6491

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

5.4. Discussion

The major finding of this study is that under microcosm-based conditions, the sibling species exhibited plastic responses in developmental success and adult phenotypic quality, that reflect their larval habitat preferences in nature (della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008). As expected, in the rice paddy environment *An. coluzzii* had higher survival and superior phenotypic quality of emerging adults compared to *An. gambiae* s.s, and vice versa in the rain puddle environment. It is interesting that this study has been successful in eliciting G × E interactions similar to those found in the natural habitats of these important malaria vector sibling species. These results lend support to the hypothesis of ecological speciation through larval adaptation to rice domestication by *An. coluzzii* (Reidenbach et al., 2012; Simard et al., 2009). Further, the reaction norms observed in this study ocurred in the absence of predators, indicating that they are inherited and not just an response to transient factors (Stearns, 1992). Crossing reaction norms are strong forms of G × E interaction, and they determine if the variation in the phenotype is heritable (Stearns, 1992).

The highlight of this study is the highly significant and crossing reaction norms found in the emergence success of the sibling species which begs to ask which characteristic(s) of the microcosm could be most important in generating this pattern. *An. coluzzii* had significantly higher survival in the more stressful rice paddy-like larval microcosm with *An. gambiae* surviving more in the supposedly less stressful rain puddle. The stressors in the paddy microcosm which include, added ammonium hydroxide, higher water depth, less light due to darker sand base, and the restriction of movement by the artificial rice plants might have elicited the expression of the stress tolerance phenotype in *An. coluzzii* (Badyaev, 2005; Bambaradeniya & Amarasinghe, 2003;

Gulisija et al., 2016; Kondrashov et al., 2017). Moreover, phenotypic plasticity is thought to commonly emerge in stressful or adverse habitats, revealing pre-existing genetic and development of organismal homeostasis (Badyaev Alexander V, 2005; Gulisija et al., 2016). An organism's ability to tolerate stress has been linked to its complexity and accumulation of unexpressed variation by genetic and phenotypic developmental systems facilitating evolutionary changes under extreme conditions (Badyaev, 2005). It has been further suggested that these plastic responses can also promote the persistence of a population under adverse conditions and aid its establishment in novel environments, before genuine genetic adaptations evolve (Gulisija, et al., 2016). The significant differences between the sibling species within different larval habitats observed in this study resulted in perfectly symmetrical crossing reaction norms, with very strong G ×E interaction, providing a strong evidence for ecological speciation. The results were reflective of the adaptive response and higher tolerance of *An. coluzzii* to ammonia in its larval habitat, which in the context of a scenario of peripatric speciation with gene flow may have been the most important driver of divergence from the ancestral *An. gambiae* s.s. (Aboagye-Antwi et al., 2015; della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008). These results were also consistent with field obtained data on the sibling species, where *An. coluzzii* prefer permanent, organically rich, predator prone habitats in contrast to its sibling species, *An. gambiae* s.s., that prefers temporal, shallow, rain-fed pools (della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008).

Adult mosquitoes that emerged from the less stressful rain puddle microcosm were significantly bigger than those from the rice paddy microcosm. *An. coluzzii* adults were equally significantly larger than *An. gambiae* s.s. across both microcosms. Previous studies have amply demonstrated that larval growth conditions influence adult body size

in *Anopheles gambiae* s.l (Aboagye-Antwi & Tripet, 2010; Takken et al., 2013; Valerio et al., 2016). Wing-length is the standard indicator of adult body size in Anophelines (Koella & Lyimo, 1996). In female Anophelines, the larger body size is positively correlated with fecundity and lifetime fitness (Takken et al., 2013). Larger males are more likely to mate and cope better with stressful abiotic conditions (Aboagye-Antwi & Tripet, 2010). The results from this study support field and laboratory studies on *An. coluzzii* and *An. gambiae* and strengthens the theory of divergent adaptations in their larval habitat (della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008). The *An. coluzzii* strain reared in CAEP, Keele laboratory appears to have maintained the reaction norms developed presumably from adaptation to rice field habitats over the years in their ability to adapt to stressful rearing conditions. Higher phenotypic quality evidenced in their larger body size compared to *An. gambiae* across both larval habitats is indicative of phenotypic plasticity (Price, et al., 2003).

The larger body size could also be as a result of overall higher mortality in *An. coluzzii* in comparison to *An. gambiae* s.s. There is the possibility that smaller mosquitoes died in the stressful rice paddy environment, leaving only stronger and larger mosquitoes to emerge. These results corroborate similar studies in laboratory conditions, where smaller adults emerged from over-crowded larval rearing trays resulting in reduced reproductive capacity, fitness and longevity (Takken et al., 2013). This further explains the significant effect of density on wing-length observed in this study as emerged adults from the 60 rearing density were significantly smaller than those from the 30 rearing density.

Time of development from first instar larvae to adult was significantly longer in the more stressful rice paddy microcosm for both sibling species. This plastic response can be linked to the effect of stressors in the environment modifying phenotypic

behaviour in these mosquito species. There were no significant differences in the duration of development between *An. coluzzii* and *An. gambiae*. This partially contrast with results from field transplantation experiments where larvae of *An. gambiae* developed faster than *An. coluzzii* in temporary water collections when predators are few, inversely larvae of *An. coluzzii* outcompetes those of *An. gambiae* in predator rich environments (Gimonneau et al., 2010). Possibly, the lack of species difference in development time was due to the absence of predators in the rice paddy microcosm, in contrast to the field rice paddy.

To foster food and economic security, production of rice in Africa has more than doubled in percentage from 1.76% between 1999-2001 to 3.96% between 2002-2013 (Sikirou et al., 2015). Subsequently, rice exports from the region have also increased in recent years with more landmass dedicated to irrigated rice farming (Nasrin et al., 2015). Advancement in agricultural technology, increased funding and support to the farmer has established rice cultivation as a year-round activity alien to its prior seasonal cycle. Other man-made hydrological schemes such as agricultural irrigation ditches and dams have further created new ecological larval niches for vector species and thus new opportunities for their specialisation and expansion into marginal habitats (Roux et al., 2014). These noble efforts to achieve self-sufficiency and food security by developing nations have further created more habitats for *An. coluzzii*, a situation that further promotes the niche expansion of this species that is highly adapted to living in human surroundings. Reports from the regions with intensive rice farming indicate the presence of vast amounts of the malaria vector all year round (Diabaté et al., 2008; Gimonneau et al., 2012). This has strong implications for existing vector control measures and the epidemiology of malaria. The results from this study will hopefully add to the literature that will serve to balance policy decisions on irrigated agriculture to mitigate the provision of additional larval

breeding sites for these vectors. The health impacts of such endeavours should be brought to the table during deliberations and policymaking.

The results from this study provides evidence that microcosms can be a useful model system for further ecological speciation studies, and other ecological and ecotoxicological aspects such as the testing for or the effect of larval predation on divergent ecological adaptation (Roeselers, et al., 2006) and bio-larvicides implementation in rice-fields. The model from this study also provides an experimental arena for mosquito ecological research with simplified miniature ecosystems that simulate natural environments and can be used to predict phenotypic responses under controlled conditions. Multiple replications due to the compact size of these systems and the ability to vary experimental conditions individually to establish a cause-effect relationship between variables and the test organism can be manipulated to provide substantial and valuable information on the ecology of *An. gambiae* s.l populations inhabiting rice-field ecosystems in the laboratory (Grenni, et al., 2012). This model study could further serve as a good compromise between actual rice-field experiments conducted in Africa that can be challenging due to high costs, environmental variability and difficult logistics; and non-microcosm laboratory experiments which are scarcely representative of the natural systems (like in Chapter 4). The advantages of this model system however are not endless as semi-field trials and then field trials will still need to be conducted before the implementation of projects, but the microcosm-based model developed here, that has revealed phenotypic differences in the sibling species, is a good starting point.

In conclusion, the major outcome of this study is the development of a laboratory model that successfully mimic species-preferred larval habitats of the *An. coluzzii* and *An. gambiae* s.s. revealing significant criss-crossing G × E interactions of biological

importance, that can be linked to rice field domestication in West Africa. The study provides evidence that support theories of ecological speciation resulting from divergent larval habitat preference among *An. coluzzii* and *An. gambiae* s.s. A consequence of *An coluzzii* larval preference is its ability to exploit more permanent, predator-rich, freshwater habitats, giving it a vectorial capacity advantage over *An. gambiae* s.s., especially in drier regions enabling it to breed and transmit *Plasmodium* all-year-round compared to its seasonal sibling (Roux et al., 2014). This information will be vital for the successful development and implementation novel vector control methods or the revision of older methods to account for the niche expansion of these vector species. It would also provide ecological information on the distribution of the sibling species in areas where inundated rice fields are associated with higher vector densities and malaria transmission. The microcosm model developed in this study can be used to investigate other adaptive traits such as oviposition site preference, the inheritance of plastic responses, longevity, male competitiveness, female fecundity of the sibling species, among other traits. It can also serve a model mini-ecosytem for mapping genes of these adaptive traits to the island of speciation within the genomes of these species.

Chapter 6

Improvement of water quality for mass Anopheline rearing: Evaluation of the impact of ammonia-capturing zeolite on larval development and adult phenotypic quality.

6.1. Introduction

Malaria is transmitted to humans through the bite of female *Anopheles* mosquitoes infected with *Plasmodium* parasites (HDV, 2017). Annual global malaria mortality is colossal, with 67% (272,000) of these deaths occurring in children under 5 years old in sub-Saharan Africa (WHO, 2019). In 2018, malaria mortality accounted for 405,000 deaths and half of the world's population remains at risk of becoming infected (WHO, 2019). WHO malaria report for 2019 records no significant progress in reducing global malaria between 2015-2018 (WHO, 2019). Further, there was a reduction in global investments in malaria control from 3.2 billion USD in 2017 to 2.7 billion USD in 2018 falling short of the 5 billion USD estimated to be required to achieve the GTS target of 40% reduction in malaria morbidity and death in 2020 (WHO, 2019). This illustrates how easily we can lose the achievements made towards malaria elimination and control if efforts are relented.

The primary vectors of malaria in sub-Saharan Africa are found in the *Anopheles gambiae* s.l species complex, with members, *An. gambiae s.s.*, *An. coluzzii*, and *An. arabiensis* transmitting malaria over vast ranges of sub-Saharan Africa and the surrounding islands (Coetzee et al., 2000; HDV, 2017). Effective control of these vectors is hindered or stalled by increasing insecticide resistance (WHO, 2017; Lees et al., 2015). There is an increasing demand for complementary or novel approaches to vector control that are effective, sustainable and environmentally friendly, to sustain the progress that has been made toward reduction and elimination of malaria transmission (WHO, 2017;

Lees et al., 2015). Alternative/complementary vector control methods currently being advocated for are the use of genetic and transgenic technologies which include, sterile insect technique (SIT), cytoplasmic incompatibility, vector-incompetence, gene-drive, and RIDL-(Release of Insects carrying a Dominant Lethal) (Alphey et al., 2013; Balestrino & Benedict, 2012). These approaches aim at population suppression or replacement through large scale releases of modified mosquito strains which rely on the availability of effective mass production structures (Balestrino & Benedict, 2012). Although research activities from the mid-1950s to mid-1980s, had established the feasibility of practical implementation of the SIT method for public health purposes, recent attention is being given to research in this area in the light of insecticide resistance challenges. The development and optimisation of mass-rearing technology are considered top priorities for the advancement vector-control-SIT to an operational (Alphey et al., 2013; Balestrino & Benedict, 2012; Bourtzis, et al., 2016; Burt, 2014; Lees et al., 2015).

Mosquito mass rearing is defined as "a large, continuous production of mosquitoes on a regular schedule resulting in adults that are comparable in specific ways to the wild mosquitoes" (Balestrino & Benedict, 2012). This implies the application of mechanisation and standardisation of rearing protocols to provide, on an industrial scale, extensive numbers of insects by an efficient, controlled and economical process (Balestrino & Benedict, 2012). Additionally, other genetically-modified mosquito deployment scenarios advocate the use of delocalized small mobile egg-to-adults rearing units, allowing for production of eggs in large production centres but rearing nearer to local targeted Anopheline populations (Tripet pers. comm., 2020). As confirmed in Chapters 3-5, and in other literature, *Anopheles gambiae s.l.* require comparatively much clean water than Culicines vector species (e.g. *Aedes aegyptis*, *Culex quinquefasciatus)*

(Gimonneau et al., 2012). The larvae of this species typically die in polluted water, either through the accumulation of toxic wastes, such as ammonia, or because of the development of anaerobic bacteria that are harmful to mosquitoes.

Ammonia is produced in aquatic ecosystems as a by-product of metabolism and organic waste decomposition by prokaryotes (Bernhard, 2010). In its unionised form, NH_3, ammonia is toxic to fish and aquatic invertebrates at concentrations above 0.2mg/l (U.S. Environmental Protection Agency, 2013). In nature, endogenously produced ammonia is largely excreted by passive diffusion or urea excretion by most aquatic organisms and subsequently converted to nitrite and then to less harmful nitrates in a process of nitrification (Figure 6.1) by microorganisms such as bacteria, archaea and fungi (Bernhard, 2010; U.S. Environmental Protection Agency, 2013). Accumulated unionised ammonia can inhibit the action of nitrifying bacteria, resulting in increased levels of ammonia in aquatic habitats thereby intensifying the deleterious effects on aquatic animals and beneficial bacteria (U.S. Environmental Protection Agency, 2013). The exact process leading to the death of Anopheline larvae in polluted water is not fully understood but could involve hypoxia, ammonia toxicity, inability to transport oxygen, pathogenicity, nutrient enrichment, and competition for food resource (Amarasinghe & Weerakkodi, 2014; Tchigossou et al., 2018; U.S. Environmental Protection Agency, 2013; Ward & Jensen, 2014).

It is not uncommon to find mosquito larval rearing trays in insectaries containing high levels of ammonia which usually result in the death of mosquito larvae (Mamai, et al., 2016). Water management in mosquito insectaries to minimise the presence of ammonia and encourage beneficial microorganisms is key to achieving optimal rearing results both for small cultures and for mass-rearing facilities (HDV, 2017; Mamai et al., 2017, 2016).

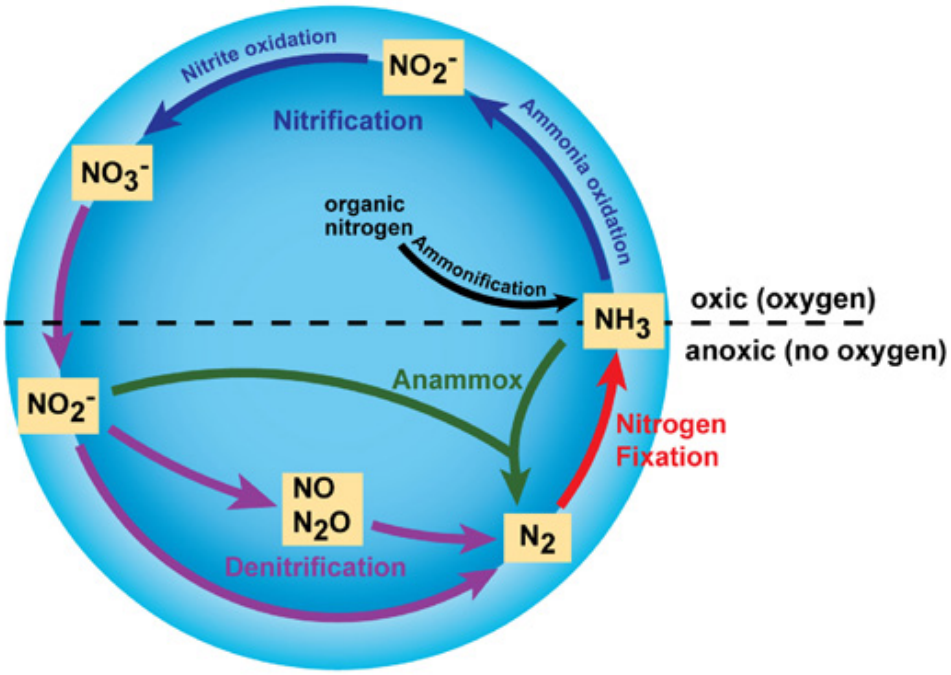

Figure 6.1. Major transformations in the nitrogen cycle (Bernhard, 2010).

This implies that rearing facilities must rely on a water circulating system with continuous quality monitoring, and other measures to reduce and prevent the development of pathogens, and feeding systems that maintain optimal diet availability (Benedict et al., 2009). The quality of mosquitoes produced from mass-rearing facilities are routinely checked as an essential aspect of an SIT programme (HDV, 2017) and the same is expected for genetically modified (GM)-mosquito production. The recommendation is to monitor mosquitoes at different life cycle stages using the following parameters: hatch rate (number of eggs laid), pupation (the percentage of larvae that successfully pupate), adult emergence (number of emerged adults), adult survival (emerged adults lasting 8-12 days), sex ratio (ratio of emerging mosquitoes, ideally 50:50), mating status (dynamic mating activity) and engorged females (females who blood-feed adequately) (HDV, 2017).

The success of an SIT or other mass-release based vector control approach relying on large-scale production of Anopheline mosquitoes, is dependent on a reliable supply of

constant water of sufficient quality (Mamai et al., 2017; Piyaratne, et al., 2005). Anopheline mosquito larvae are filter feeders, they receive all essential macro- and micronutrients and oxygen through the water in which they are reared (Gimnig et al., 2002; Kaufman et al., 2006). If only clean water were to be used for this purpose, huge amounts of water will be required. For example, for large SIT production centres the FAO/IAEA recommended larval rearing rack (which holds up to 200,000 *Anopheles* larvae) use approximately 250l of water daily (HDV, 2017; Mamai et al., 2017). Approximately 100,000l of water is required to produce 10,000,000 sterile males per week (Mamai et al., 2017). Many countries where mass-release methods might be applied are located in arid regions where clean water is scarce and unreliable (Mamai et al., 2017). Over one billion people currently reside in regions where water is a scarce commodity, and this number could increase to 3.5 billion by 2025 (FAO, 2012; Mamai et al., 2017). Increasing world populations will further mount pressure on these scarce water resources. Anthropogenically induced global warming further increases the chances for conditions that lead to extended periods of drought (FAO, 2012; Mamai et al., 2017). This increasing demand for access to clean water especially in arid regions with vector-borne disease (VBD) endemicity, necessitated the use of alternative water treatment for the implementation of mass-release based VBD control approaches (Mamai et al., 2017). To be sustainable and environmentally friendly, mass-rearing systems need to be cost effective. Methods of water conservation is therefore integral in the successful implementation of sustainable mass-release vector control programmes (Mamai et al., 2017).

Investigations on reuse and recycling of larval rearing water for successive generations of mosquito larvae has been initiated by the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

(Mamai et al., 2017). The first study considered the reuse of larval rearing water ("dirty water" henceforth) for rearing subsequent batches of *An. arabiensis* larvae without treating the water (Mamai, et al., 2016), using FAO/IAEA recommended mass rearing protocols (Balestrino & Benedict, 2012; HDV, 2017). Results from this study demonstrated that *An. arabiensis* can be reared in reused larval water without any effect on hatch rate, larval development time and mortality (Mamai, et al., 2016). On the downside, there was a negative carry-over effect on the body size, fitness and longevity of emerged adults, which may potentially hamper the success of the SIT component (Mamai, et al., 2016). The authors concluded that although mosquito production was not adversely affected by "dirty" water reuse, the quality of subsequent adults was significantly affected; and they recommended water treatment before reuse (Mamai, et al., 2016).

Multiple technologies have been employed in water treatment for recycling purposes such as irradiation, ultrafiltration (UF) ultrasonic treatments, heat treatment, autoclaving and reverse osmosis (RO) (Backer et al., 2002; Bohdziewicz, et al., 2003). UF and RO are commonly used pressure-driven membrane-based filtration processes that involve forcing water molecules through a semi-permeable membrane to produce high-quality recycled water (Mamai et al., 2017). Whilst the UF method is used to remove colloids, virus, suspended and macromolecular matter; RO even removes bacteria, viruses, dissolved species, low-molecular organic compounds and mineral substances (Mamai et al., 2017). As a follow-up to the earlier study on reusing untreated water, a subsequent study sponsored by FAO/IAEA, investigated the reuse of water treated by UF and RO for rearing *An. arabiensis* (HDV, 2017; Mamai et al., 2017). The authors reported success in rearing *An. arabiensis* in treated (recycled) water for at least one subsequent generation. Although the treatment of water with UF and RO resolved the issue with

decreased mosquito adult size and longevity observed in the previous study where dirty water was reused; the authors agree that this method is not a single specific technology for water treatment and processing high volumes of water during an overall mass-rearing process. They also acknowledged that the cost of treatment is significant, which will ultimately increase the operational costs of running the mass-rearing facility (Mamai et al., 2017).

Whilst these achievements towards water conservation in mosquito mass-rearing are laudable, cheaper alternative treatments, but also quick, easily applied to large volumes of water, are needed to ensure reduced operational costs, so scarce resources available for research can be extended. The use of probiotics in aquaculture is one of such water treatment alternatives. Probiotics, which are basically live, dead or component microbial cell are administered to rearing water or as a feed supplement. They confer health benefits to the aquatic organisms by improving disease resistance (Irianto & Austin 2002; Newaj-Fyzul et al., 2007; Silva et al., 2012), growth performance (Boonthai, et al., 2011; Kumar, et al., 2006), feed utilization, stress response or general vigour which is achieved through the enhancement of the hosts microbial balance or the microbial balance of the host's ambient environment (Merrifield et al., 2010). Traditionally, ammonia has been removed from aquaculture water systems by biofilters containing nitrifying microorganisms. Ammonia (NH_3) oxidising bacteria (AOB) convert ammonia to nitrite (NO^{2-}), which is then converted to less harmful nitrate (NO^{3-}) by nitrite oxidizing bacteria (NOB) (Costa, et al., 2006). Probiotics have also been shown to improve water quality by reducing the number of pathogenic bacteria (Dalmin, et al., 2001). As with any method, the use of probiotics to improve water quality and production in an aquatic ecosystem have some shortfalls. Over-dosage and prolonged administration of probiotics can induce immune suppression of continuous responses of the hosts (Sakai, 1999).

Moreover, the fate of live probiotics in aquatic environments is uncertain. There is the possibility of horizontal gene transfer to other animals including humans (Newaj-Fyzul et al., 2014), and this concern may be more serious with the use of probiotics in rearing vectors (*An. gambiae* s.l) of human diseases. Furthermore, nitrifying bacteria are slow growing microorganisms whose natural development in aquaculture systems could take up to 3 months (Keuter et al., 2017). Even when probiotics are inoculated, studies have shown that it may take 14 days for the full development of nitrifying bacteria; a period during which ammonia can reach critical levels (Skleničková et al., 2020; Son, et al., 2000). This hysteresis in the efficiency of probiotics in ammonia reduction has necessitated research into alternative/complementary ammonia absorbent material to improve water quality in aquaculture systems (Son et al., 2000). In the light of these constraints in the use of probiotics in fish aquaculture, its application for mosquito rearing will require further extensive investigations to ascertain its safety, cost effectiveness, ease of application especially for mass production facilities.

The use of zeolite might be another water treatment alternative that is relatively cheaper compared to UF and RO; and can be easily applied compared to probiotics. Zeolite has been widely applied in fish and crustacean aquaculture to improve water quality and feed quality as well as reduce the negative environmental impacts of aquaculture and improve the quality of seafood (Abdel-rahim, 2017). Zeolites are microporous crystalline aluminosilicates with chemically neutral basic honeycomb-like structures that originate from volcanic rocks (Ghasemi, et al., 2018). They are inert, and structurally stable even at extreme temperatures up to 750ºC (Abdel-rahim, 2017). There are more than 60 types of naturally occurring zeolites with 150 synthetic types formulated with improved efficiency (Ghasemi, et al., 2018). Clinoptilolite is one of the purest, effective and cheapest type of natural zeolite (Ghasemi et al., 2018; Skleničková et al.,

2020), it is not volatile and is insoluble in water (Ghasemi, et al., 2018). The zeolite structure consists of tetrahedric $(SiO_4)^{4-}$ and $(AlO_4)^{5-}$ groups connected by oxygen bridges. When silicon (Si) is replaced by aluminium (Al) in the zeolite framework, a negative charge is formed on the overlying oxygen atom. This negative charge is compensated by cations, mostly alkali metals (Li^+, Na^+, K^+) and by alkaline earth metals (Ca^{2+}, Mg^{2+}) (Querol et al., 2002). As a result of this chemical structure, zeolites have excellent capability to absorb cations and they prefer cations with greater radius and monovalent charge, hence their affinity for cations such as ammonium ion (NH^{4+}) (Kuronen, et al., 2000). This high selection for NH^{4+} forms the basis for most applications of zeolite in aquaculture systems (Figure 6.2) (Ghasemi, et al., 2018). The chemical structure of zeolite forms a network of channels and cavities allowing easy penetration of micron-sized molecules which are filtered according to size, polarity and shape, thereby serving as an efficient filter absorbing various substances that come in contact with it (Abdel-rahim, 2017). Due to its porous nature, the ion exchange occurs not only at the surface but also deep within the zeolite structure, further enhancing its efficiency (Hartman & Fogler, 2007; Inglezakis, 2012). Following saturation, zeolite can be recharged (when incorporated in filters) by soaking in 10% NaCl solution, thus renewing their capacity and can then be reused (Ghasemi et al., 2018; Hartman & Fogler, 2007; Inglezakis, 2012).

Zeolites have been extensively applied in fish and crustacean aquaculture because of their selective absorption of ammonia and toxic heavy metals as well as an additive in fish food (Ghasemi, et al., 2018). Water quality is improved when the zeolite is used because it can adsorb ammonia, heavy metals, pesticides, smells, radioactive cations and many other toxins (Abdel-rahim, 2017). Its efficiency in ammonia removal is greater than 83% when initial ammonia content is less than or equal to 1.0ppm.

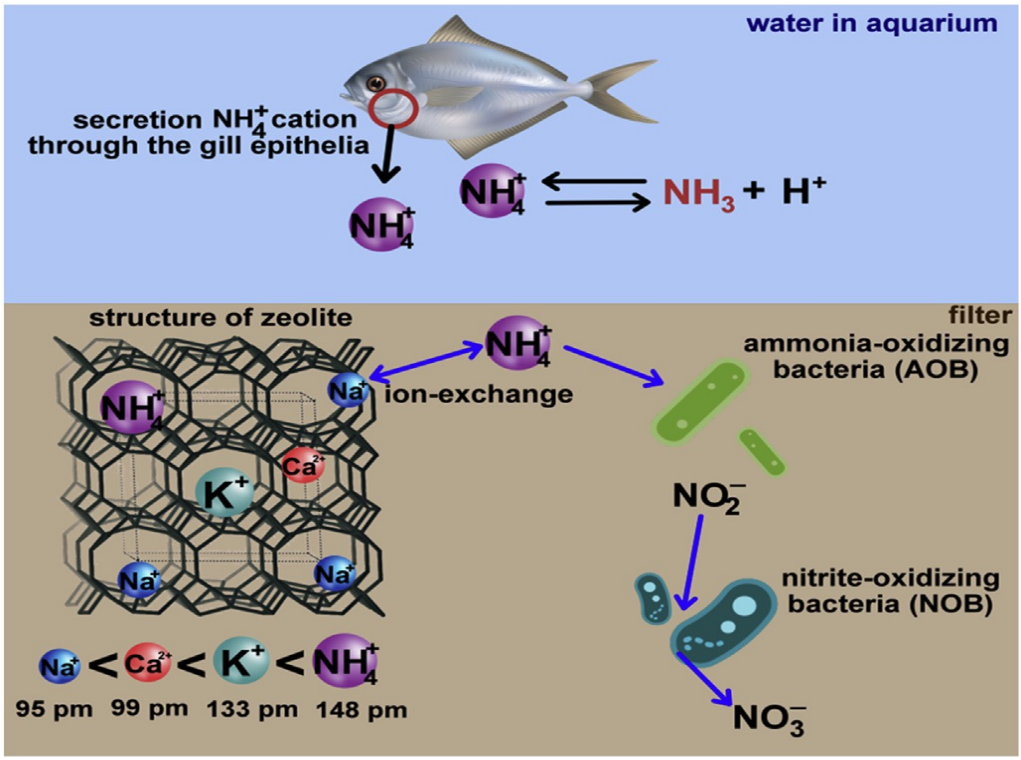

Figure 6.2: Competition for NH_4^+ cations between zeolite material and nitrifying microorganisms in an aquarium filter (Ghasemi, et al., 2018).

The greater the concentration of initial ammonia, the lesser the ammonia removal efficiency (Abdel-rahim, 2017; Aly, et al., 2016; Emadi, et al., 2001). Particle size and application method also affect the efficiency of ammonia removal in aquaculture systems. Decreasing zeolite particle size improves ammonia removal. There is no standard dose of application as dosage will depend on fish biomass, the protein content of the feed, feed stability and water quality. The recommendation is to add zeolite based on the expected and/or measured quantity of nitrogenous by-products. Average ammonia absorption capacity is 25mg NH_4^+ /g of zeolite (Abdel-rahim, 2017; Ghasemi et al., 2018). When using zeolite in aquaculture other factors to be considered that might impact on its efficiency include; conductivity, pH, temperature, initial concentration of cations in the water; concentration, particle size and exposure time of zeolite (Ghasemi et al., 2018; Skleničková et al., 2020).

Another important factor to consider in the use of zeolite to improve water quality in closed aquaculture systems is that by absorbing ammonium ions (NH_4^+), zeolites can greatly influence the abundance and development of nitrifying microorganisms (Motesharezadeh, et al., 2015). Several studies have shown that the use of zeolite in biological filters while able to reduce coliform bacteria and other pathogens, absorb ammonia and nitrates more quickly than nitrifying bacteria which are then unable to use NH_4^+ for their metabolic necessities, thus negatively impacting the population of these beneficial microorganisms (Montalvo et al., 2014; Sirakov et al., 2015; Skleničková et al., 2020).

The use of natural zeolites and their modified forms has many advantages including low cost, abundance in many parts of the world and being environmentally friendly. However, for successful application to obtain optimal production, users must consider zeolite particle size, chemical composition of water, dosage and balance these factors to accommodate beneficial bacteria in these aquatic ecosystems (Ghasemi, et al., 2018).

In this study, we experimentally evaluated the use of zeolite treatment in rearing the Mopti strain of *An. coluzzii* in comparison with a continuous flow system (simulated by daily water changes). Larval development, adult emergence and body size were recorded to score success in both approaches. Results show that treating rearing water (1000ml) with zeolite (1g/l) improved mosquito adult emergence and could be a cheaper alternative to more expensive techniques such as ultra-filtration, reverse osmosis filters which is often part of continuous water flow systems; and could complement the application of probiotics.

6.2. Materials and Methods

6.2.1. Mosquito strain

The Mopti strain of *An. coluzzii*, colonized 17 years ago in 2003 by the Lanzaro Laboratory (UC Davis) from the village of N'Gabacoro droit near Bamako, Mali, West Africa, were used for the experiments. The strains were maintained by the Tripet group in dedicated insectaries of the Centre of Applied Entomology and Parasitology (CAEP), Keele University, UK, as described in Chapter 2.

6.2.2. Experimental design.

First instar larvae of *An. coluzzii* were reared at two larval rearing densities (200 and 400 larvae per tray), under four different water treatment regimes, and using two contrasted feed regimes. This resulted in a fully balanced 2 x 4 x 2 design and 16 larval trays per replicate with a total sample size of 19200 larvae for four replicates (Figure 6.3). Trays were identified with coloured tapes codes and fully randomized in terms of their positions on the insectary shelves.

Experimental factor 1: Experiments were conducted using two larval densities- 200 and 400 (Figure 6.3).

Experimental factor 2: Larvae were reared in four water treatment groups:

A. Control- first instar larvae were initially trayed in 500ml of mineral water and received an additional 500ml of mineral water on day 5 (Figure 6.3)

B. Zeolite without water replacement- On day 1, first instar larvae were trayed in 500ml of mineral water, on day 4, 1g of finely ground zeolite powder (Minerals-Water, Unit 6, Orwell close, Fairview industrial park, Rainham, RM13 8UB, United Kingdom), was added to the rearing water and on day 5, 500ml of additional mineral water was added to

the rearing trays. This group will be subsequently referred to as the zeolite group (Figure 6.3)

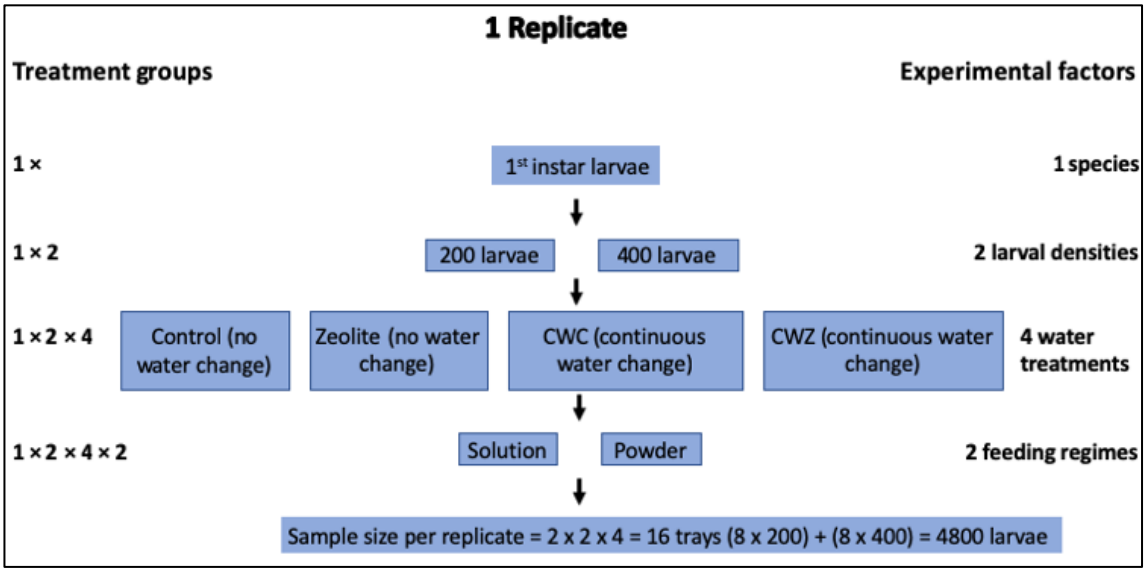

Figure 6.3: Experimental design showing experimental factors combined in one replicate resulting in 2 larval densities, four experimental water treatments (Zeolite, Control, CWC, CWZ) and 2 feeding regimes.

C. Zeolite with continuous water replacement: first instar larvae were trayed in 500ml of mineral water on day 1; 1g of finely ground zeolite powder was added to the rearing water on day 4; on day 5, 400ml of water was gently drained from the trays using a low pressured water pump (same used for picking pupae as described in Chapter 2) through a filter net to prevent mosquito larvae escaping into the pump after which 900 ml of fresh mineral water was added to the tray. This process of gently draining rearing water and replacing with fresh water was repeated daily from day 5 until all mosquitoes in the tray had pupated. This group will be subsequently referred to as CWZ (continuous water changes with zeolite) (Figure 6.3)

D. Continuous water replacement without zeolite: first instar larvae were trayed in 500ml of mineral water on day 1; on day 5, 400ml of water was gently drained from the trays using a low pressured water pump (described in chapter 2) through a filter net to

prevent mosquito larvae escaping into the pump after which 900 ml of fresh mineral water was added to the tray. This process of gently draining rearing water and replacing with fresh water was repeated from day 5 until all mosquitoes in the tray had pupated. This group will be subsequently referred to as CWC (continuous water change) (Figure 6.3)

Experimental factor 3: Contrasted feeding regimes- larvae were fed with two standardised feeding regimes (solution and powder feed). Powder feeding regime consists of daily rations of ground fish food, using a spatula to spread on the water surface: 0.1µl of Liquifry liquid fish food (Interpret Ltd, Surrey, UK) on day 1, 20mg on days 2–3, 40mg on day 4, and 90 mg on day 5 until pupation. Solution feeding regime consist of the same food quantity dissolved in deionized water (0.1µl of Liquifry on day 1, 1ml of 6mg/10ml of TetraMin Baby on days 2–3, 1ml of 30mg/10 ml of TetraMin Baby on day 4, and 1ml of 60mg/10 ml of TetraMin Baby on day 5 until pupation) and injected into the larval trays using a pipette (Figure 6.3)

6.2.3 Data collection

Mosquitoes pupating from larval trays were picked using a 3ml plastic pipette and transferred to styrofoam cups containing mineral water (at the low density of ~ 20 pupae in 100ml), then placed in adult cages (as described in chapter 2) for emergence. Larval survival was scored by deducting total numbers of pupae from the initial numbers of first instar larvae placed in the tray. Pupal mortality is the number of mosquitoes that died following pupation. Adult emergence is the total number of adults that emerged from the tray from the initial number of first instar larvae placed in the tray. Development time was recorded as the time from day 1 of the experiment when first instar larvae were placed in the trays until the day mosquitoes emerged as adults. Emerged adults were collected using a mouth aspirator, sexed and stored in 75% ethanol for subsequent wing-

length measurement. A total of 1280 emerged adults equivalent to 40 males and 40 females per treatment were randomly sampled for wing-length measurements, following the procedure detailed in Chapter 2 for wing measurement.

6.2.4. Water quality analysis

Measurements for ammonia (NH_3) were taken using a Handheld Colorimeter kit (Hanna Instruments, Inc. Highland Industrial Park, 584 Park East Drive, USA), nitrate was measured using API aquarium test kits (Mars Fishcare North America, Inc, 50E Hamilton street, Chalfont, PA, 18914, USA), on days 4, 6, 8, and 10 (if larvae were still alive in the tray) following experimental set-up. There was a steady build-up of ammonia, rising above toxicity threshold on day 4 and reaching a peak on day 8 when most larvae pupate (Appendix B).

6.2.5. Statistical analysis

All data collected were analysed using the software JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA). All data were checked for deviations from normality and heterogeneity of variance, and analyses were conducted using parametric and non-parametric methods as appropriate. The 2 x 4 x 2 design of the experiment allowed for fully-balanced multivariate statistical models. Data from all replicates were used for analysis. In multivariate analyses, replicate effects were tested but were only reported when significant. Interactions between independent variables were tested using a step-wise approach and only those significant were retained in the final models. For analyses of proportion of larvae, pupae, adults, and sex ratio likelihood odds ratios were used for *post-hoc* pairwise group comparisons following logistic regressions. Goodness of fit tests (50:50) were used to test sex ratio of emerged adults. Body size was analysed through

general linear models followed by Tukey's HSD *post-hoc* pairwise comparisons. Developmental times (day of emergence) were analysed by Cox Proportional-Hazard models with likelihood odds ratios for *post-hoc* pairwise comparisons. Finally, ammonia and nitrate measurements were analysed through generalised linear model using standard least squares.

6.3. Results

6.3.1. Physicochemical properties of larval trays

There was a significant ($P < 0.0001$) impact of water treatment on ammonia concentrations in larval trays, which increased significantly ($P < 0.0001$), with time from day 4 to day 8 (Table 6.1, Figure 6.4). In Control and Zeolite treatments, there was a steady build-up of ammonia, rising above toxicity threshold (0.2mg/l) on day 4 and reaching a peak on day 8 when most larvae pupate (Figure 6.4). Ammonia was significantly lower in Zeolite treatment compared to Control. Ammonia levels were significantly lower and relatively stable in CWC and CWZ compared to treatments without water change (Control and Zeolite) (Figure 6.4).

Inversely, nitrate levels increased with time in CWC and CWZ and were significantly ($P < 0.0001$), higher compared to Zeolite and Control. However, nitrate levels in Zeolite were significantly higher than those in Control (Figure 6.4; Table 6.1). Among all treatment trays, nitrate concentrations were significantly higher in powder feed compared to solution feed (Table 6.1; Figure 6.5).

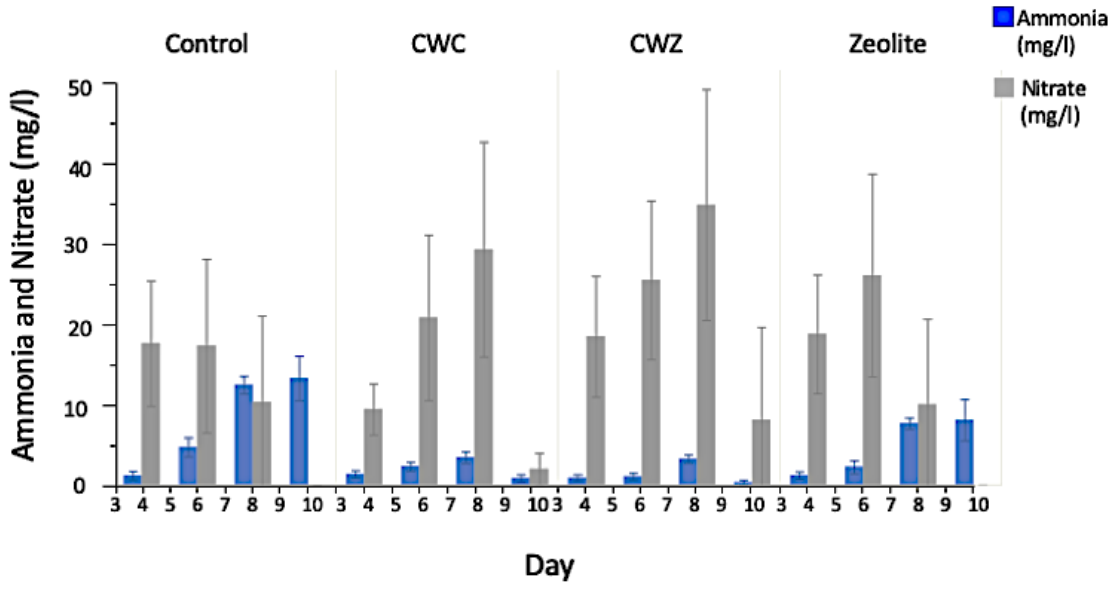

Figure 6.4: Ammonia (blue bars) and nitrate (grey bars) concentration across treatment trays from day 4 to day 10. Whiskers represent 95% confidence intervals (CI).

Table 6.1: General linear model of ammonia and nitrate concentrations across treatments

Parameter	Source	df	F-ratio	P-value
Ammonia(mg/l)	Feed	1	0.605	0.4374 ^{ns}
	Density	1	0.4077	0.5238^{ns}
	Water treatment	3	88.361	<0.0001***
	Day	1	171.397	<0.0001***
	Treatment*Day	3	56.165	<0.0001***
	Day*Day	1	82.230	<0.0001***
Nitrate (mg/l)	Feed	1	40.497	<0.0001***
	Density	1	3.167	0.0764 ^{ns}
	Water treatment	3	12.992	<0.0001***
	Day	1	128.072	<0.0001***
	Treatment*Day	3	6.202	0.0005**
	Day*Day	1	61.472	<0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

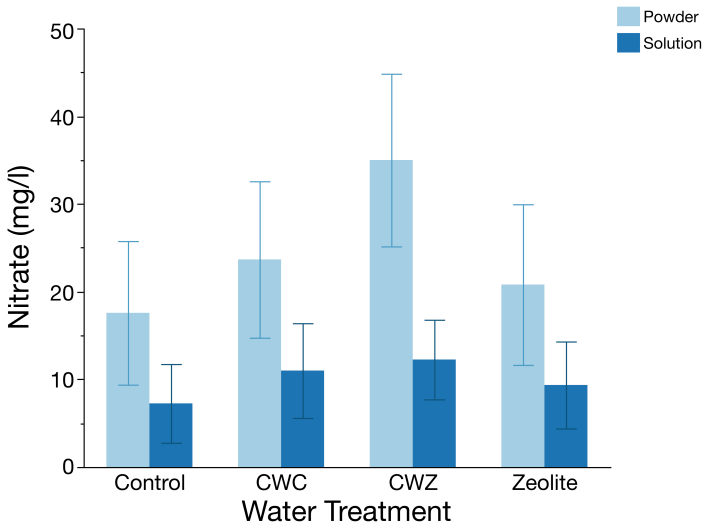

Figure 6.5: Nitrate concentration by feed across treatment trays, light blue bars show powder feed, dark blue bars show solutionr feed. Whiskers represent 95% confidence intervals (CI).

6.3.2. Effect of density, water treatment, and feed type on larval survival

Percentage larval survival of *An. coluzzii* was significantly impacted by treatment type, feed, density and the interactions between these variables (Table 6.2). Overall, larval survival was significantly higher in the CWC treatment (66%) for both rearing densities and feed in comparison to other treatments (Control – 55%, CWZ – 52%, Zeolite – 52%). (Table 6.4). Larval survival was 10% higher in powder feed solution feed compared to solution feed and at 200 larval rearing density compared to 400 larval rearing density (Figure 6.6; Tables 6.2, 6.3, 6.4).

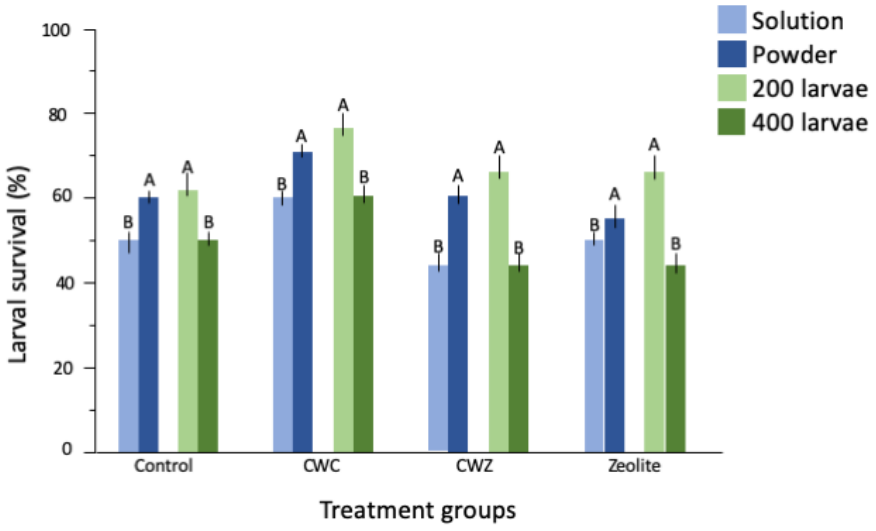

Figure 6.6: Percentage larval survival across water treatments for two larval rearing densities(light green bars-200 larvae, dark green bar-400 larvae) and feed (light blue bars-solution, dark blue bars-powder).Whiskers represent 95% confidence intervals (CI). Within water treatment groups significant differences are represented by different letters.

At 200 larval rearing density, larval survival significantly differed among treatments where survival was highest in CWC (77%) followed by CWZ (67%), then Zeolite (67%) and Control (63%) (Figure 6.6; Table 6.3). Larval survival was also significantly different at 400 larval rearing density with 61% surviving in the CWC followed by 51% in control, with no significant difference in survival between CWZ (44%) and Zeolite (45%) (Figure 6.6; Table 6.3).

Table 6.2: Logistic regressions (Effect Likelihood Ratio Tests) of mosquito survival among treatments

Parameter	Source	DF	Likelihood ratio	P-value
Larval survival	Density	1	610.267	<0.0001***
	Feed	1	195.915	<0.0001***
	Treatment	3	221.067	<0.0001***
	Treatment*Feed	3	39.440	<0.0001***
	Treatment*Density	3	31.839	<0.0001***
	Feed*Density	1	7.118	0.0076*
Pupal mortality	Density	1	5.007	0.0252*
	Feed	1	2.049	0.1523^{ns}
	Treatment	3	76.424	<0.0001***
	Treatment*Feed	3	42.522	<0.0001***
	Treatment*Density	3	8.433	0.0379*
	Feed*Density	1	7.885	0.0050*
Adult emergence	Density	1	544.058	<0.0001***
	Feed	1	187.584	<0.0001***
	Ttreatment	3	258.443	<0.0001***
	Feed*Density	1	4.801	0.0285*
	Treatment*Feed	3	44.096	<0.0001***
	Treatment*Density	3	46.674	<0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

Table 6.3: Odds ratios for the effect of density and feed on larval survival within treatments

Source	Parameter	Odds ratio	P-value
Density (200 larvae)	CWC vs Control	0.517	<0.0001***
	CWZ vs Control	0.832	0.0141*
	CWZ vs CWC	1.608	<0.0001***
	Zeolite vs Control	0.842	0.0210*
	Zeolite vs CWC	1.627	<0.0001***
	Zeolite vs CWZ	1.011	0.8817^{ns}
Density (400 larvae)	CWC vs Control	0.655	<0.0001***
	CWZ vs Control	1.311	<0.0001***
	CWZ vs CWC	2.001	<0.0001***
	Zeolite vs Control	1.290	<0.0001***
	Zeolite vs CWC	1.968	<0.0001***
	Zeolite vs CWZ	0.984	0.7440^{ns}
Feed (Solution)	CWC vs Control	0.601	<0.0001***
	CWZ vs Control	1.213	0.0022**
	CWZ vs CWC	2.016	<0.0001***
	Zeolite vs Control	0.991	0.8897^{ns}
	Zeolite vs CWC	1.648	<0.0001***
	Zeolite vs CWZ	0.818	0.0014**
Feed (Powder)	CWC vs Control	0.564	<0.0001***
	CWZ vs Control	0.900	0.1051^{ns}
	CWZ vs CWC	1.596	<0.0001***
	Zeolite vs Control	1.095	0.1628^{ns}
	Zeolite vs CWC	1.942	<0.0001***
	Zeolite vs CWZ	1.217	0.0031**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

Solution feed impacted significantly on larval survival among treatments, with highest larval survival in CWC (60%) , followed by Control (50%), Zeolite (49%) and CWZ (43%) (Figure 6.6; Table 6.3). Larval survival for powder feed also significantly differed among treatments with the highest in CWC (72%), then CWZ (61%), Control (60%), and Zeolite(55%) (Figure 6.6; Table 6.3).

Table 6.4: Mosquito survival at life history stages across treatments

Feed	Larval density	Treatment	%Larval survival	%Pupal mortality	%Adult emergence
Solution	200	Control	61 (57-64)	16 (13-18)	45 (42-49)
		CWC	75 (71-77)	7 (5-9)	68 (65-71)
		CWZ	62 (58-65)	8 (6-10)	54 (51-58)
		Zeolite	62 (59-65)	6 (4-8)	56 (53-60)
Powder	200	Control	66 (63-69)	8 (6-10)	58 (54-61)
		CWC	79 (76-82)	10 (8-12)	69 (66-73)
		CWZ	73 (70-76)	6 (4-8)	67 (64-70)
		Zeolite	72 (69-75)	9 (7-11)	63 (60-67)
Solution	400	Control	44 (42-46)	10 (9-12)	34 (31-36)
		CWC	53 (51-56)	4 (3-5)	49 (47-52)
		CWZ	34 (32-36)	4 (4-6)	30 (27-32)
		Zeolite	43 (41-45)	9 (8-11)	34 (32-36)
Powder	400	Control	58 (55-60)	10 (9-12)	48 (45-50)
		CWC	69 (66-71)	9 (8-11)	60 (57-62)
		CWZ	55 (52-57)	6 (5-7)	49 (46-51)
		Zeolite	46 (44-49)	8 (7-9)	38 (36-41)

Ninety-five percent confidence intervals are in brackets. Larval survival, pupal mortality and adult emergence were calculated out of the initial sample sizes of 800 (200 larval density) and 1600 (400 larval density).

6.3.3. Pupal mortality by density, feed regimes and water treatments

Overall, pupal mortality was significantly impacted by larval rearing density, water treatment and the interactions between these variables. Although interactions between feed and other variables were significant, feed had no significant impact on pupal mortality across treatments (Table 6.2). Highest pupal mortality (11%) was observed in the Control with the least mortality occurring in CWZ.

Within treatments, at 200 larval rearing density, pupal mortality significantly differed among treatments. The highest mortality (12%) was found in the Control group, followed by CWC (8%), CWZ (7%), and Zeolite (7%) (Figure 6.7; Table 6.5).

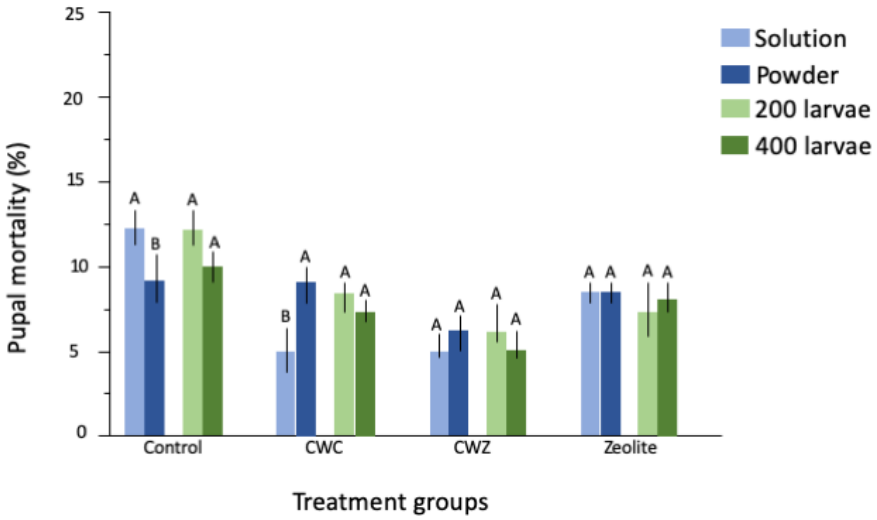

Figure 6.7: Pupal mortality across water treatments for two larval rearing densities (light green bars-200 larvae, dark green bar-400 larvae) and feed (light blue bars-solution, dark blue bars- powder). Whiskers represent 95% confidence intervals (CI). Within treatments and for independent variables, significant differences are represented by different letters.

At 400 larval rearing density, there was a significant effect of density on pupal mortality across water treatments. Pupal mortality was highest in the Control group (10%) followed by 8% in Zeolite, 7% in CWC and 5% in CWZ (Figure 6.7; Table 6.5). Pupal mortality differed significantly among treatment as a result of significant interactions between feed by treatment (Table 6.2). Pupal mortality was highest (12%) in Control when solution feed was used, followed by Zeolite (8%), then 5% for CWC and CWZ (Table 6.5; Figure 6.7). Pupal mortality was insignificant for powder feed among Control, CWC and Zeolite, with significantly lower mortality in CWZ (Table 6.5; Figure 6.7).

Table 6.5: Odds ratios for the effect of density and feed regimes on pupal mortality within treatments

Source	Parameter	Odds ratio	P-value
Density (200 larvae)	CWC vs Control	1.483	0.0014**
	CWZ vs Control	1.820	<0.0001***
	CWZ vs CWC	1.227	0.1373^{ns}
	Zeolite vs Control	1.654	<0.0001***
	Zeolite vs CWC	1.116	0.4184^{ns}
	Zeolite vs CWZ	0.909	0.4986^{ns}
Density (400 larvae)	CWC vs Control	1.722	<0.0001***
	CWZ vs Control	2.022	<0.0001***
	CWZ vs CWC	1.174	0.1481^{ns}
	Zeolite vs Control	1.223	0.0206*
	Zeolite vs CWC	0.710	0.0006**
	Zeolite vs CWZ	0.605	<0.0001***
Feed (Solution)	CWC vs Control	2.644	<0.0001***
	CWZ vs Control	2.362	<0.0001***
	CWZ vs CWC	0.893	0.3903^{ns}
	Zeolite vs Control	1.838	<0.0001***
	Zeolite vs CWC	0.695	0.0048**
	Zeolite vs CWZ	0.778	0.0454*
Feed (Powder)	CWC vs Control	0.966	0.7452^{ns}
	CWZ vs Control	1.557	0.0002**
	CWZ vs CWC	1.612	<0.0001***
	Zeolite vs Control	1.101	0.3822^{ns}
	Zeolite vs CWC	1.140	0.2258^{ns}
	Zeolite vs CWZ	0.707	0.0040**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

6.3.4. Adult emergence of *An. coluzzii* across water treatments

Adult emergence across treatment trays were significantly impacted by rearing density, feed type and the interactions of these independent variables (Table 6.2). As before, adult emergence which embodies overall mosquito survival was highest in CWC. Emergence was also significantly higher in powder feed across treatments (Table 6.2). Within

treatments, adult emergence differed significantly among treatment groups by density and feed regime. At 200 larval rearing density, adult emergence was highest (69%) in CWC followed by 60% in both CWZ and Zeolite, then 51% in control (Figure 6.8; Table 6.6).

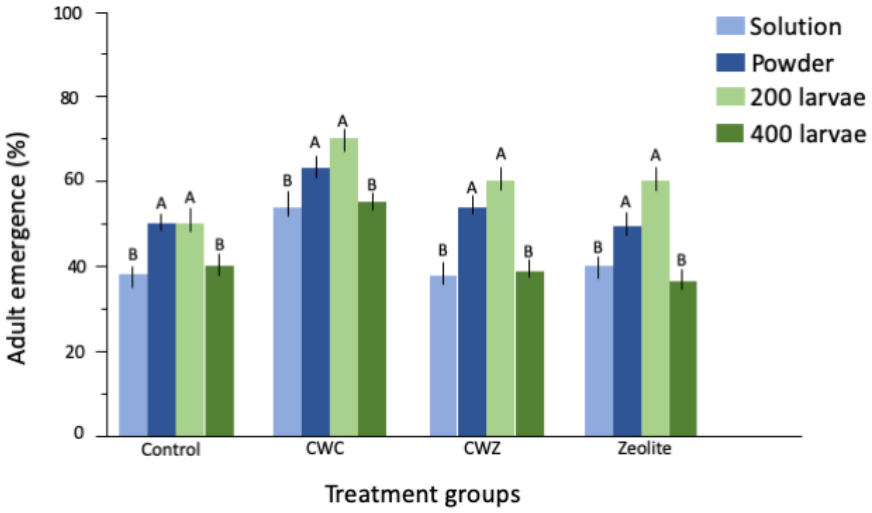

Figure 6.8: Adult emergence across water treatments for two larval rearing densities(light green bars-200 larvae, dark green bar- 400 larvae) and feed (light blue bars-solution, dark blue bars- powder).Whiskers represent 95% confidence intervals (CI). Within treatments and for independent variables, significant differences are represented by different letters.

Mosquito adult emergence at 400 larval rearing density was highest in CWC (54%), followed by Control (41%), (39%) in CWZ then (36%) Zeolite (Figure 6.8; Table 6.6). A similar pattern was observed for feed type with the highest adult emergence for solution feed in CWC (55%) followed by Zeolite (41%) then 38% for both CWZ and Control (Figure 6.8; Table 6.6).

Table 6.6: Odds ratios for the effect of density and feed regimes on adult emergence within treatments

Source	Level	Odds ratio	P-value
Density (200 larvae)	CWC vs Control	0.480	<0.0001***
	CWZ vs Control	0.683	<0.0001***
	CWZ vs CWC	1.422	<0.0001***
	Zeolite vs Control	0.713	<0.0001***
	Zeolite vs CWC	1.484	<0.0001***
	Zeolite vs CWZ	1.043	0.5592^{ns}
Density (400 larvae)	CWC vs Control	0.571	<0.0001***
	CWZ vs Control	1.086	0.1142^{ns}
	CWZ vs CWC	1.900	<0.0001***
	Zeolite vs Control	1.210	0.0002**
	Zeolite vs CWC	2.117	<0.0001***
	Zeolite vs CWZ	1.114	0.0386*
Feed (Solution)	CWC vs Control	0.451	<0.0001***
	CWZ vs Control	0.921	0.1925^{ns}
	CWZ vs CWC	2.043	<0.0001***
	Zeolite vs Control	0.798	0.0003**
	Zeolite vs CWC	1.769	<0.0001***
	Zeolite vs CWZ	0.866	0.0223*
Feed (Powder)	CWC vs Control	0.609	<0.0001***
	CWZ vs Control	0.805	0.0006**
	CWZ vs CWC	1.322	<0.0001***
	Zeolite vs Control	1.081	0.2133^{ns}
	Zeolite vs CWC	1.776	<0.0001***
	Zeolite vs CWZ	1.343	<0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

6.3.5. Mosquito survival by sex across water treatments

Overall, there were no significant differences in the sex of surviving mosquitoes across treatment groups, rearing densities and feed type (Table 6.7). Within treatment groups, the sex ratio of surviving mosquitoes, did not significantly deviate from the expected

50:50 ratio except at CWZ/400 larval density/powder feed and Zeolite/400 larval density/powder feed; where females significantly survived more than males (Table 6.8).

Table 6.7:Logistic regression (Effect Likelihood Ratio Tests) of mosquito survival by sex

Source	DF	Likelihood ratio	P-value
Treatment	3	0.470	0.9255^{ns}
Feed	1	3.001	0.0832^{ns}
Density	1	0.141	0.7075^{ns}

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

Table 6.8: Likelihood ratio test of probabilities (50:50) of *An. coluzzii* sex ratio across treatments

Treatment	Density	Feed	Sex	% Overall survival	Sample size	Chi-square	df	P-value
Control	200	Solution	Female	52 (47 - 57) *187*	361	0.4682	1	0.4938^{ns}
			Male	48 (43 - 53) *174*				
		Powder	Female	54 (49 – 58) *248*	462	2.5044	1	0.1135^{ns}
			Male	46 (42 – 51) *214*				
	400	Solution	Female	51 (47 – 55) *277*	540	0.3630	1	0.5468^{ns}
			Male	49 (45 -53) *263*				
		Powder	Female	50 (47 – 54) *386*	765	0.0641	1	0.8002^{ns}
			Male	50 (46 -53) *379*				
CWC	200	Solution	Female	50 (46 – 54) *273*	543	0.0166	1	0.8976^{ns}
			Male	50 (46 – 54) *270*				
		Powder	Female	50 (46 – 54) *281*	558	0.0287	1	0.8655^{ns}
			Male	50 (46 – 54) *277*				
	400	Solution	Female	50 (47 – 53) *395*	790	0	1	1^{ns}
			Male	50 (47 – 53) *395*				
		Powder	Female	52 (50 – 55) *496*	951	1.7682	1	0.1836^{ns}
			Male	48 (45 – 51) *455*				
CWZ	200	Solution	Female	51 (47 – 56) *222*	433	0.2795	1	0.5970^{ns}
			Male	49 (44 – 53) *211*				
		Powder	Female	49 (45 – 54) *265*	537	0.0913	1	0.7626^{ns}
			Male	51 (46 – 55) *272*				
	400	Solution	Female	48 (44 – 53) 228	472	0.5425	1	0.4614^{ns}

Treatment	Density	Feed	Sex	% Overall survival	Sample size	Chi-square	df	P-value
			Male	52 (47 – 56) 244				
		Powder	Female	55 (51 – 58) 425	778	6.6728	1	0.0098*
			Male	45 (42 – 49) 353				
Zeolite	200	Solution	Female	52 (47 - 56) 232	450	0.4356	1	0.5092^{ns}
			Male	48 (44 – 53) 218				
		Powder	Female	51 (47 – 56) 259	506	0.2846	1	0.5937^{ns}
			Male	49 (44 – 53) 247				
	400	Solution	Female	49 (45 – 53) 265	542	0.2657	1	0.6062^{ns}
			Male	51 (47 – 55) 277				
		Powder	Female	55 (51 – 59) 337	614	5.8726	1	0.0154*
			Male	45 (41 – 49) 277				

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

6.3.6. Effect of water treatments on adult wing-length

Across all larval trays, adult wing-length was significantly impacted by larval rearing density and the water treatment type in which they were reared. Adult wing-length significantly differed by sex; females were significantly larger than males. Although feed type had no significant impact on wing-length, the interaction between treatment and feed significantly impacted adult wing-length (Tables 6.9, 6.10, 6.12).

Pairwise comparison of means revealed significantly larger adult wing-length in the CWC treatment compared to CWZ and Zeolite but not Control. No significant difference in wing-length was observed between CWZ and Control, as well as between CWZ and Zeolite, but wing-length in the Control group significantly differed from Zeolite. (Figure 6.9; Table 6.10). Within water treatments, adults reared in 200 larval density were significantly larger than those reared in 400 larval density (Figure 6.9). Adult mosquitoes emerging from the CWC at 200 larval rearing density were significantly larger than those from CWZ and Zeolite but not Control. There were no significant differences between CWZ, Zeolite and Control. (Figure 6.9; Table 6.11)

Table 6.9: General linear model of wing-length across treatments

Parameter	Source	df	F-ratio	P-value
Wing length	Density	1	17.106	<0.0001***
	Feed	1	1.973	0.1603^{ns}
	Water treatment	3	9.852	<0.0001***
	Sex	1	232.853	<0.0001***
	Treatment*Feed	3	2.998	0.0298*

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. Abbreviation: df, degrees of freedom

Table 6.10: Tukey's (post-hoc) pairwise comparison of wing-length means between treatments.

Source	Level	t-ratio	P-value
Wing-length	CWC vs Control	-1.36	0.5258^{ns}
	CWZ vs Control	2.26	0.1077^{ns}
	Zeolite vs Control	3.59	0.0020**
	CWZ vs CWC	3.62	0.0017**
	Zeolite vs CWC	4.94	<0.0001***
	Zeolite vs CWZ	1.32	0.5479^{ns}

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

At 400 larval rearing density, adult wing-length was significantly (P = 0.0470) larger in CWC compared to Zeolite. Wing-length in CWZ, and Control did not differ significantly from the Zeolite group (Table 6.11). Females reared in the CWC and Control groups were significantly larger than those reared in Zeolite but not with CWZ. There was no significant difference in wing-length of females in Zeolite and CWZ. (Table 6.11). Males emerging from CWC were significantly larger than those from CWZ and Zeolite but not from Control. There was no significant difference in wing-length of adults from CWZ, Zeolite and Control (Table 6.11).

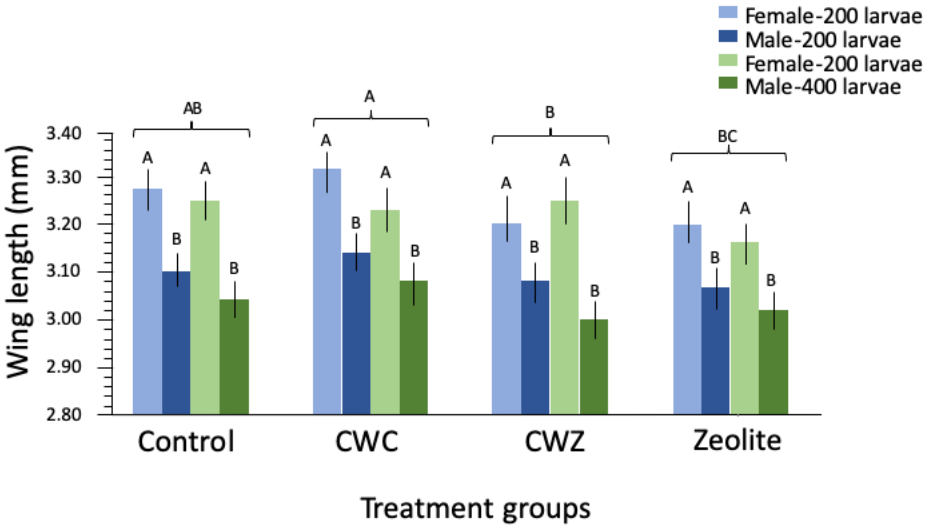

Figure 6.9: Mean wing-length of emerged mosquitoes across water treatments for two larval rearing densities(light green bars-200 larvae, dark green bar-400 larvae). Whiskers represent 95% confidence intervals (CI). Significant differences among treatments and independent variables within treatments are represented by different letters.

Table 6.11: Turkey's (post-hoc) pairwise comparison of wing-size means between water treatments by sex and density.

Source	Level	t-ratio	P-value
Density (200 larvae)	CWC vs Control	-1.73	0.3100^{ns}
	CWZ vs Control	2.00	0.1892 ^{ns}
	Zeolite vs Control	2.26	0.1085 ^{ns}
	CWZ vs CWC	3.73	0.0012**
	Zeolite vs CWC	3.99	0.0004**
	Zeolite vs CWZ	0.26	0.9938 ^{ns}
Density (400 larvae)	CWC vs Control	-0.07	0.9999 ^{ns}
	CWZ vs Control	1.01	0.7464 ^{ns}
	Zeolite vs Control	2.53	0.0569 ^{ns}
	CWZ vs CWC	1.08	0.7030 ^{ns}
	Zeolite vs CWC	2.60	0.0470*
	Zeolite vs CWZ	1.52	0.4255 ^{ns}
Sex (Female)	CWC vs Control	-0.19	0.9977^{ns}
	CWZ vs Control	1.51	0.4317^{ns}
	Zeolite vs Control	3.55	0.0024**
	CWZ vs CWC	1.70	0.3259^{ns}
	Zeolite vs CWC	3.73	0.0012**

Source	Level	t-ratio	P-value
Sex (Male)	Zeolite vs CWZ	2.04	0.1751^{ns}
	CWC vs Control	-1.85	0.2525^{ns}
	CWZ vs Control	1.72	0.3128^{ns}
	Zeolite vs Control	1.42	0.4898^{ns}
	CWZ vs CWC	3.57	0.0022**
	Zeolite vs CWC	3.27	0.0062*
	Zeolite vs CWZ	-0.31	0.9895^{ns}

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

Table 6.12: Mean wing-length of surviving adult *An. coluzzii* across treatments.

Water treatment	Larval density	Sex	Mean wing length (mm)
Control	200	Female	3.27 (3.23 – 3.32)
		Male	3.10 (3.07 – 3.14)
	400	Female	3.25 (3.21 – 3.29)
		Male	3.05 (3.01 – 3.08)
CWC	200	Female	3.31 (3.26 – 3.35)
		Male	3.15 (3.11– 3.18)
	400	Female	3.23 (3.18 –3.27)
		Male	3.08 (3.03 – 3.12)
CWZ	200	Female	3.20 (3.16 – 3.25)
		Male	3.08 (3.04 – 3.12)
	400	Female	3.25 (3.20 – 3.30)
		Male	3.00 (2.95 – 3.04)
Zeolite	200	Female	3.20 (3.15 – 3.25)
		Male	3.07 (3.03 – 3.11)
	400	Female	3.16 (3.11 - 3.20)
		Male	3.02 (2.98 – 3.06)

Notes: Ninety-five percent confidence intervals are in parentheses and the sample row is 80.

6.3.7. Impact of density, water treatment and feed regimes on development time

The duration of development from first instar larvae until adult emergence differed significantly among treatment groups (P = 0.0004) by density (P<0.0001) and feed type (P<0.0001) (Table 6.13). Interactions between treatment by feed, and treatment by

density, significantly impacted on development time (Table 6.13). Development time was significantly longer in CWZ compared to CWC and Control but not with Zeolite.

Table 6.13: Cox Proportional-Hazard analyses of development time

Parameter	Source	df	Chi-Square	P-value
Day of emergence	Density	1	614.460	<0.0001***
	Feed	1	142.292	<0.0001***
	Water treatment	3	18.179	0.0004**
	Water treatment*Feed	3	8.365	0.0390*
	Water treatment*Density	3	21.040	0.0001**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

There were no significant differences in development time between Zeolite and CWC or Control (Table 6.14). Mosquito development was completed in a shorter time in 200 larval rearing density, compared to 400 larval rearing density (Tables 6.13, 6.15 Figure 6.10). Overall, development time was significantly longer in solution feed compared to powder feed (Tables 6.13, 6.15; Figure 6.10).

Table 6.14: Risk ratio comparison of means of development time among treatment groups

Level	Risk-ratio	P-value
CWC vs Control	1.008	0.7863^{ns}
CWZ vs Control	0.900	0.0007**
CWZ vs CWC	0.893	<0.0001***
Zeolite vs Control	0.954	0.1374^{ns}
Zeolite vs CWC	0.947	0.0611^{ns}
Zeolite vs CWZ	1.061	0.0563^{ns}

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05.

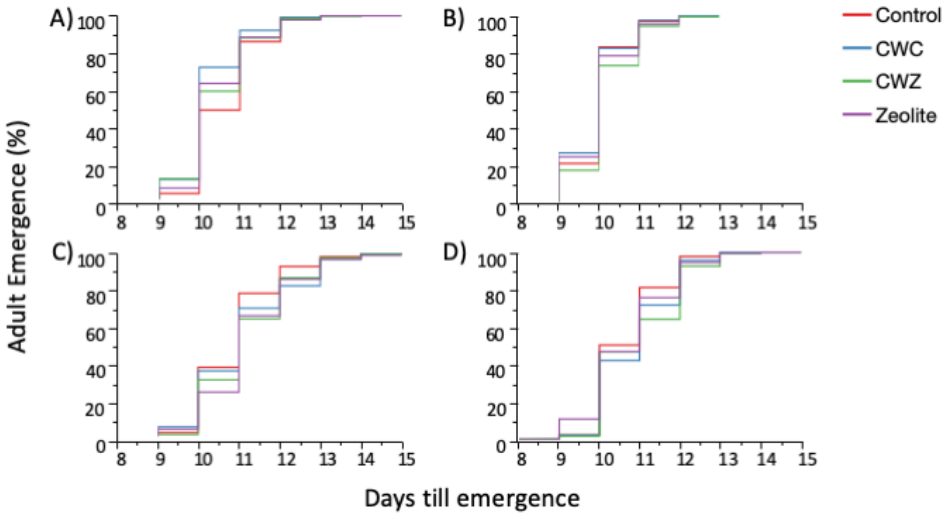

Figure 6.10: Survival curves of mosquito larvae in water treatment types by densities and feed regimes, A) 200 larvae/Solution feed; B) 200 larvae/Powder feed; C) 400 larvae/Solution feed; D) 400 larvae/Powder feed.

Table 6.15: Mean development time of *An. coluzzii* across water treatments.

Treatment	Larval density	Feed	Days till emergence
Control	200	Solution	10.64 (10.56 - 10.73) *361*
		Powder	10.00 (9.93 – 10.06) *462*
	400	Solution	10.92 (10.83 – 11.00) *540*
		Powder	10.70 (10.63 – 10.76) *765*
CWC	200	Solution	10.27 (10.20 – 10.34) *543*
		Powder	9.95 (9.89 – 10.01) *558*
	400	Solution	11.11 (11.02 – 11.20) *790*
		Powder	10.89 (10.83 – 10.95) *951*
CWZ	200	Solution	10.45 (10.36 – 10.53) *433*
		Powder	10.17 (10.10 – 10.24) *537*
	400	Solution	11.21 (11.10 – 11.31) *472*
		Powder	10.97 (10.89 – 11.05) *778*
Zeolite	200	Solution	10.46 (10.38 – 10.55) *450*
		Powder	10.02 (9.95 – 10.09) *506*
	400	Solution	11.25 (11.15 – 11.35) *542*
		Powder	10.74 (10.65 – 10.83) *614*

Notes: Ninety-five percent confidence intervals are in parentheses and the sample sizes are italicised.

6.4. Discussion

As expected, mosquitoes reared in the trays where water was continuously refreshed provided a better environment for optimal mosquito growth and development. Consistently lower ammonia concentrations and higher nitrate concentrations in these trays indicate efficient conversion of toxic ammonia to harmless nitrate (Florescu et al., 2011). Mosquito survival and adult body size were maximised in these treatment groups due to the absence or minimal presence of toxic compounds such as ammonia (Hargreaves, 1998). Nitrogenous wastes are known to be poisonous to aquatic organisms above certain concentrations. Unionised ammonia which is a by-product of protein metabolism by aquatic animals is toxic to fish and other freshwater animals above 0.2mg/l, in closed aquatic systems (Florescu et al., 2011; Skleničková et al., 2020; U.S. Environmental Protection Agency, 2013). In larval trays without water replacement, ammonia concentrations increased steadily, exceeding toxicity threshold on the fourth day, and reaching a peak on the tenth day. Zeolite treatment significantly decreased ammonia concentrations compared to Control trays where zeolite was not applied. Nitrate concentrations were equally higher in Zeolite treatment without water change compared to Control. The cause of overall higher mortality in *Anopheles* larval trays without water replacement (Control and Zeolite) in comparison to those with water replacement (CWC and CWZ) could range from hypoxia, ammonia toxicity, inability to transport oxygen, pathogenicity, nutrient enrichment, and competition for food resource (Amarasinghe & Weerakkodi, 2014; Tchigossou et al., 2018; U.S. Environmental Protection Agency, 2013; Ward & Jensen, 2014)

Although not observed for overall mosquito survival, the impact of ammonia-absorbing zeolite in improving water quality in larval trays without water replacement was evident at the 200 larval rearing density. Adult emergence was significantly higher

in Zeolite treatment compared to Control at the 200 larval density, validating the ability of zeolite to improve water quality in an aquaculture system, in this case mosquito larval rearing trays (Abdel-rahim, 2017; Ghasemi et al., 2018). However, at higher larval density (400), the effect of zeolite was not evident for mosquito adult emergence, possibly due to two factors; first, ammonia concentration produced by in the 400-larval-density-trays were above the saturation compared to amount of zeolite applied in this study, and secondly, the effects of intra-specific competition both for food and space and cannibalism among larval instars, outweighed any benefits from ammonia reduction (Epopa et al., 2018).

For the latter factor (intra-specific competition), the larval stage is the only developmental stage in which density dependent competition occurs in anopheline mosquitoes (Gimonneau et al., 2014; Juliano, 2009; Kivuyo et al., 2014; Valerio et al., 2016). Intra-instar larval predation and competition have been reported for *An. coluzzii* in nature and the laboratory and may have accounted for the reduced survival and adult body size in the trays with 400 larvae (Gimonneau et al., 2012; Munga et al., 2006). An increase in larval density, given the same amount of food, results in overcrowding, leading to competition for food which leads to higher mortality of larvae that are unable to secure adequate food required for growth (Gimonneau et al., 2014; Koenraadt & Takken, 2003; Paaijmans et al., 2009). While rare in nature, overcrowded larval breeding in insectaries is possible due to lack of space, a good balance between the density of larvae, the size of larval rearing trays and the amount of food provided is important for optimal emergence rates of adult mosquitoes with desirable phenotypic qualities (Epopa et al., 2018).

Additionally, overcrowded trays in this study must have resulted in the production relatively higher amounts of toxic ammonia due to the increased metabolism and waste

production. When zeolite has been used for improving water quality in fish and crustacean aquaculture, reports reveal that the greater the concentration of initial ammonia, the lesser the ammonia removal efficiency (Abdel-rahim, 2017; Aly, et al., 2016; Emadi, et al., 2001). Although larval density did not have a statistically significant impact on ammonia content in the larval trays, it was higher at 400 larval rearing density, providing a possible explanation for the reduced effect of ammonia adsorption by zeolite in these trays since the same amount of zeolite was used at both rearing densities. Exposure time of zeolite might have also impacted on the effectiveness of zeolite at this rearing density (Farhangi, et al., 2013; Farhangi & Rostami-Charati, 2012). Although the scope of this study did not include extensive physio-chemical analysis and assay of the effect of the individual parameters on survival and adult body size, they may have contributed to the survival and quality of adults from the zeolite group at higher larval densities and increasing the amount of zeolite application can improve mosquito rearing outcomes (Abdel-rahim, 2017; Ghasemi et al., 2018). These results also corroborate findings from field studies that show that the physio-chemical properties of larval habitats such as pH, temperature, ammonia concentration, initial concentration of cations in the water, particle size, nitrate and sulphate determine the presence, development and survival of immature Anophelines (Briegel, 2003; Mutero, et al., 2004; Muturi et al., 2008; Mwangangi et al., 2007).

A crucial factor to consider on the effect of zeolite used in this study to improve water quality for increased mosquito production without water replacement is that zeolites can greatly influence the abundance and development of nitrifying microorganisms (Motesharezadeh, et al., 2015). Unionised ammonia can inhibit the action of nitrifying bacteria, resulting in increased levels of ammonia in aquatic habitats thereby intensifying the deleterious effects on aquatic animals and beneficial bacteria

(U.S. Environmental Protection Agency, 2013). In one study, zeolite was able to absorb ammonia more quickly than the nitrifying bacteria which were then unable to use NH_4^+ for their metabolic necessities (Montalvo et al., 2014). In another study it was reported that zeolite reduced ammonia and nitrite concentrations, maintaining them at safe levels but negatively impacted nitrifying bacteria in the biological filter (Skleničková et al., 2020). In yet another study, microbiological testing of water in recirculation systems with and without zeolite showed a rapid decline in coliform bacteria as well as other pathogens where natural zeolite was used, although it did not significantly impact bacteria species of the Enterobacteriaceae family (Sirakov, et al., 2015). In this study, experimental trays with reduced nitrate levels generally had higher ammonia content and hence higher mosquito mortality and reduced body size. This dynamics of nitrification (conversion of ammonia to nitrate by nitrifying bacteria) is indicative of the bacteria communities that are likely varied between experimental trays. Natural larval environments of *Anopheles* typically contain algae, bacteria and other micro-organisms and this might have reflected in mosquito survival and development in this study. Microorganisms serve as food, immunity boosters, nitrogen-converters and could also be inimical pathogens to aquatic invertebrates (Mamai, et al., 2016). To better understand the function and mechanism of microbial population in mosquito larval rearing trays, the next chapter of this thesis (Chapter 7) is focused on the characterisation of the bacteria communities in the treatment trays in this study (Chapter 6) by sequencing and quantification of microbial communities present in the rearing trays. The overall adult emergence rate was equivalent to ~56% and was low across all treatment groups but much higher at 70% at 200 larval density. This can be attributed to intra-specific larval competition, zeolite dosage, and the impact of zeolite on the bacteria communities in the trays. For future applications/experiments this

effect of zeolite on nitrifying bacteria has to considered in the design for water quality improvements to ensure maximum mosquito yield and adult phenotypic quality.

In this study, the type of feed regime which *An. coluzzii* larvae were fed significantly impacted overall mosquito survival and duration of development. Powder feed significantly improved larval survival, adult emergence and development time was significantly shorter, compared to solution feed across water treatment types. This is likely due to the higher nitrate concentrations in trays where solution feed was used compared to powder feed. Higher nitrate levels indicate greater ammonia conversion in powder feeding thus higher mosquito survival (Bernhard, 2010).

Zeolite water treatment also favourably impacted on the duration of mosquito development time. Development time was not significantly longer in Zeolite treatment compared to the more effective continuous change CWC group. This allowance for synchronous hatching and pupation with the use of zeolite is the ideal condition both for smaller insectaries and mass-rearing facilities (Mazigo, et al., 2019). Any feed regime and or additive that can shorten pre-imaginal development time is welcome as it will reduce labour costs and enhance accelerated production of adults (Epopa et al., 2018). This is particularly desirable in the mass rearing of adult mosquitoes for vector control/research programmes where large releases of adult mosquitoes are required for mass rearing of adult mosquitoes (Epopa et al., 2018). Staggered hatching or pupation in mosquitoes has been linked to environmental and genetic conditions such as stress caused by inadequate nutrition, competition or predation (Naylor, et al., 2014) which subsequently creates an inefficient system when insects are needed for experiments or mass-release.

For future implementation of zeolite in mosquito mass production programmes, it is of important note that sex ratio of surviving mosquitoes in this study did not

significantly deviate from the expected 50:50 ratio (HDV, 2017). An essential component for mosquito rearing whether in a small insectary culture or mass-rearing facility, is keeping track of the input-output of resources to foresee potential problems and tackle them in time. Monitoring mosquito life cycle (hatch rate, pupation, adult emergence, adult survival), sex ratio, mating status, engorged females, amongst others, are key parameters for judging the quality and efficiency of a mosquito rearing facility (HDV, 2017). Ideally, the sex ratio should be 50:50 to ensure mating efficiency for males and high production in females (HDV, 2017).

For concerns regarding water conservation and cost reduction in mass rearing, it is of important note that at the 200 larval rearing density, mosquito survival in the Zeolite group was significantly higher than that of the Control group with no water change and adult body size was not significantly lower compared to CWC. At an average ammonia adsorption capacity of 25mg NH_4^+/g of zeolite (Abdel-Rahim, 2017), and simple provision for reuse of zeolitic materials (zeolite imbedded biofilters), it might be more economically viable to consider using zeolite to treat water before reuse instead of the more expensive ultrafiltration or reverse osmosis methods (HDV, 2017; Mamai et al., 2017). This could be especially important when planning mass release vector control programmes in malaria endemic countries with very low GDIs (Gross Domestic Income) and also in arid regions (Chima et al., 2003; FAO, 2012; Mamai et al., 2017). Following saturation, zeolitic materials can be recharged by soaking in 10% NaCl solution, thus renewing their capacity and can subsequently be reused (Hartman & Fogler, 2007; Helfrich & Libey, 1990; Inglezakis, 2012). Ultrafiltration systems on the other hand are expensive, costing between 10,000 USD for small gallon per minute of water flow to 20 million USD for high flow demand in municipal water systems (Guo, et al., 2014). These costs are not inclusive of other costs associated with running these systems such as

operating costs, shipping costs, space requirements, regulation fees, system engineering and design (Guo, et al., 2014). Estimated capital costs for reverse osmosis system in 2012 was 5,750 USD and annual running cost of 1000USD for 8.33m^3/day without pre-treatment (Englehardt, et al., 2013). Conversely, cost estimates of a private investment project for a zeolite-based water purification system in Hungary projects a 40% reduction in installation cost and 20% reduction in operational costs compared to the currently used RO and UF filtration systems (StradiSound Strategy, 2020). Moreover, one study has demonstrated that zeolite and probiotics (nitrifying bacteria) can be used together as complementary water additives to control ammonia and nitrite content in closed aquaculture systems (Motesharezadeh, et al., 2015). The use of natural zeolites and their modified forms therefore has many advantages which includes low cost, its abundance in many parts of the world and being environmentally friendly. For successful application leading in optimal production of mosquitoes, users must consider zeolite particle size, chemical composition of water and balance these factors to accommodate beneficial bacteria in these aquatic ecosystems.

In conclusion, in settings where water is very scarce and/or water replacement or treatment options are too onerous, the use of zeolite for the rearing of *An. coluzzii* can improve mosquito survival at a lower cost. However, zeolite dosage, larval density, feed type, physio-chemical properties of rearing water and impact on beneficial bacteria will need to be further improved/optimised to maximise the economic potential of its use.

Chapter 7

Improvement of water quality for mass Anopheline rearing II: Dynamics of bacterial communities under different water treatments revealed by 16S ribosomal RNA gene ultra-sequencing.

7.1. Introduction

Mosquitoes of the *Anopheles gambiae* s.l. complex are the major vectors of human malaria in sub-Saharan Africa. The recently re-classified sibling species *Anopheles gambiae* s.s. and *Anopheles coluzzii* are the most abundant and widespread in the vector complex, transmitting malaria in most of the sub-Saharan African region (Coetzee et al., 2013; Gimonneau et al., 2014). Although the sibling species are morphologically identical and share similar resources such as vertebrate hosts, adult resting sites and freshwater larval habitats; various studies have documented divergent ecological adaptations between these species (Costantini et al., 2009; della Torre et al., 2005; Gimonneau et al., 2014; Simard et al., 2009). *An. coluzzii* has been shown to preferentially develop in nutrient rich, predator-prone, permanent/ semi-permanent larval habitats such as rice fields and recently in water polluted with decaying organic matter and inorganic wastes in urban centres, while *An. gambiae* prefers shallow, rainfed, nutrient poor water clusters in the surrounding relatively pristine rural settings (della Torre et al., 2005; Diabaté et al., 2009; Kamdem et al., 2012; Lehmann & Diabate, 2008). This selective distribution in different aquatic habitats is indicative of divergent adaptations such as ammonia tolerance and has been documented as a factor that contributes to their ongoing speciation (Tene Fossog et al., 2013).

The immature stages of Anopheline mosquitoes comprising four larval instars and a pupal stage develop in aquatic environments that typically contain algae, bacteria, fungi and protozoa (Mamai, et al., 2016). Mosquito larvae explore these micro-organisms

present in their habitats as a primary food source through filter-feeding thereby acquiring microbial communities in their mid-gut and some community members are carried over to the adult stage (Coon et al., 2014; Gimnig et al., 2002; Kaufman et al., 2006). These microbial communities are required for the growth and development of most mosquito species until adult emergence whether in the field or laboratory (Coon et al., 2014) since they contribute to digestion, nutrition, reproduction and mosquito immune responses from pathogens (Gimonneau et al., 2014). The microbiota acquired at the larval stage can modulate the immune response of *Anopheles gambiae* s.l, and influence its vector competence to human pathogens, in addition to the innate immunity of the mosquito. (Dennison et al., 2014; Dong et al., 2009; Kumar et al., 2018). Several studies have shown that microbial communities in different mosquito larval habitats vary with physicochemical parameters (Coon et al., 2014; Dickson et al., 2017; Onchuru et al., 2016). A study characterizing microbial communities associated with *An. coluzzii* and *An. gambiae* in Yaounde, Cameroon from 5 different aquatic habitats in 3 localities in peri-urban areas of showed that mosquitoes emerging from different aquatic habitats harbour different microbial communities (Gimonneau et al., 2014). Samples obtained from the aquatic surface microlayers and subsurface water of mosquito larval habitats, larvae midgut, epithelia of freshly emerged adults, midguts, ovaries and salivary glands revealed correlations between the bacterial content of sample mosquito microbiota and the larval habitat, indicating that the breeding site is crucial in shaping the microbiome composition of adult mosquitoes (Gimonneau et al., 2014). Aside from bacteria being important components of a mosquito microbiome and of their external larval environment, and the two being interdependent, in the context of mosquito Anopheline mass-rearing, bacterial communities also play an important role because their dynamics

can lead to rapid death of larval stages hence they could constrain the production capacity of rearing facilities and their management is paramount.

In closed aquaculture systems such as fish ponds or mosquito larval rearing tray, microorganisms play major roles in productivity, nutrient cycling, nutrition of cultured organisms, water quality and disease control (Moriarty, 1997). Nitrogen is an essential element in these aquaculture systems and often limits production of reared aquatic animals, whether it is fish or in this case, mosquito larvae (Ward & Jensen, 2014). Owing to this requirement for nitrogen in biological macromolecules of aquatic organisms, the acquisition, and cycling of nitrogen is key in the structuring of microbial communities in closed aquatic ecosystems like a mosquito larval tray (Ward & Jensen, 2014). Nitrogen cycle primarily involves four microbiological processes, such as, nitrogen fixation, mineralisation (decomposition), nitrification and denitrification (Hayatsu, et al., 2008). A wide variety of microorganisms ranging from archaebacteria, proteobacteria, Gram-positive eubacteria and fungi are responsible for the functioning of these microbial processes (Ye & Thomas, 2001). The efficient management of the activities of microbes in the food web and nutrient cycling in closed aquaculture system such as a fish pond and for this study, a mosquito larval tray, is necessary for optimising production (Moriarty, 1997).

Under insectary conditions, it is not uncommon to find Anopheline larval rearing trays in insectaries containing high levels of ammonia which usually result in the death of mosquito larvae (Mamai, et al., 2016). Ammonia is produced in mosquito rearing trays as a by-product of insect metabolism and decomposition of food waste, and has been shown to be toxic to fish and other aquatic invertebrates especially in its unionised form (Hargreaves, 1998). Furthermore, the nutritional bioavailability of nitrogen in the mosquito larval trays can be reduced through the accumulation of ammonia in these trays.

This can happen via competition for available biologically available nitrogen or, dissolved oxygen in the water column, and both situations have been proven to be detrimental to aquatic organisms (Mamai, et al., 2016). Ideally, by the process of nitrification, this ammonia should be converted to nitrite and then to nitrate by bacteria and other microorganisms (Ye & Thomas, 2001).

Nitrite is also a potentially-toxic nitrogenous compound that may accumulate in fish culture ponds. It is released as an intermediate product of nitrification and denitrification. Nitrite toxicity is expressed in its ability to competitively bind with haemoglobin to form methemoglobin which does not have the ability to transport oxygen (Amarasinghe & Weerakkodi, 2014; Tchigossou et al., 2018; Ward & Jensen, 2014; Ye & Thomas, 2001). Oxygen is vital for the survival of all organisms including insects and a decrease in the availability of dissolved oxygen (DO), that is usually associated with eutrophic conditions, is detrimental to aquatic organisms (Moniz, 2013). Even though the situation in a mosquito larval tray might not be of eutrophic proportions, it has been shown that moderate chronic hypoxia can significantly reduce survival in fish embryos (Bardon-Albaret & Saillant, 2016). *Anopheles* larvae like fish embryos depend on cutaneous respiration and their surface area is limited; and although breathing tubes are present, they are rudimentary in the immature stages (Bardon-Albaret & Saillant, 2016). Many authors have demonstrated that the presence or absence, and the abundance of *Anopheles* larvae is positively associated with the concentration of DO in oviposition sites (Dejenie, et al., 2011; Oyewole et al., 2009). *Anopheles* larvae were shown to be adapted to oligotrophic, low nutrient waters with higher oxygen levels and when DO drops, larval and adult survival were significantly reduced (Oyewole et al., 2009). Microorganisms that play a role in decomposition (saprophytes or heterotrophs) could be harmless, pathogenic or beneficial (Moriarty, 1997; Zhou, et al., 2009). Whilst the

harmless and beneficial ones decompose organic matter to meet the growth requirements of cultured species, maintain the eco-equilibrium, inhibit the proliferation of harmful organisms and disintegrate harmful chemicals (such as unionised ammonia NH_3 and nitrites), the pathogenic microbes cause diseases (Zhou et al., 2009). To maintain a healthy closed aquatic ecosystem, there has to be a balance of the microecology to ensure beneficial microbes are present in sufficient amounts to suppress the population and effect of pathogens. Beneficial microorganisms (probiotics) have been successfully applied to improve water quality in aquaculture systems. Probiotics have been shown to reduce organic matter accumulation (Rengpipat, et al., 1998; Verschuere, et al., 2000) mitigate nitrogen (Wang & Gu, 2010) and phosphate pollution (Wang & He, 2009), control ammonia and nitrite concentrations (Cha, et al., 2013) as well as the reduction of pathogenic bacteria (Dalmin et al., 2001; Park et al., 2000).

Water management in mosquito insectaries to minimise the presence of ammonia and nitrites as well as encouraging the proliferation of beneficial microorganisms is key to achieving optimal rearing results both for small cultures and for mass-rearing facilities (HDV, 2017; Mamai et al., 2017, 2016). To better understand the microbial contribution to Anopheline survival and phenotypic quality in the context of mass-rearing, microbial communities present in these trays would need to be characterised over the course of larval development into adulthood. Currently there is a paucity of literature on bacterial community dynamics in relation to water management in the context mass-rearing toward mosquito release programmes.

One way to address this knowledge gap would be via studies of microbial communities present in the mosquito rearing environment through ultra-sequencing of the 16S rRNA gene. Over the past decade, this approach has become standard for the identification and enumeration of bacteria phylotypes present in samples in a cost-

effective manner and has the advantage of being cultivation-independent (Caporaso et al., 2011; Gloor et al., 2010; Klindworth et al., 2013). Bacterial profiling constitutes an important first step in the determination of important bacterial and protist organisms in a given sample and can generate important information about their interaction with their growth environment (Gloor et al., 2010). It involves the combination of high-throughput sequencing technologies, and advanced computational tools that can exploit metadata (sample description) to relate hundreds of samples to one another so as to reveal biological patterns (Caporaso et al., 2011). Bacterial profiling usually employs the 16S rRNA gene as the phylogenetic marker. This process usually involves the sequencing of PCR amplified variable region of the bacterial 16S and of the protistan small subunit of ribosomal RNA genes (Caporaso et al., 2011; Gloor et al., 2010).

Although there are alternative techniques such metagenomics which provide insight into all of the genes and possibly gene function present in a given community, 16S rRNA-based studies remain the preferred choice for bacterial analysis because of its extraordinary capacity to document unexplored diversity and ecological characteristics of either whole communities or individual bacterial taxa (Caporaso et al., 2011). The 16S rRNA gene is approximately 1500bp long and consist of 9 (hyper) variable regions named V1 – V9, interspersed with more conserved regions (Winand et al., 2020). It is particularly suitable for bacterial analysis for the following reasons: firstly, the gene is universally distributed, allowing the analysis of phylogenetic relationships among important taxa and secondly, it is a functionally indispensable part of the bacterial core gene set and thus only weakly affected by horizontal gene transfer which further supports its use for phylogenetic studies (Acinas, et al., 2004; Daubin, et al., 2003; Větrovský & Baldrian, 2013). Further advantages of the 16S rRNA gene suitability for phylogenetic studies is based on it being subject to variation especially in certain variable regions.

Whilst these variable regions allow for sufficient diversification to provide a classification tool, the 16S rRNA gene also contains conserved regions that allow for the design of suitable PCR primers or hybridisation probes for various taxa at different taxonomic levels ranging from individual strains to whole phyla (Acinas et al., 2004; Větrovský & Baldrian, 2013). Despite the wide application of 16S rRNA amplicon based sequencing, its use for the description of bacterial diversity is however limited by the presence of variable copy numbers in bacterial genomes and sequence variation within closely related taxa or within a genome (Klappenbach, et al., 2000; Větrovský & Baldrian, 2013). While it allows for reliable identification of bacteria genera (>99%) it can potentially misguide identification of bacteria species (86% reliability) (Winand et al., 2020). This limitation can often be mitigated with the choice of variable region to be amplified for sequencing. For several bacteria species that have sequence variations between multiple 16S rRNA gene copies, these copies are identical in certain regions. For instance, *Escherichia coli* (ATCC 70096), has 7 copies of 16S rRNA, of which 6 are different over the full gene length, but all 7 are identical when considering the V4 region (Edgar, 2018).

The Illumina sequencing platform is the preferred sequencing technique employed for microbial characterisation compared to the earlier Sanger and 454 sequencing methods. This preference is due to Illumina's reduced per base costs, comparatively high sequencing depths and its ability to detect rare phylotypes (Caporaso et al., 2011; Klindworth et al., 2013). The Illumina platform is however limited in that it produces relatively shorter reads (75-100bp in a single read- although paired reads can provide 150 – 200bp from a single molecule), initially believed to be too short to transverse any of the 16S rRNA variable regions (Caporaso et al., 2011; Gloor et al., 2010). A recent study has however demonstrated that this limitation can be circumvented

by identifying maximally informative sites for specific groups of organism, and that fragments of the 16S rRNA as small as 100bp were sufficient to resolve microbial community differences (Liu et al., 2007; Tremblay et al., 2015; Winand et al., 2020)

Datasets generated from 16S rRNA sequencing of environmental samples and microbiome analyses using high-throughput sequencing (HTS) are compositional and not absolute because they have an arbitrary total imposed by the instrument being used (Gloor, et al., 2017). The total read count observed in a HTS run is of a fixed size and only a random sample of the relative abundance of the molecules in the underlying ecosystem (Gloor et al., 2017; Jian, et al., 2020). The read counts cannot be related to the absolute number of molecules in the input sample as implicitly acknowledged when microbiome datasets are converted to relative abundance values, or normalised or rarefied (Weiss et al., 2017). Therefore, an assumption of independence cannot be made for HTS experiments because the sequence instruments can only deliver reads up to the capacity of the instruments, generating compositional data that are proportions or probabilities but contain absolute information about relationships between compositional parts (Gloor, et al., 2017). These limitations restrain the use of relative abundance data from next generation sequence (NGS) for absolute quantification of microbial diversity. The challenges encountered with NGS data analysis include; changes of components that are mutually dependent which lead to misinterpretations of microbial community structures because the increase of one taxon leads to a concurrent decrease of others in compositional data; high false discovery rates which occur when compositional data are analysed using traditional methods; and correlation analysis of relative abundance being strongly subject to negative correlation bias and spurious associations, particularly hampering the interpretation of microbial changes in longitudinal studies, such as interventions (Jian et al., 2020). Without NGS-independent experiments to validate

sequence data, it is difficult to determine which taxon was impacted by a treatment or to identify the actual target organism for a specific treatment. Absolute quantification of microbial abundances can be achieved either by adding a cell (flow cytometry, spike-in-bacteria) or DNA-based approach (synthetic DNA and qPCR) into standard NGS workflow (Jian et al., 2020) or having it as an additional validation step. In biosciences and medical research, the real time PCR (quantitative PCR, qPCR) is an established method for the detection and quantification of different microbial organisms which is used for such validation (Kralik & Ricchi, 2017). qPCR is used to measure the number of copies of a gene of interest in a community or an environmental sample (Brankatschk et al., 2012). The method (as described in detail in chapter 2) is based on the real-time monitoring of the exponential amplicon formation associated with PCR using a reporter molecule (such as SYBR Green dye) (Brankatschk et al., 2012).

In the previous chapter (Chapter 6) of this thesis, we have shown that water treatment (zeolite and water change) aimed at ammonia reduction improved water quality in larval trays, hence the survival rates of mosquito larvae and adult quality (wing-length). It was however unclear the role, if any, that microbial communities present in the larval trays played in mosquito development and survival. Based on the evidence presented in the 1st sections of the introduction and the findings of Chapter 6, we hypothesised that bacterial communities were likely to vary between rearing trays and treatment types (Control, Zeolite, CWC, CWZ) in a way that impacted mosquito developmental success and phenotypic quality. Bacterial communities from larval rearing trays were therefore characterized using environmental tray DNA extraction followed by sequencing of the 16S rRNA gene. Faced with a very large and diverse assemblage of bacteria identified, we applied three filters designed to link bacteria species to health indicators in the larval trays (ammonia, nitrate and percentage survival).

Candidate bacteria species established by the filtering systems were subsequently quantified by qPCR. Results obtained reveal that the abundance and diversity of bacteria species in larval trays with no-water-replacement (NWR-Control and Zeolite) were significantly higher than those with water-replacement (WR-CWC, CWZ) and this likely impacted on mosquito developmental success and adult phenotypic quality. Filtering of the 1031 species detected via bacterial profiling using information on functional ecological criteria as well as correlations with ammonia levels and mosquito emergence rates led to the identification of several bacteria taxa that are either directly responsible for larval death or indicator species associated with a change to anaerobic toxic conditions in polluted rearing trays. This is the 1st comprehensive study focusing on the dynamics of bacterial community in the mosquito larval rearing habitat. These findings can inform rearing water management strategies and pave the way towards functional characterisation of specific bacterial taxa via experimental inoculation of pure cultures into larval trays to confirm their impact on larval development.

7.2. Materials and Methods

7.2.1. Sampling for bacterial DNA extraction

To determine the bacterial content in mosquito rearing trays, 100ml of water was collected in a completely randomized pattern from all the treatment groups from Chapter 6 (Control, Zeolite, CWC, CWZ), from 32 experimental conditions in four replicates (a total of 128 samples) as described in chapter 6. The collections were made in two batches of 50ml from each tray using sterile falcon tubes (Thiery, et al., 1991) on days 8 and 10 from the first day of the experiment to reflect the peak period of ammonia build-up in the trays as described in chapter 6. Also important in the choice of collection date is that most pupation in *Anopheles* larval tray occur on day 8, and by day 10, pupation is almost complete in these trays. Falcon tubes were centrifuged at 3000 × g for 15 minutes at room temperature (Maynard et al., 2005), and supernatant carefully discarded. Pellets were resuspended in 500µL of phosphate-buffered saline (PBS), transferred into sterile 1.5ml Eppendorf tubes and stored in -20ºC until DNA extraction (Caldwell & Lattemann, 2004).

7.2.2. Bacterial DNA extraction

Total genomic DNA was extracted from the water samples using Qiagen DNeasy® blood and tissue extraction kit. Samples were defrosted and excess PBS discarded. To ensure that DNA extraction was efficient for both gram-positive bacteria (have thick peptidoglycan cell wall with teichoic acids) and gram-negative bacteria, samples were pre-treated with an enzymatic lysis buffer (lysozyme). The enzymatic lysis buffer contained 20mM Tris Cl, pH 8.0, 2mM Sodium EDTA, 1.2% Triton® X-100 and immediately before extraction, 20mg/ml of lysozyme is added to improve cell lysis. The pellets were re-suspended in 180µl of enzymatic lysis buffer and incubated for 30 minutes

in a 37ºC pre-heated heating block. Subsequently 25 µl of proteinase K and buffer AL (Qiagen blood and tissue kit) was added to the sample, samples were vortexed and then incubated at 56 ºC for 30 minutes. To achieve a homogenous solution, 200 µl of 100% ethanol was added to the sample, then samples were thoroughly mixed by vortexing, and then transferred into the DNeasy mini spin columns placed in 2ml collection tubes. Samples were centrifuged at 6000 × g for 1 minute at room temperature and the flow-through discarded with the collection tubes. DNeasy mini spin columns were placed in a new 2 ml collection tube, 500 µl Buffer AW1 was added, then centrifuged for 1 min at 6000 × g. Flow-through and collection tubes were discarded. The DNeasy Mini spin columns were placed in a new 2 ml collection tube, 500 µl Buffer AW2 added, and then centrifuged for 3 min at 20,000 × g to dry the DNeasy membrane. Flow-through and collection tubes were discarded. The DNeasy mini spin columns were placed in a clean 1.5 ml or 2 ml microcentrifuge tubes, 100 µl Buffer AE was pipetted directly into the DNeasy membrane. Tubes were incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000 × g to elute. To ensure maximum DNA yield, the last step was repeated using new 1.5ml microcentrifuge tubes. Tubes containing extracted bacteria DNA were stored in -80ºC. Following nanodrop spectrophotometric quantification, samples with DNA concentration below 300ng/µl were concentrated using speed-vac and re-eluted in 50 µl of AE (Appendix N).

7.2.3. 16S rRNA gene amplicon sequencing library preparation for Illumina MiSeq system.

The sequencing was carried out by the investigator at the Vector Functional Genomics and Microbiology Laboratory in the Institute of Biotechnology of the São Paulo State University “Júlio de Mesquita Filho” (UNESP), Botucatu Campus, São Paulo, Brazil.

Samples were stored on ice and transported by air to the host laboratory in Brazil. To characterize the bacterial communities in the larval rearing trays, 16S rRNA gene was sequenced from bacteria DNA extracted as described in the previous section. In this study, the variable V4 region of the 16S rRNA gene were targeted for sequencing using a combination of benchtop sample preparations, onboard primary analysis, secondary analysis using BaseSpace and CLC genomics workbench (Appendix K). The workflow for library preparation and sequencing include:

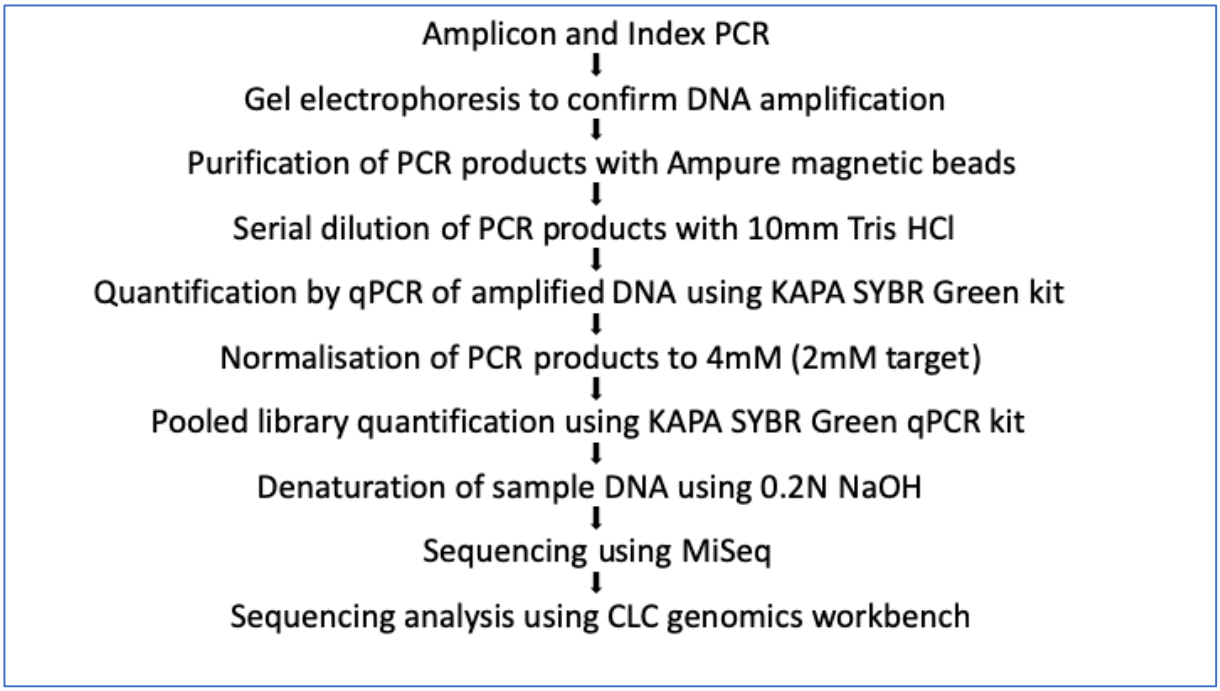

Figure 7.1: Workflow for 16S rRNA library preparation and sequence on MiSeq system.

Amplicon and Index PCR

Bacterial 16S rRNA gene V4 variable regions were amplified by PCR using universal forward and reverse primers developed (Caporaso et al., 2011) to yield optimal community clustering. Primer sequences were divided into 5 regions; the first region is the Illumina adapter (in orange), the second is a barcode region with unique sequence (in black), the third (green) and fourth regions are stabilizing and linker sequences that are

not homologous to any 16S rRNA sequence (red), and the fifth region (purple) is targeted to the conserved region of the 16S rRNA gene. The primer design and subsequent PCR product are illustrated in Figure 7.2.

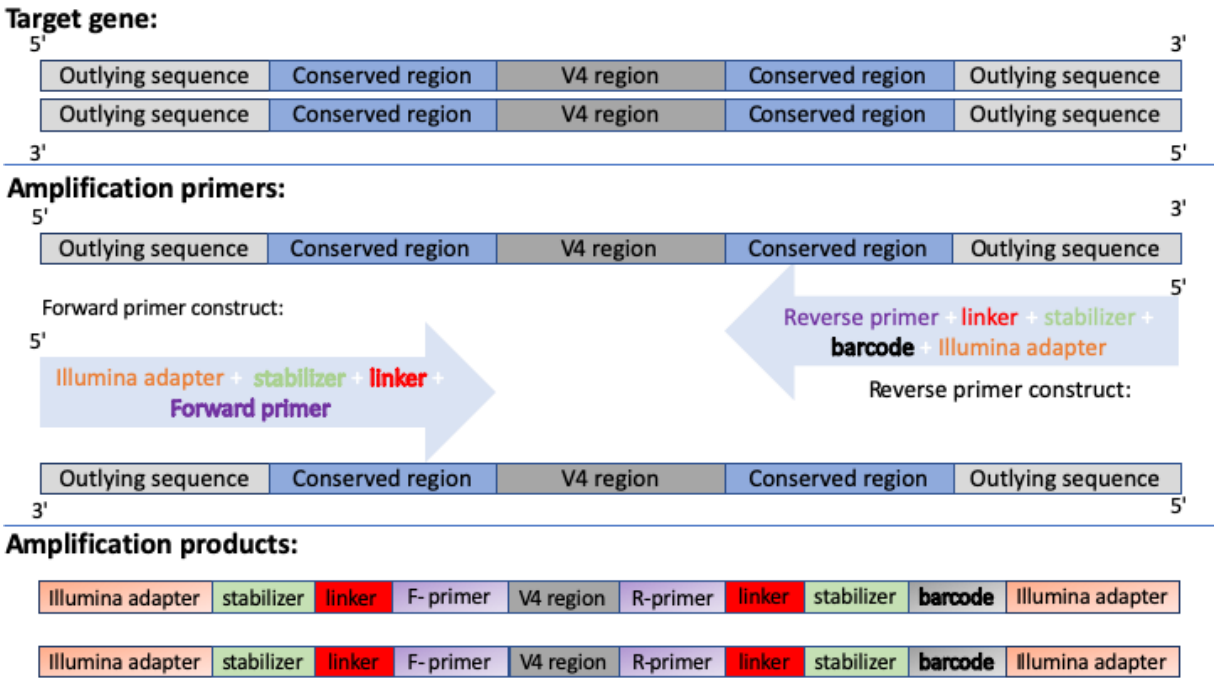

Figure 7.2: Primer construct for amplification and indexing of bacterial DNA for 16S rRNA gene sequencing.

The forward primer sequence used for PCR amplification is (F515 5' - AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMG CCGCGGTAA –3 ') (Caporaso et al., 2011). Unique identifier universal reverse primers for each sample are listed in Appendix C. Amplicons were generated in three replicates of 25µl per reaction. Each PCR master mix contained 2.5 µl of DNA buffer, 1.5 µl MgCl, 0.5 µl dNTP, 0.5 µl forward primer, 0.5 µl unique reverse primer (per sample), 0.2 µl Taq polymerase and 17.8 µl of DNA-free water. To prevent contamination of PCR reaction, PCR mix was performed under a biological safety hood following ultraviolet (UV) disinfection. Gloves, pipette tips and pipettes were also disinfected with UV prior to PCR reagents mixing. Template DNA (2 µl) was added in a separate laboratory space

from where PCR master mix was done. The PCR cycle for amplification included a denaturation step at 94ºC for 3 minutes, followed by 40 cycles of 94 ºC for 45 minutes, 60 ºC for 1 minute, and 72 ºC for 1 minute, then a final extension of 42 ºC for 10 minutes. Following amplification, 5 µl of PCR product was passed through a gel electrophoresis step to confirm the presence of the amplified product which was approximately 460bp.

Purification of PCR products

PCR products were purified by a magnetic bead DNA purification system following the Agencourt® Ampure® PCR purification protocol (Agencourt Biosciences Cooperation, A Beckman Coulter Company, 500 Cummings Center, Suite 2450, Beverly Massachusetts, USA) (Appendix O). The three replicates of amplified PCR reaction per sample were pooled (60 µl) into a well on a 96 well PCR plate. Following purification (as described in the Agencourt protocol- Appendix P), 40 µl of DNA suspended solution was carefully transferred to sterile wells on a 96 well PCR plate.

Quantification of DNA by qPCR

Prior to qPCR quantification, samples were serially diluted in ten-fold dilutions (1:10, 1:100, 1:1000, 1:10,000) using 10mm Tris HCL (containing Tween 0.05%), on a 96 well PCR plate. Diluted samples were thoroughly homogenised using vortex and micro-pipette at each dilution stage. DNA in diluted samples (1:1000, 1:10,000) were then quantified in triplicates using KAPA Biosystems Library Quantification Kit and protocol for Illumina platforms (Appendix L).

Normalization and re-quantification of DNA

Following quantification, purified undiluted samples were normalized to 4nM with DNA-free water using the formula below:

$$C_1V_1 = C_2V_2$$

$$V_1 = (4 \times 100) \div Concentration\ of\ DNA$$

To create a pooled sample that will be sequenced, 4 µl of each normalized sample was pipetted into a sterile Eppendorf tube. This pool was homogenised and diluted as before, then quantified using KAPA Biosystems Library Quantification Kit for Illumina platforms for 1:1,000, 1:10,000, and 1:100,000 dilutions to confirm normalization (Appendix L).

Sequencing on Illumina MiSeq System

The pooled normalised sample was corrected to 2nM with DNA free water using the $C_1V_1 = C_2V_2$ formula. To denature the samples, 5µl of pooled sample and 5 µl of NaOH @ 0.2N were pipetted into a sterile 1.5ml Eppendorf tube and left to incubate at room temperature for 5 minutes. Subsequently, 990µl of defrosted Hyb (buffer -part of the illumine sequence kit) was added into the denatured pooled sample. The final library was then prepared in a new sterile 1.5ml Eppendorf tube consisting of 400µl of (denatured pool + hyb) and 120µl of phiX (10pM) (a known control library that will not interfere with index). The mixture was then transferred into the sequencing cartridge. Other reagents (3.4µl of forward primer at 100pM, 3.4µl of index, and 3.4µl of reverse primer) were then pipetted into specified wells in the cartridge (Appendix K). Sample metadata (sample description) and index sequence were programmed into the MiSeq sequencer system and then sample was allowed to run for approximately 65 hours. Paired-end

sequenced reads were downloaded from the BaseSpace account of the host laboratory and then downstream analysis of fastq sequence reads were performed on the CLC genomics workbench.

7.2.4. Sequence data analysis using CLC genomics workbench

Paired-end sequence reads in the fastq file format were downloaded from the BaseSpace account of the host laboratory. To optimize the read lengths, sequenced reads were trimmed and subsequently filtered, with a minimum of 100bp length. Following read optimization steps, the reads were clustered into Operational Taxonomic Units (OTUs) using the global alignment method which groups reads into clusters based on taxonomic distribution of indexed tags against a 16S reference database (SILVA v119). OTUs represent taxa-specific associations with greater than or equal to 97% similarity, all reads that do not meet this minimum identity threshold were discarded from further analysis. OTUs with low abundance were also removed from further analysis. To visualize the OTU abundance table, a metadata table (a description of samples – replication, conditions, treatment) is linked to the OTU table. Using the MUSCLE tool, OTUs were aligned against the reference sequence on the SILVA v119 database to create a phylogeny tree. Secondary analyses were performed using the filtered OTU tables and phylogeny tree to determine the number of species in given sample (Alpha diversity), and the differences in species diversity between samples (Beta diversity). Further similarities between samples were visualized by heat map construction. Statistical differences between OTUs were determined by Permanova analysis (Qiagen 2018- Appendix M).

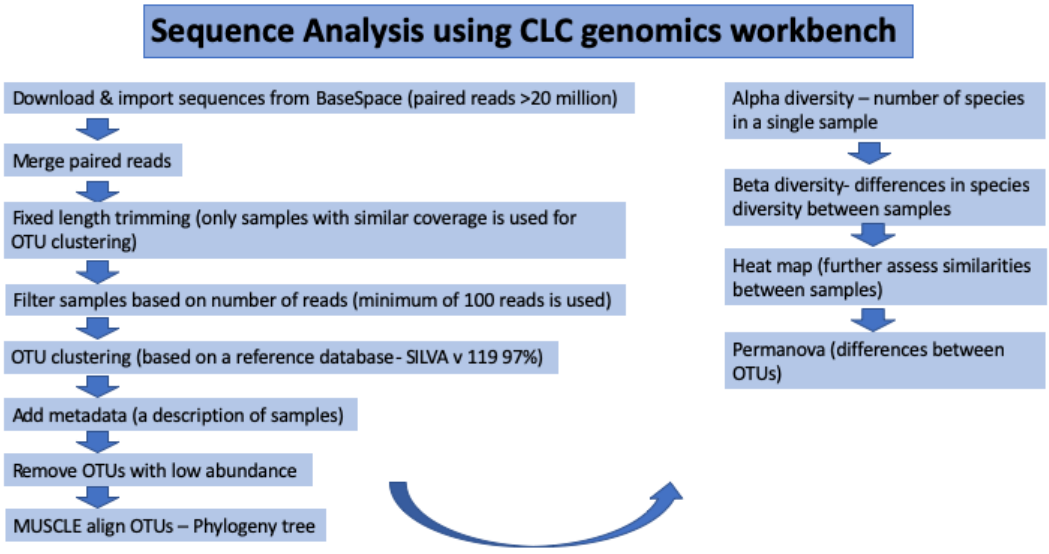

Figure 7.3: A flowchart summary of analysis of sequence reads on CLC genomic workbench.

7.2.5. Filters for the identification of beneficial and detrimental candidate bacteria species in *An. gambiae* s.l. insectary.

Following 16S rRNA sequencing of water samples from the *An. gambiae* s.l. larval rearing trays in the mosquito insectary-based experiment at CAEP, Keele University, as described in chapter 6, a total of 1031 bacteria species were identified. Four filtering systems were designed to correlate bacteria species to experimental trays to aid in narrowing the species list to candidate bacteria species that might be beneficial or detrimental to mosquito larvae.

Filter 1: Based on biology and abundance of bacteria species

Two criteria were used for this filter, firstly, bacteria species were ranked by OTU abundance of bacteria species in treatment groups with lowest mosquito adult emergence (Control and Zeolite). Subsequently, the most abundant species in the "cleaner trays", CWZ and CWC were also considered. Further filtering was conducted based on literature search conducted involving the following keywords -pathogenicity, ammonia, nitrate

conversion, denitrification, nitrogen fixing. A total of 17 bacteria families were listed in filter 1 as possible suspect species impacting on mosquito development in larval trays (Table 7.9).

Filter 2: Based on Spearman's correlation analysis of OTU abundance and mosquito survival parameters.

This group of filters consisted of 3 sub-filters where the OTU abundance of samples were correlated with adult emergence (2A), ammonia content (2B) and nitrate content (2C) in larval trays. Sequencing data obtained from Day 8 (sampling of water samples for bacteria DNA extraction) were excluded as they were basically a replication of Day 10. The first sub-group (Filter 2A) contains two sub-filters based on positive and negative correlations between OTU abundance of bacteria species and percentage adult emergence from mosquito larval rearing trays. Species that negatively correlated with adult emergence above 30% (correlation coefficient of -30 and above) were categorized as detrimental to mosquito larvae as their population were higher with decreasing adult emergence. Those that positively correlated with adult emergence above 30% (correlation coefficient of +30 and above) were classed as beneficial to mosquito larvae as their population increased with increasing adult emergence (Rumsey, 2016). The species list was ranked from the highest correlation to the least correlation. Spearman's non-parametric statistical test provided an R-value and a P-value.

Filter 2B also consisted of two sub-filters based on positive and negative correlations between OTU abundance of bacteria species and ammonia content in the larval rearing trays. Species that negatively correlated with ammonia above 30% (correlation coefficient of -30 and above) were categorized as being beneficial to mosquito larvae as their population where higher with decreasing ammonia content in the

larval trays. Those that positively correlated with ammonia above 30% (correlation coefficient of +30 and above) were classed as detrimental to mosquito larvae as their population increased with increasing ammonia (Rumsey, 2016). The species list was ranked from the highest correlation to the least correlation. Spearman's non-parametric statistical test provided an R-value and a P-value.

The third sub-group in this filter (Filter 2C) was based on the positive correlation (above 30%) between OTU abundance of bacteria species and nitrate content in mosquito larval trays. These species were classed as beneficial to mosquito larvae as they increase with increasing nitrate concentrations. There were no negative correlations above 30% for this group.

Filter 3: Based on a comparison of the Log_{10} ratio of OTU abundance in clean trays vs dirty trays

This filter was designed to isolate species that thrive in control conditions (dirty water) compared to those in better larval rearing conditions (CWC and Zeolite). The ratio of OTU abundance was Log_{10} transformed such that all Log ratios above 0 indicate a change towards "bad" bacteria, and those above Log = 1 indicate a 10-fold change or more in the same direction. Taxa in the category for Control vs CWC comparison were highlighted and those for Control vs Zeolite are also highlighted. Finally, data were combined with the assumption that the taxa involved in both categories were the candidate detrimental bacteria. A graph was then plotted with a reference line at Log_{10} = 1 showing the subset of bacteria that were overgrowing for both comparisons. The taxa in the right area of the graph were listed as candidate bacteria possibly detrimental to mosquito larvae. This was repeated for the two larval densities (200-filter 3A and 400-filter 3B) investigated in the zeolite experiment as described in chapter 6.

The three sets of filters described above were fitted into Venn diagrams (Figures 7.5a, 7.5b) to isolate candidate bacteria species that were detrimental to mosquito larvae and those were beneficial. Bacteria species found in the intersect of two or three Venn diagrams were listed as candidate bacteria for subsequent quantification by qPCR.

7.2.6. Primer design and optimization for quantification of candidate bacteria species by qPCR.

Following the earlier described filters, 10 bacteria species were selected for quantification by qPCR. The selection was based on the following parameters; species occurring in two or three filters with the highest correlation either as detrimental or beneficial, species that have been cultured and have their full genome available on the National Center for Biotechnology Information (NCBI) database, and for whom species-specific primers could be designed using Primer-BLAST tool. Accession numbers (unique identification numbers) of bacteria species were used on the NCBI (National Center for Biotechnology Information) database to find the genome of the species and then the primer-BLAST tool was employed for primer design. Primers were designed with the following criteria- length of PCR product between 50-150bp, primer sequence length between 18-30 nucleotides, GC content of primer sequence between 40-60%, melting point of primers between $58 - 60℃\left(T_m = 2℃ \times (A + T) + 4℃ \times (C + G)\right)$ and finally the five nucleotides at the 3' end had no more than two G and/or C bases (Johnson et al., 2013). Designed primers were re-checked for specificity using the microbial-BLAST tool to ascertain if other species with the same product would be amplified. The primer-BLAST tool was also used as a final check by imputing primer sequences instead of the required parameters to ascertain the possibility of amplifying other non-specific products.

Three sets of primers were designed for a particular species and primers were obtained from Eurofins genomics. Primers obtained were optimised by PCR, to determine the primer set that best amplify the desired product. One PCR reaction contained 2.5µl of PCR buffer (MgCl inclusive), 0.5 µl of dNTP, 0.5 µl of forward and reverse primers, 0.2 µl of Taq polymerase, 18.8 µl of DNA-free water and 2 µl of genomic DNA. The PCR reaction was done in a PCR MAX Alpha thermal cycler, at 94℃ for 3 minutes to denature the DNA, 40 cycles of 94℃ for 45 seconds, 50℃ for 30 seconds and 72℃ for 10 seconds, then a final extension at 72℃ for 3 minutes. Amplified products were viewed by gel electrophoresis on 1% agarose gel. Primers with clear bands at the desired product length were carried over for qPCR quantification (Table 7.1).

Table 7.1: Primer sequences for qPCR quantification of candidate bacteria species.

S/N	Accession number	Species	Primer pair sequence (5'->3')	Product length (bp)
1	ARCM01000002.993920.995384	*Ancylobacter* sp. FA202	Forward- ACTCACTGAACGAGTGGCTG Reverse- CGCAGACATATCCGTCGTCA	77
2	JF706531.1.1366	*Xylophilus* sp. PDD-37_7j_hv_b-3	Forward- CCGTCTTGTAGATGTGCAGC Reverse- GGTGATGAACGTCATCGTGG	137
3	AF144383.1.1437	*Ramlibacter tataouinensis*	Forward- GGTCATCCCTTCAAGGTGGA Reverse- CCGGATAGTCGACGAGTTCA	101
4	FJ390462.1.1455	*Bacillus weihenstephanensis*	Forward- CCGCTGTAGCTGGATGAAAC Reverse- AAGAACCTTAGCCTACGGGG	63
5	EF465533.1.1436	*Pseudoxanthobacter* soli-DSM 19599	Forward- AACACATGCCCGGATGAAAC Reverse- AGGACCTATAAGCCCCCTCA	124
6	JX879739.1.1388	*Nubsella* sp. EsD18	Forward- GCCTGTAAAGCCGGATCTTG Reverse- GTTCTTGGCCGAAGCCTATG	51
7	DQ166946.1.1431	*Aeromonas hydrophila*	Forward- AAAAGGAGGGGATTGGCAGA Reverse- AAACTGGCCTGTCACTCTCA	112
8	EU434572.1.1389	*Brevundimonas diminuta*	Forward- GATGACGTCGTTCGTCAAGG Reverse- GATCTTGGCGGGTTTCATCC	141
9	EU730907.1.1387	*Sphingomonas wittichii*	Forward- CAAGAGGTCGAATGTGCCG Reverse- GAAGAAGTCTTCGATCTCGGTG	117
10	CP002959.3819378.3820883	*Turneriella parva* DSM 21527	Forward- ATGTTGCACCTCCTTCGC Reverse- GGCGGTGCAAGAACTCAT	53

7.2.7. Quantification of candidate bacteria species by qPCR

Amplification and detection of genomic DNA extracted from water samples in the zeolite experiment (Chapter 6) by qPCR were performed with an Applied Biosystems StepOnePlus™ qPCR instrument, using optical grade 96 well plates and the KAPA SYBR® qPCR master kit (Appendix J). Quantification was done for the 10-candidate bacteria species in all experimental conditions sampled on day 10 of the zeolite treatment assay (Chapter 6-methodology) and for the four biological replicates, resulting in a total of 64 qPCR reactions per species, totaling 1920 (640 × 3 technical replicates) reactions. To create a standard curve, 4µl of extracted DNA per sample was pipetted into a 1.5 µl Eppendorf tube creating a pool of genomic DNA for a replicate. The pooled samples were serially diluted in four 10-fold (10^1 – 10^4) dilutions with PCR-grade water. Three technical replicates of each pooled sample dilution were quantified, with each qPCR reaction consisting of 3.6µl of PCR-grade water, 5 µl of KAPA master mix, 0.2 µl of forward primer, 0.2 µl of reverse primer, 0.2 µl of Rox high (KAPA mix) and 1 µl of diluted genomic DNA. Three no-template controls were included for each qPCR plate run. The thermal cycle used for the qPCR run was a 3-minute denaturing step at 94℃, 40 cycles of 94℃ for 45 seconds, 50℃ for 30 seconds, 72℃ for 10 seconds, and a final extension at 72℃ for 3 minutes. To ensure the specificity of the amplified product, a melting curve stage was added to the run for melting curve dissociation analysis to determine the amplification of primer-dimers or non-target products. Wells without a definitive melt curve peak were excluded from further analysis. A total of 40 standard curves were plotted using the average quantification cycle *Cq* values and genome size of the target species. Following DNA amplification, average *Cq* values (eliminating values from technical replicates that differed significantly), were plotted against the Log_{10} of the copy numbers of the bacteria genome of the given species. The resulting regression

equation was used to determine the copy number in any given sample using this standard curve. The copy numbers of a particular species were calculated using the formula below:

$$Target\ (copies/\mu l) = \frac{DNA\ concentration\ in\ ng/l \times Avogadro's\ number(6.022 \times 10^{23})}{Length\ of\ genomic\ DNA(bp) \times Average\ weight\ of\ dsDNA(660\ g/mol)}$$

Regression equation:$y = kx + C$ .Where y = Cq values, k = regression coefficient or slope, x = concentration/amount of target and C = intercept (the Cq value when one copy number should be detected) (Brankatschk et al., 2012; Kralik & Ricchi, 2017). The linearity of the standard curves was determined by R^2 values, standard curves with R^2 values lower than 0.9 were discarded.

Individual samples were quantified in technical triplicates and for the four biological replicates using the regression equation of a given standard curve for that biological replicate and species (Figure 7.4). Thus, the number of copies of DNA of a particular species in a sample (experimental tray) is in relation to the total number of bacteria speciesDNA numbers in a given pooled biological replicate. The qPCR reaction mix for individual samples is the same as for the standards, the 1:10 dilution of genomic DNA was used for sample amplification.

7.2.8. Statistical analysis

Following OTU clustering and Alpha diversity analysis, permutational multivariate analysis of variance (non-parametric MANOVA) were conducted on beta diversity to measure the size effect and significance of grouped variables using the CLC genomics workbench. All qPCR related data were analysed using the software JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA).

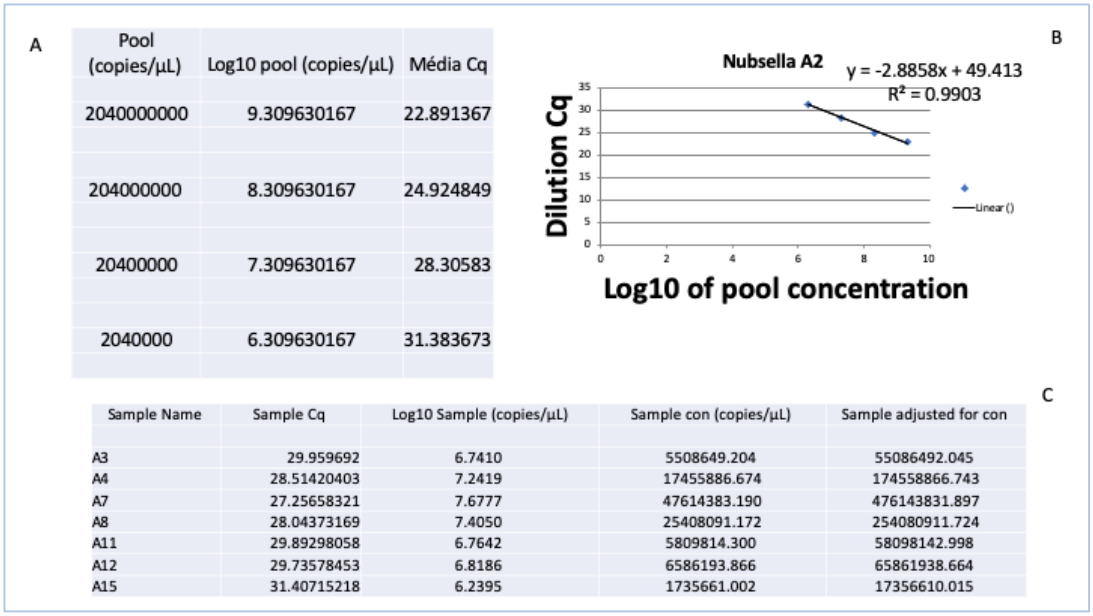

Figure 7.4: A sample excel page used for conversion of *Cq* values to copy numbers. A- Copy numbers of 10-fold serial dilutions, the Log_{10} of those values and the *Cq* values at which they were amplified. B- A sample standard curve used to quantify *Nubsella* sp. for one replicate. C- Individual *Cq* values of samples converted to copy numbers using the regression equation of the standard curve.

Mean Log_{10} of copy numbers of samples were pooled from four treatment groups earlier described in chapter 6 (Zeolite, CWC, CWZ and Control) to two pairs of treatment classes: Treatment class 1: water-replacement (WR), consisting of CWC & CWZ, and no-water-replacement (NWR) consisting of Zeolite & Control. Treatment class 2: zeolite treatment (ZT) consisting of CWZ & Zeolite and none-zeolite treatment (NZT), consisting of CWC & Control). Data from all replicates were used for analysis, replicate effects were tested but were only reported when significant. All data were checked for deviations from normality and heterogeneity (using normal distribution tool and Levene's test), and analyses of variance of mean values were conducted using parametric (Pooled t-Test) and non-parametric (non-parametric t-test) methods where appropriate. Mean comparison between species and within treatment groups were conducted using Turkey-Kramer HSD pairwise comparisons.

7.3. Results

7.3.1. Microbiome analysis of *An. coluzzii* larval rearing trays in the insectary

Overall, Proteobacteria was the most abundant bacteria phylum (60%), followed by Bacteriodetes (25%), Firmicutes (6%), Actinobactera (4%), Deinococcus Thermus (3%), Verrucomicrobia (2%), other bacteria phylum- Armatimonadetes, Cyanobacteria, Planctomycetes and Gemmatimonadetes, were less than 1% relative to total bacteria abundance (Figure 7.5, Table 7.2).

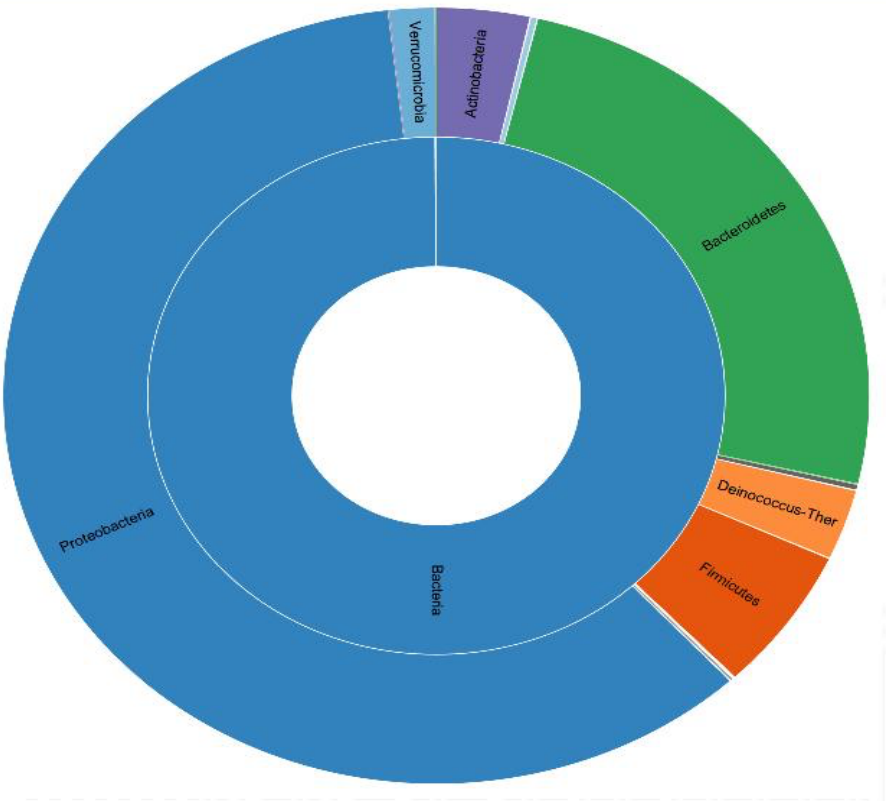

Figure 7.5: Relative OTU abundance of bacteria species in all treatment groups.

Within groups, relative abundance of bacteria phylum followed a similar trend with Proteobacteria having the highest abundance in all groups but subsequent phylum varied in percentage abundance within groups (Table 13, Figure 7.6).

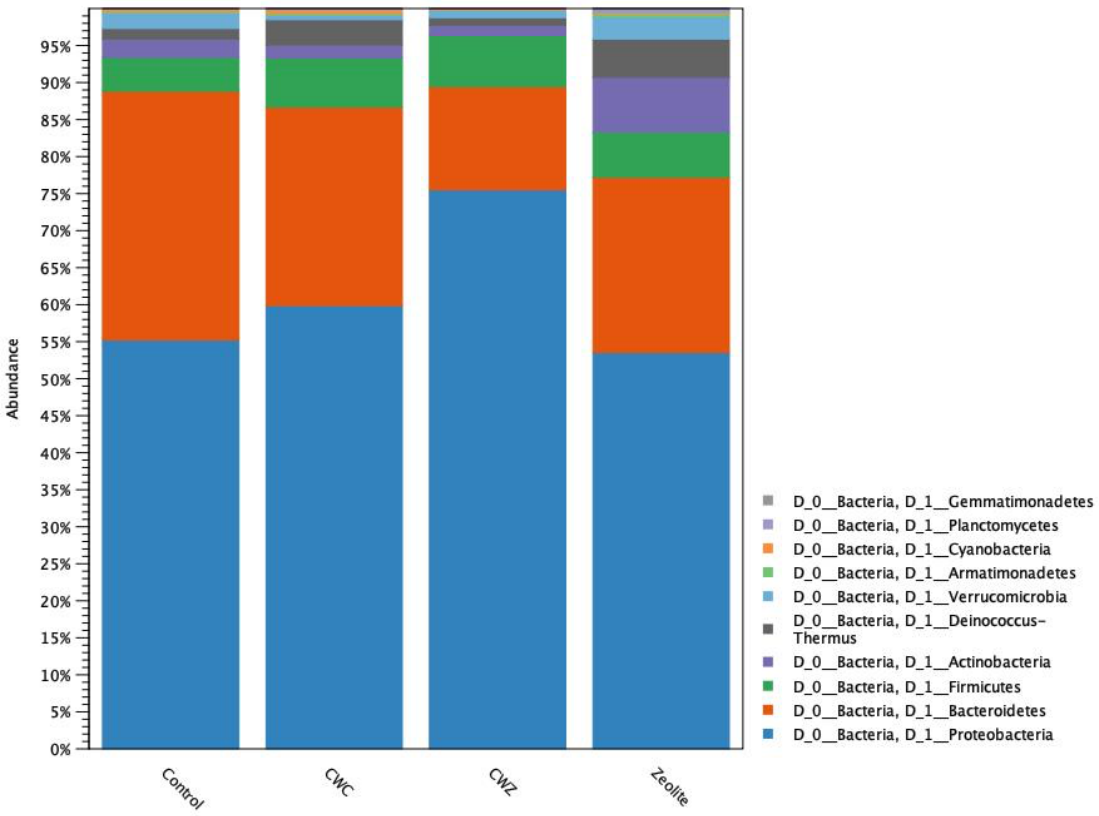

Figure 7.6: Relative abundance of bacteria phylum by treatment groups. Vertical axis of the bar chart is showing the relative abundance of OTUs at the phylum level, horizontal axis showing the treatment groups in which the bacteria phyla occurred.

Table 7.2: Relative abundance of bacteria phyla within treatment groups

Bacteria Phylum	Relative abundance within treatment groups			
	Control	CWC	CWZ	Zeolite
Proteobacteria	55% (519,113)	60% (624,606)	76% (605,828)	56% (609,860)
Bacteriodetes	34% (315,937)	30% (279,818)	14% (111,940)	25% (269,675)
Firmicutes	5% (42,944)	7% (68,973)	7% (55,204)	6% (69,494)
Actinobacteria	2% (23,321)	2% (18,274)	1% (11,246)	8% (84,555)
Deinococcus Thermus	1% (13,370)	3% (35,846)	1% (8,180)	5% (58,815)
Verrucomicrobia	2% (19,598)	0.6% (6,625)	0.9% (7,409)	3% (33,935)
Armatimonadetes	0.2% (2,160)	0	0	0
Cyanobacteria	0.3% (2,507)	0.5% (5,295)	0	0
Planctomycetes	0	0	0	0
Gemmatimonadetes	0	0	0	0
Total	**938,950**	**1,039,437**	**799,807**	**1,092,399**

Notes: OTU numbers are in parenthesis.

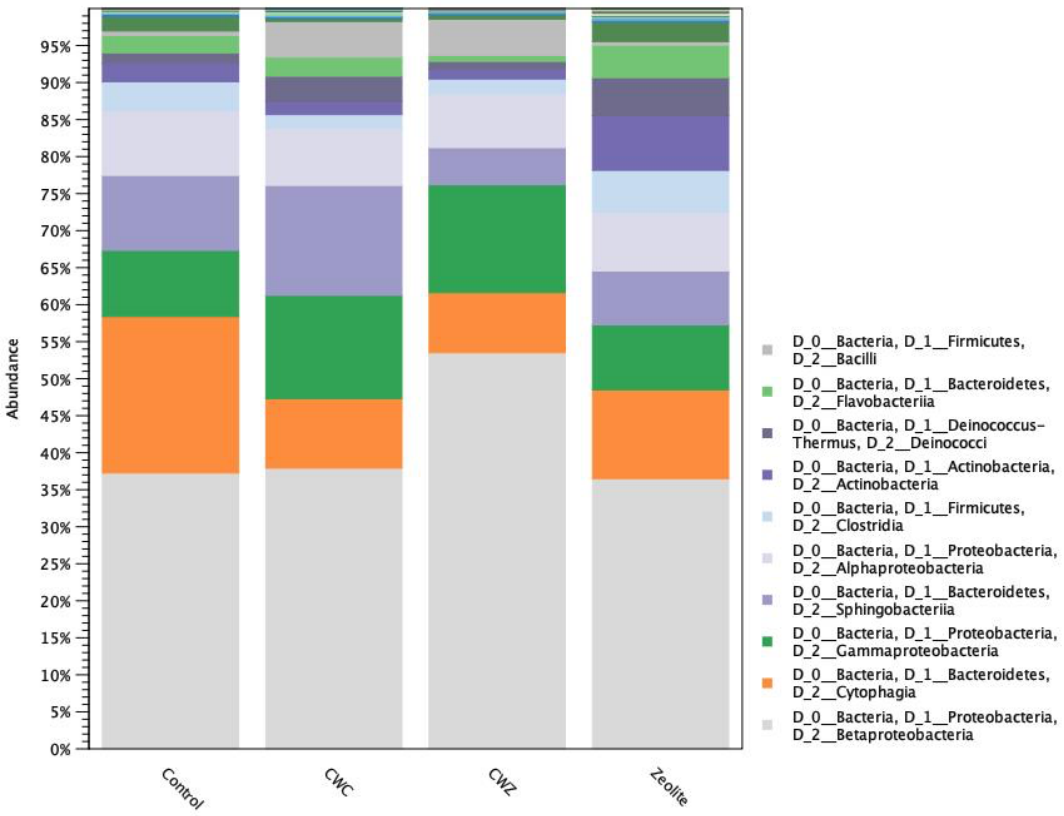

Figure 7.7: Relative abundance of bacteria class by treatment groups. Vertical axis of the bar chart is showing the relative abundance of OTUs at the class level, horizontal axis showing the treatment groups in which the bacteria classes occurred.

Within treatment groups the relative abundance of major bacteria taxonomic groups (class, order, family, and genus) varied in decreasing measures from the bottom of the graph, upwards (Figure 7.7, 7.8, 7.9). Filters were then created (as described in section 7.2.5) to isolate candidate bacteria species that are linked to treatment groups.

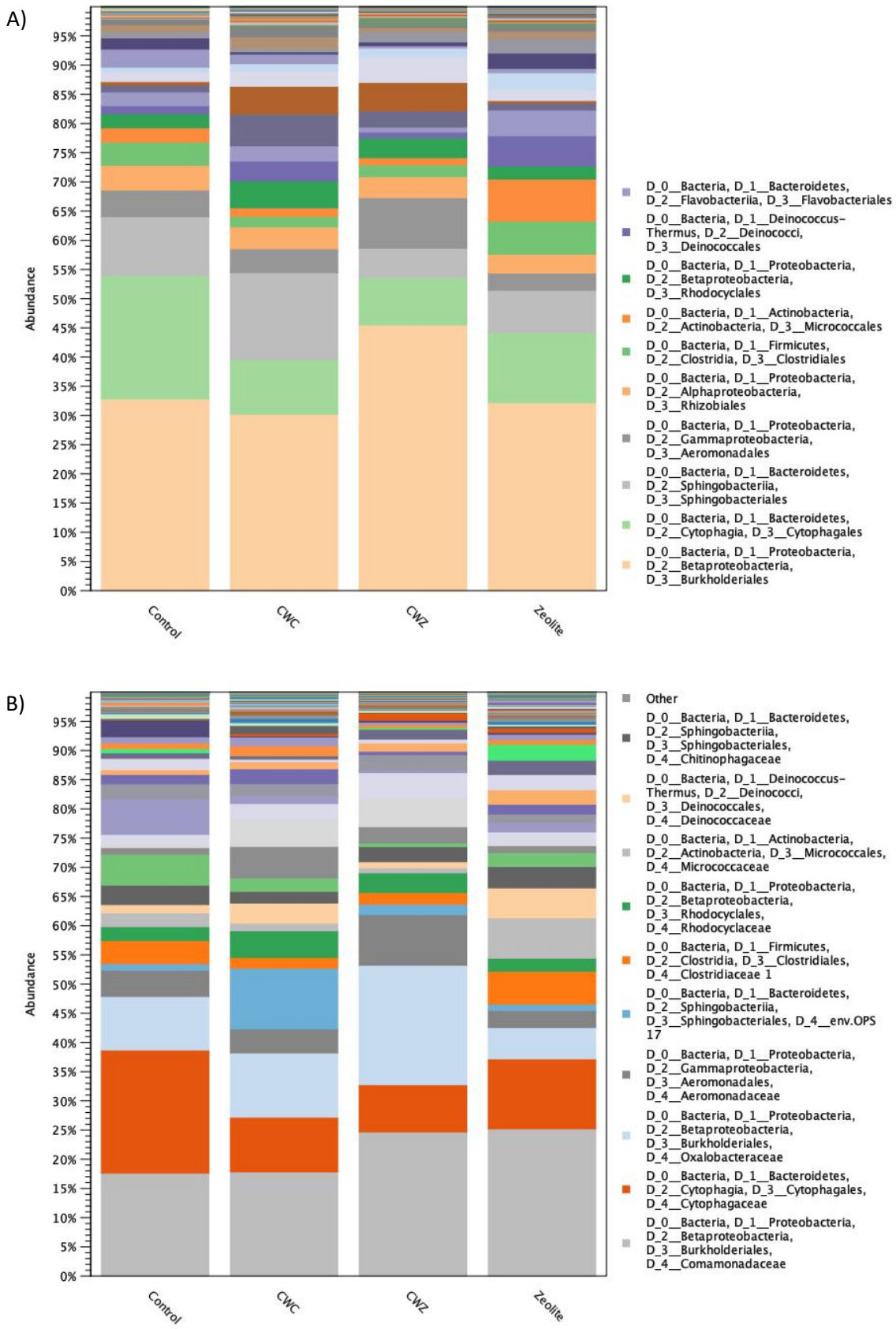

Figure 7.8: Relative abundance of bacteria **A)** order, and **B)** family by treatment groups. Vertical axis of the bar chart is showing the relative abundance of OTUs at the family level, horizontal axis showing the treatment groups in which the bacteria families occurred.

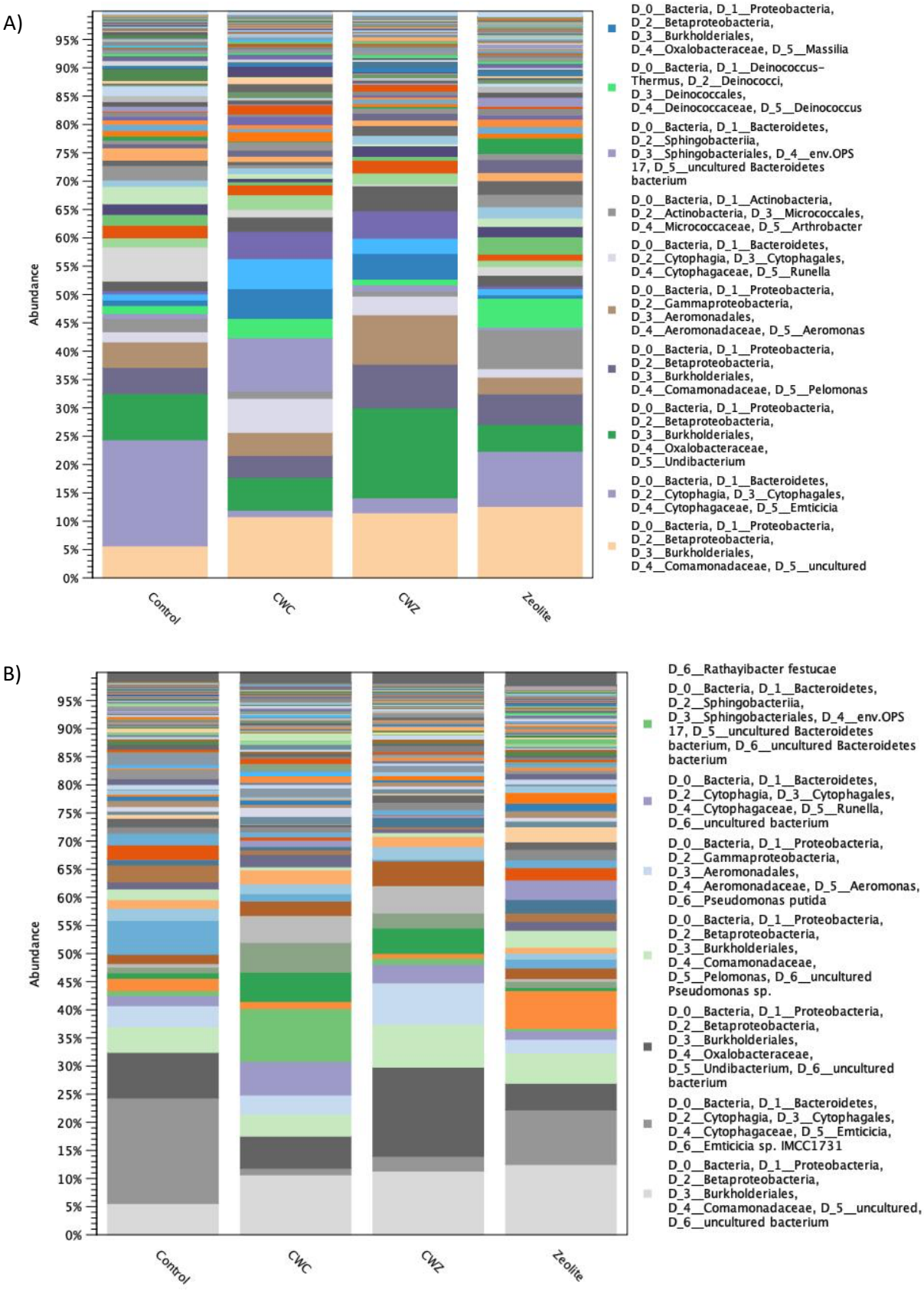

Figure 7.9: Relative abundance of bacteria **A)** genus, and **B**) species by treatment groups. Vertical axis of the bar chart is showing the relative abundance of OTUs at the species level, horizontal axis showing the treatment groups in which the bacteria species occurred.

The number of species (species richness) within each sample is represented by individual lines on the Alpha diversity rarefaction chart (Figure 7.10). The separate, heterogenous peaks of the samples indicated that the sampling depth was adequate to reveal bacteria diversity as heterogeneity did not decrease with increasing number of reads. Furthermore, each sample presented a plateau stage, which indicated that the sequencing efficiently resolved the microbial diversity of the sampled larval habitats (Qiagen, CLC genomic workbench version 11). The alpha diversity results were estimates and not absolute number of species since abundance table generated by the taxonomic profiling tool was used for the rarefaction analysis (Qiagen, CLC genomic workbench version 11). The Simpson's index was used for this rarefaction analysis and it measures the presence/absence of taxa and additionally accounts for the number of times that each taxon was observed (Lozupone & Knight, 2008).

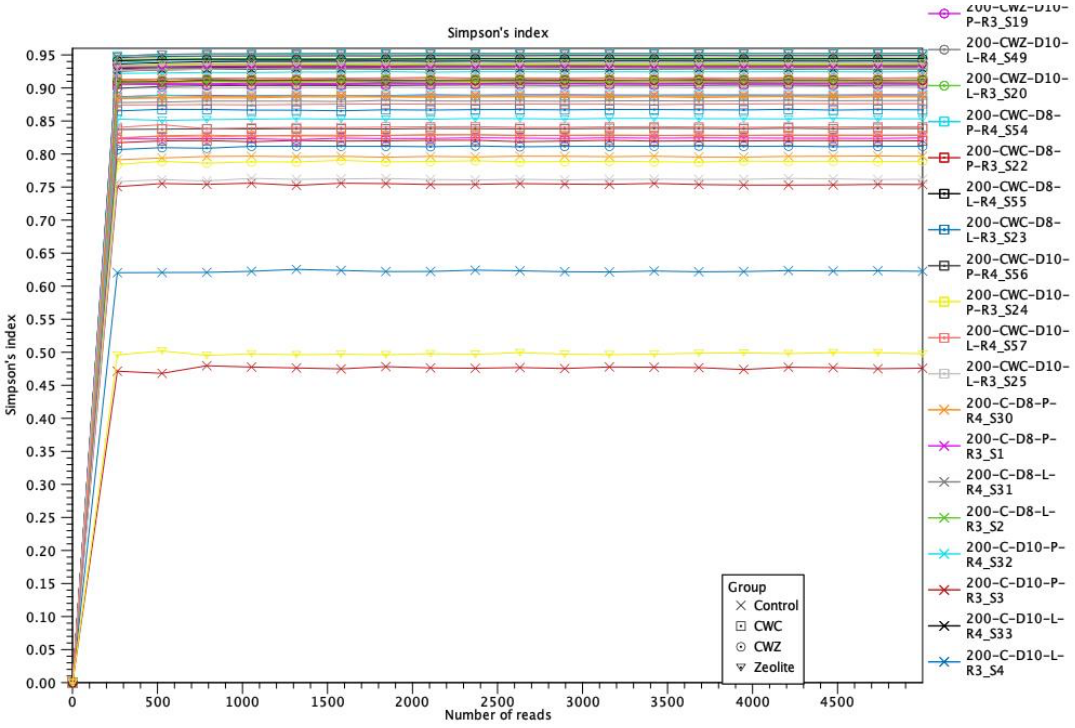

Figure 7.10: Alpha diversity chart showing the Simpson's diversity index on the vertical axis and the number of reads generated by Illumina MiSeq sequencing of mosquito larval rearing water samples on the horizontal axis. The chart was generated from the OTU abundance table and the phylogenetic tree.

Beta diversity analyses were conducted to reveal the changes in species diversity among samples. Firstly, the distance between samples performed with quantitative (weighted UniFrac) and qualitative (unweighted Unifrac) beta analysis measures revealed that the microbial composition of samples differed by treatments. Subsequently, principal coordinates from a principal coordinate analysis were plotted against each other to summarize the microbial community compositional differences between samples and the distance between the points represent how compositionally different the samples were from each other (Goodrich, et al., 2016). Qualitative (presence or absence of species) beta analysis based on phylogeny revealed differences in species composition between samples, with major clusters around the PCo3 (Figure 7.11).

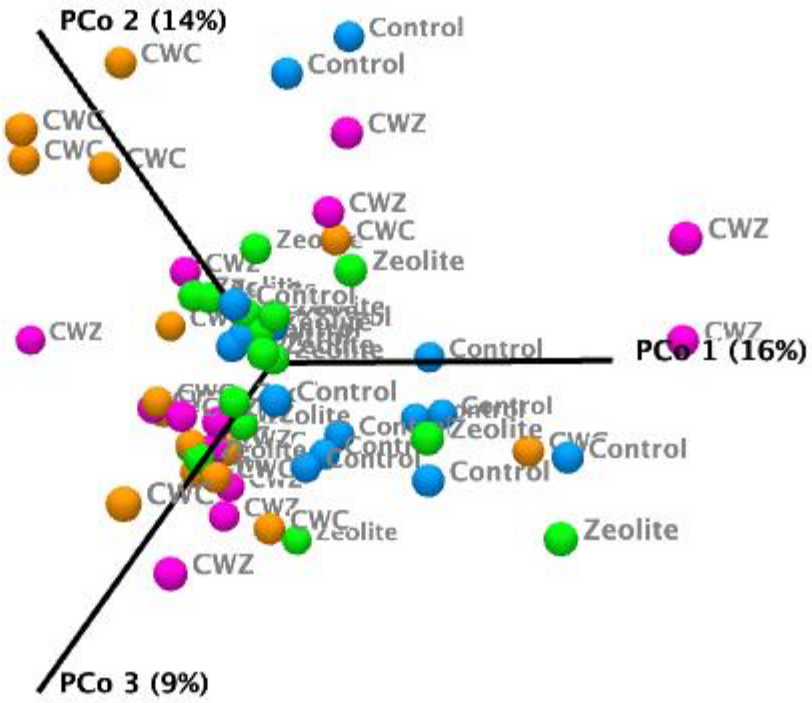

Figure 7.11: Beta-diversity of OTU sequences from *An. coluzzii* larval trays (Control-blue, CWC-yellow, CWZ-pink, Zeolite-green) summarising the microbial community composition between samples. Each point represents a single sample, and the distance between points represent how compositionally different they are from other samples. Unweighted UniFrac (qualitative and phylogenetically based) metrics were used to perform Principal coordinate analysis.

Quantitative beta analysis (using sequence abundance) not based on phylogeny, show an even spread of species composition within samples, with the exception of some outliers around the PCo 1. (Figure 7.12).

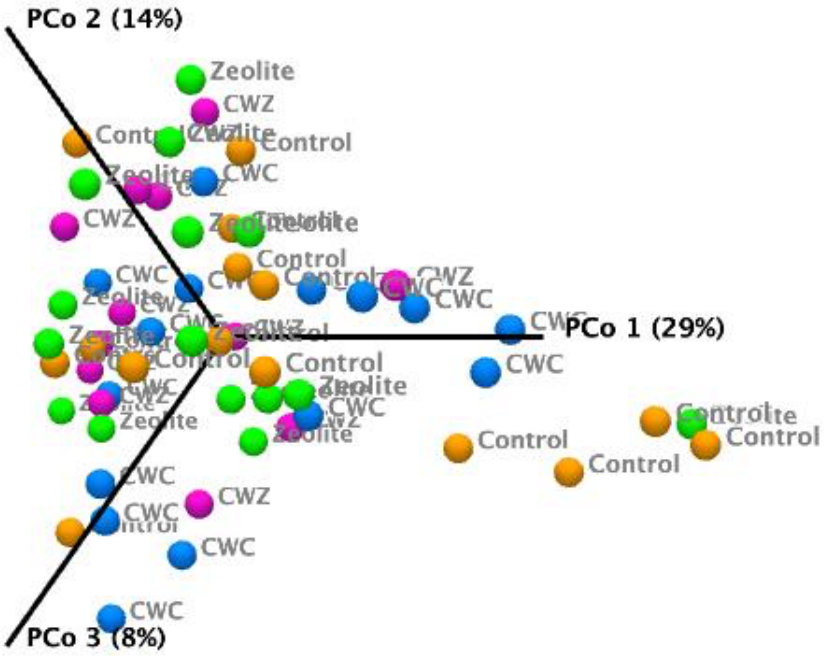

Figure 7.12: Beta-diversity of OTU sequences from *An. coluzzii* larval trays (Control-blue, CWC-yellow, CWZ-pink, Zeolite-green), summarising the microbial community composition between samples. Each point represents a single sample, and the distance between points represent how compositionally different they are from other samples. Weighted UniFrac (quantitative and not phylogenetically based) metrics were used to perform Principal coordinate analysis.

Non-parametric MANOVA revealed significant differences ($P < 0.0001$) in the species composition (beta diversity) among larval rearing trays (treatment groups). *Ad-hoc* comparisons between groups show significant differences between treatment groups with water replacement (CWC and CWZ) and those without water replacement (Control and Zeolite) (Tables 7.3a, 7.3b). No significant differences were observed between treatment groups without water replacement and equally between those with water replacement.

Table 7.3a: Bray-Curtis PERMANOVA analysis of beta diversity

Variable	Treatment groups	Pseudo-f statistic	P-value
Treatment groups	Control, CWC, CWZ, Zeolite	2.17707	P < 0.0001***

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05.

Table 7.3b: Bray-Curtis PERMANOVA analysis of beta diversity comparison between treatments.

Treatment	Pseudo-f statistic	P-value	P-value(Bonferroni)
Control vs CWC	2.79379	0.00103 **	0.00618 *
Control vs CWZ	2.81086	0.00043 **	0.00258 **
CWC vs CWZ	1.52650	0.10884 ns	0.65304 ns
Control vs Zeolite	1.25121	0.22065 ns	1.00000 nd
CWC vs Zeolite	2.55531	0.00277 **	0.01662 *
CWZ vs Zeolite	2.13558	0.00838 *	0.05028 *

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05.

7.3.2. Filters for identification of candidate bacteria species in *An. gambiae* s.l. insectary.

Filter 1 contains 17 bacteria families based on the biology of bacteria species ranked by OTU abundance as described in the methods section 7.2.5. (Table 7.4). Filter 2 consists of 3 sub-filters based on positive and negative correlations to biotic (adult emergence) and abiotic (ammonia and nitrate) within larval trays. A total of 587 bacteria species were isolated in this filter with 130 species negatively correlating with adult emergence (Filter 2Ai-Table 7.5a), 112 species positively correlating with adult emergence (Filter 2Aii-Table 7.5b), 155 species positively correlating with ammonia (Filter 2Bi- Table 7.6a), 96 species negatively correlating with ammonia (Filter 2Bii- Table 7.6b) and 94 species positively correlating with nitrate concentrations (Filter 2C- Table 7.7) in the larval trays.

Table 7.4.: Filter 1-Biological characteristics of bacteria families by OTU abundance (Full table in Appendix D)

S/N	Bacteria family	Criteria	OTU abundance in Treatment groups			
			Control	Zeolite	CWC	CWZ
1	Cytophagaceae (Cytophagales)	Found in organically rich material, also adapt to low nutrient, gram negative, may be aerobic, microaerophilic, capnophilic (CO2 requiring) or facultatively anaerobic are organotrophs, able to degrade biomacromolecules like proteins, chitin, pectin, agar, starch. (McBride, et al., 2014)	198,501	136,604	97,857	65,058
2	Comamonadaceae (Burkholderiales)	Include plant and human pathogen. Order is phenotypically, metabolically & ecologically diverse. Includes strictly aerobic & facultatively anaerobic chemoorganotrophs. Include obligate & facultative chemolithotrophs, nitrogen fixing organisms (Garrity, et al., 2005)	165,623	287,350	186,086	198,021
3	Oxalobacteriaceae (Burkholderiales)	Mostly aerobic, microaerobic to facultatively anaerobic. Found in diverse environmental habitats like water, soil, plant associated. Some species are mild plant pathogens, some are opportunistic human pathogens. Gram-negative. Mesophilic, with some psychrophilic (Baldani et al., 2014)	85,999	61,010	114,740	163,769
4	Burkholderiaceae *Pseudomonas* sp.	Several species are pathogenic for humans, other warm blooded animals, fish, eels as well as other vertebrates & invertebrates such as leeches (Garrity, et al., 2005)	56,863	18,120	14,349	3,110

Notes: Table continued in Appendix D

Table 7.5a: Filter 2Ai-OTU abundance vs emergence @ Spearman's correlation -30. (Full table in Appendix E)

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	FR667304.1.1357	*Roseomonas* (UB-Acetobacteraceae)	207	-0.6636	<0.0001***
2	EU773989.1.1373	Clostridiaceae1 (UB)*	15	-0.5918	0.0007**
3	KC432217.1.1330	*Clostridium* sensu stricto 3 (UB-Clostridiaceae1)*	7,024	-0.5909	0.0007**
4	HM778794.1.1387	Gammaproteobacteria-aaa34a10-UB	448	-0.5905	0.0007**
5	HM778860.1.1375	*Aeromonas* (uncultured Aeromonadaceae)*	80	-0.5813	0.0009**
6	DQ166946.1.1431	*Aeromonas hydrophila** (Aeromonadaceae)*	112	-0.5786	0.0010**
7	AB487832.1.1332	*Clostridium* sensu stricto 10*(Clostridiaceae1)	19	-0.5762	0.0011**
8	HM779015.1.1417	*Shewanella* (UB- Shewanellaceae)	228	-0.5507	0.0020**
9	JF808900.1.1502	*Tolumonas* sp. (UB- Aeromonadaceae)*	7,676	-0.5408	0.0025**
10	FJ205850.1.1269	*Clostridium* sensu stricto 1* (Clostridiaceae1)	27	-0.5089	0.0048**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species/family/groups. Table continued in Appendix E

Table 7.5b: Filter 2Aii- OTU abundance vs adult emergence @ Spearman's positive correlation +30 (Full table in Appendix E)

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	AB255079.1.1492	*Variovorax* (UB- Comamonadaceae)*	63	0.6933	<0.0001***
2	KF037634.1.1519	*Phaselicystis* (UB-Phaselicystidaceae)	2,586	0.6345	0.0002**
3	EU730907.1.1387	*Sphingomonas wittichii* (Sphingomonadaceae)*	56	0.5981	0.0006**
4	FJ374243.1.1485	*Comamonas* (UB- Comamonadaceae)*	73	0.5844	0.0009**
5	KF010745.1.1485	Comamonadaceae-UB*	85,589	0.5736	0.0011**
6	AY695728.1.1430	*Nitratireductor* (UB-Phyllobacteriaceae)*	47	0.5831	0.0009***
7	EU704796.1.1279	*Aquabacterium* sp. (UB-Comamonadaceae)*	4,706	0.5727	0.0012**
8	HM277954.1.1356	Chitinophagaceae -UB*	10,053	0.5714	0.0012**
9	HQ166654.1.1466	*Leptothrix* (UB-Comamonadaceae)*	1,162	0.5591	0.0016**
10	AB539840.1.1439	*Spirosoma* sp. APU1a (Cytophagaceae)	241	0.5555	0.0018**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species/family/groups. Table continued in Appendix E.

Table 7.6a: Filter 2Bi- OTU abundance vs NH_3 @ Spearman's correlation +30 (positive correlation) (Full table in Appendix F)

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	ARCM01000002.993920.995384	*Ancylobacter* sp. FA202 (Xanthobacteriaceae)	14,777	0.7764	<0.0001***
2	KC254734.1.1308	*Kaistia hirudinis* (Rhizobiaceae)	961	0.7210	<0.0001***
3	HG529104.1.1303	*Prosthecobacter* (UB-Verrucomicrobiaceae)*	16,796	0.6787	<0.0001***
4	GQ263646.1.1467	*Pedobacter* (UB-Sphingobacteriaceae)*	234	0.6616	<0.0001***
5	HM274274.1.1354	Chitinophagaceae-UB*	1,421	0.6606	<0.0001***
6	AF144383.1.1437*	*Ramlibacter tataouinensis* (Comamonadaceae)*	489	0.6468	0.0001**
7	HM341157.1.1356	*Variovorax* (uncultured Comamonadaceae)*	41	0.6303	0.0002**
8	GQ158268.1.1407*	*Lampropedia* (UB-Comamonadaceae)*	154	0.6272	0.0003**
9	JN868977.1.1523*	Comamonadaceae - UB	48	0.6190	0.0003**
10	KF150693.1.1413	*Pedobacter* sp. THG-G12 (Sphingobacteriaceae)	245	0.6157	0.0004**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species/family/groups. Table continued in Appendix F

Table 7.6b: Filter 2Bii- OTU abundance vs NH_3 @ Spearman's correlation -30 (negative correlation)

(Full table in appendix F)

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	FN563004.1.1426	*Noviherbaspirillum* (photoautotrophic bacterium GMMC _photoauto_1) Oxalobacteraceae	49	-0.6787	<0.0001***
2	FJ802311.1.1212	Comamonadaceae (iron reducing enrichment culture clone FEA_2_E4)	74	-0.6528	0.0001**
3	JF429369.1.1488	*Acidovorax* (Comamonadaceae-UB)*	6,575	-0.6194	0.0003**
4	JF222253.1.1350	*Leptothrix* (UB-Comamonadaceae)*	86	-0.6089	0.0005**
5	EF018753.1.1401	*Acidovorax* (Comamonadaceae-UB)*	119	-0.5944	0.0007**
6	AB599879.1.1412	Comamonadaceae-USH1-UB*	100	-0.5835	0.0009**

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
7	DQ664240.1.1431	*Aquabacterium*-IMCC1721 (Comamonadaceae)*	42	-0.5759	0.0011**
8	JN217068.1.1494	*Acidovorax* sp. (Tepidicella-Comoamonadaceae)*	218	-0.5744	0.0011**
9	CP002959.3819378.3820883	*Turneriella parva* DSM 21527 (Leptospiraceae)	17	-0.5715	0.0012**
10	AY792258.1.1537	*Variovorax* (UB- Comamonadaceae)*	19	-0.5693	0.0013**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species/family/groups. Table continued in Appendix F

Table 7.7: Filter 2C- OTU abundance vs Nitrate @ Spearman's positive correlation +30. (Full table in Appendix G)

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	KC747739.1.1382	*Vibrio cholerae* (Vibrionaceae)*	2,122	0.6312	0.0002**
2	EU537205.1.1383	*Comamonas* (UB-Comamonadaceae)*	57	0.6162	0.0004**
3	HM779429.1.1454	MB19-Aeromonadales-UB	20	0.5952	0.0007**
4	FJ823923.1.1498	*Acidovorax* (UB- Comamonadaceae)*	936	0.5807	0.0010**
5	FJ347719.1.1277	Comamonadaceae-UB*	85	0.5704	0.0012**
6	FJ562171.1.1242	*Nitrobacter* (UB- Bradyrhizobiaceae)*	177	0.5672	0.0013**
7	JX521628.1.1499	*Acidovorax* (UB- Comamonadaceae)*	123	0.5635	0.0015**
8	EF555457.1.1469	*Achromobacter xylosoxidans* (Alcaligenaceae)	172	0.5606	0.0016**
9	FJ193907.1.1439	Comamonadaceae-UB*	20	0.5463	0.0022**
10	EF019908.1.1335	*Bradyrhizobium* (UB-Bradyrhizobiaceae)*	10	0.5425	0.0024**

P- value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species. Table continued in Appendix G

Filter 3 consists of two sub-filters with a total of 26 species (19 species at 200 larval density and 7 species at 400 larval density) based on the filter description in method section 7.2.5 as possible candidate bacteria that were detrimental to mosquito larvae in the experimental trays (Tables 7.8a and 7.8b).

Table 7.8a: Filter 3A: Highest bacteria abundance Log ratio between Control vs CWC & Control vs Zeolite at 200 larval density. (Full table in Appendix H)

S/N	Species number	Name	OTU abundance
1	FN436071.1.1449	*Ochrobactrum*- UB (Brucellaceae)	288
2	FJ375362.1.1346	*Azospirillum*- UB (Rhodospirillaceae)	21,218
3	FJ393109.1.1505	*Variovorax*- UB (Comamonadaceae)	437
4	KC633571.1.1346	*Bergeyella*- UB (Flavobacteriaceae)	171
5	FJ375487.1.1458	*Cloacibacterium*- UB (Flavobacteriaceae)	6,048
6	FJ418700.1.1325	*Sinorhizobium* sp. CCBAU 51063 (Rhizobiaceae)	52
7	APMI01000355.1.1237	Wastewater metagenome -UB (Chitinophagaceae)	1,966
8	HE583162.1.1377	*Delftia*-UB (Comamonadaceae)	6,019
9	AM403225.1.1369	Myroides (Flavobacteriaceae bacterium D11-24b1)	2,699
10	ARCM01000002.993920.995384	*Ancylobacter* sp. FA202 (Xanthobacteraceae)	14,777

Notes: Table continued in Appendix H

Table 7.8b: Filter 3B: Highest bacteria abundance Log ratio between Control vs CWC & Control vs Zeolite at 400 larval density

S/N	Species number	Name	OTU abundance
1	FJ375362.1.1346	*Azospirillum*- UB (Rhodospirillaceae)	21,218
2	AY856847.1.1449	*Sphingobacterium* sp. 62 (Sphingobacteriaceae)	56,741
3	AB240273.1.1475	*Haloferula*-UB (Verrucomicrobiaceae)	4,002
4	JN391803.1.1495	*Variovorax*-UB (Comamonadaceae)	445
5	FN668067.1.1483	*Sphingobacterium* sp. -UB NS11-12 Marine group	7,031
6	JF217076.1.1347	*Perludicibaca*-UB (Moraxellaceae)	6,700
7	JX489898.1.1487	Uncultured soil bacterium NS11-12 Marine group-Sphingobacteriales	251

Venn-diagrams fitting all filters to reveal possible detrimental (Figure 7.13) and beneficial (Figure 7.14). Bacteria at the intersect of two or three Venn diagrams were prioritised in the final selection of candidate bacteria species to be validated by qPCR.

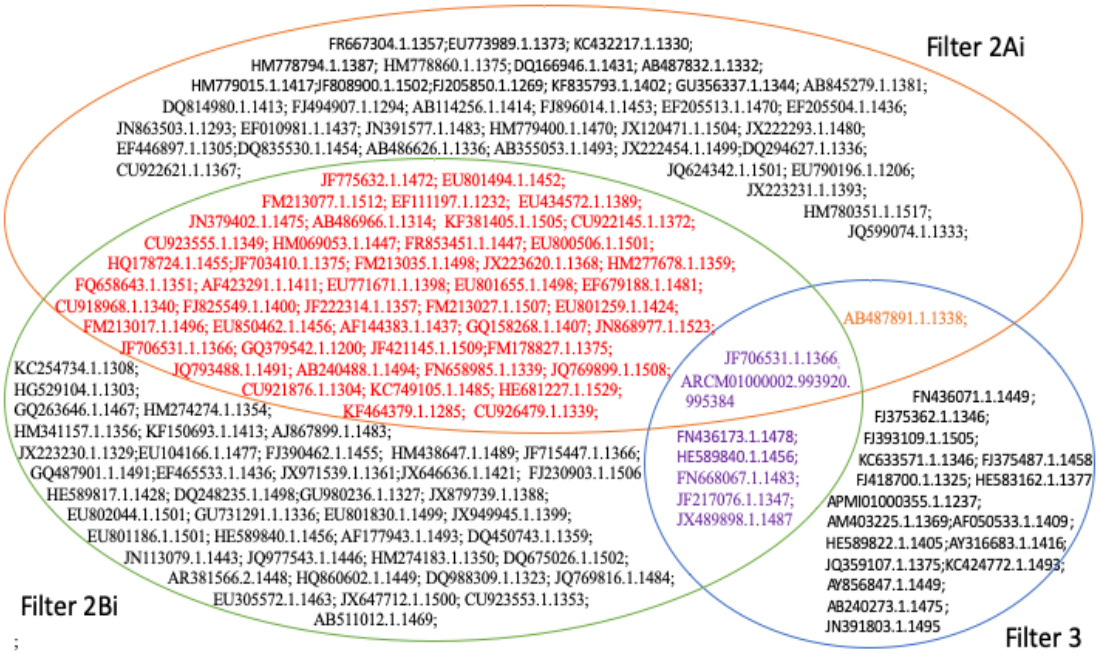

Figure 7.13: Venn diagrams showing detrimental candidate bacteria species associated with Filter 2Ai (negative correlation between adult emergence and OTU abundance- orange circle), Filter 2Bi (positive correlation between OTU abundance and ammonia) and Filter 3 (species associated with dirty water groups). Species in red and purple, occurring between two filters are prioritized as prime candidates.

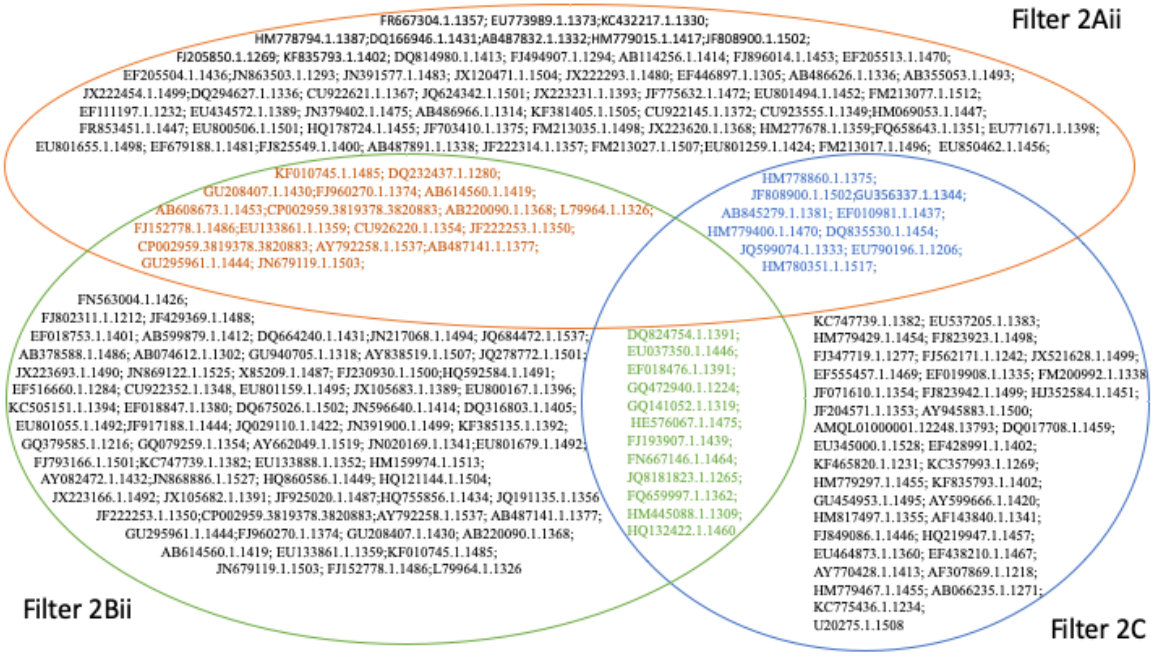

Figure 7.14: Venn diagrams showing candidate beneficial bacteria species associated with Filter 2Aii (positive correlation between adult emergence and OTU abundance- orange circle), Filter 2Bii (negative correlation between OTU abundance and ammonia) and Filter 2C (positive correlation between nitrate and OTU abundance). Species in orange, green and blue, occurring between two filters are prioritized as prime candidates.

A total of 10 bacteria species were selected based on the above described filters, Venn diagram streamlining, availability of full genome sequence on NCBI database and the limited financial resources and time allowed for this study (Table 7.9). The first 8 bacteria species were deemed detrimental species while the last 2 (in blue ink) are selected as beneficial bacteria species.

Table 7.9: Candidate bacteria species for quantification by qPCR.

S/N	Accession number	Species	OTU abundance	Spearman's R	P-value
1	ARCM01000002.993920.995384	*Ancylobacter* sp. FA202 (Xanthobacteraceae)	14,777	0.7764	<0.0001***
2	JF706531.1.1366	*Xylophilus* sp. PDD-37_7j_hv_b-3 (Comamonadaceae)	55,011	0.5971	0.0006**
3	AF144383.1.1437	*Ramlibacter tataouinensis*(Comamonadaceae)	489	0.6468	0.0001**
4	FJ390462.1.1455	*Bacillus weihenstephanensis* (Bacillaceae)	13	0.5977	0.0006**
5	EF465533.1.1436	*Pseudoxanthobacter* soli-DSM 19599 (Xanthobacteraceae)	296	0.5613	0.0015**
6	JX879739.1.1388	*Nubsella* sp. EsD18 (Sphingobacteriaceae)	4,977	0.4889	0.0071**
7	DQ166946.1.1431	*Aeromonas hydrophila*(Aeromonadaceae)	112	-0.5786	0.0010**
8	EU434572.1.1389	*Brevundimonas diminuta* (Caulobacteraceae)	60	-0.4739	0.0094**
9	EU730907.1.1387	*Sphingomonas wittichii* (Sphingomonadaceae)	56	0.5981	0.0006**
10	CP002959.3819378.3820883	*Turneriella parva* DSM 21527 (Leptospiraceae)	17	-0.5715	0.0012**

Notes: 1-8- correlate with larval mortality; 9-10 correlate with larval survival. P- value and Spearman's R are derived from non-parametric correlation analysis with OTU abundance. P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant). Spearman's R and P values- Spearman's rank positive correlation to: percentage ammonia (filter 2Bi)/ ^{2}- negative correlation to adult emergence (filter 2Ai)/ ^{3}- positive correlation to adult emergence (filter 2A)/^{4}- negative correlation to ammonia. Species in blue are candidate beneficial bacteria.

7.3.3. Quantification of candidate bacteria species in *An. coluzzii* larval rearing trays by qPCR.

Overall, candidate bacteria species differed in DNA abundance across treatment groups (Table 7.11a Figure 7.15) within *An. coluzzii* larval trays. The three most abundant bacteria species were *Xylophilus* sp. PDD-37_7j_hv_b-3 (Comamonadaceae) followed by *Sphingomonas wittichii* (Sphingomonadaceae), *Turneriella parva* DSM 21527 (Leptospiraceae) whilst the least abundant species were *Brevundimonas diminuta* (Caulobacteraceae), then *Bacillus weihenstephanensis* (Bacillaceae) and *Ramlibacter tataouinensis* (Comamonadaceae) (Tables 7.10, 7.11a, Figure 7.16).

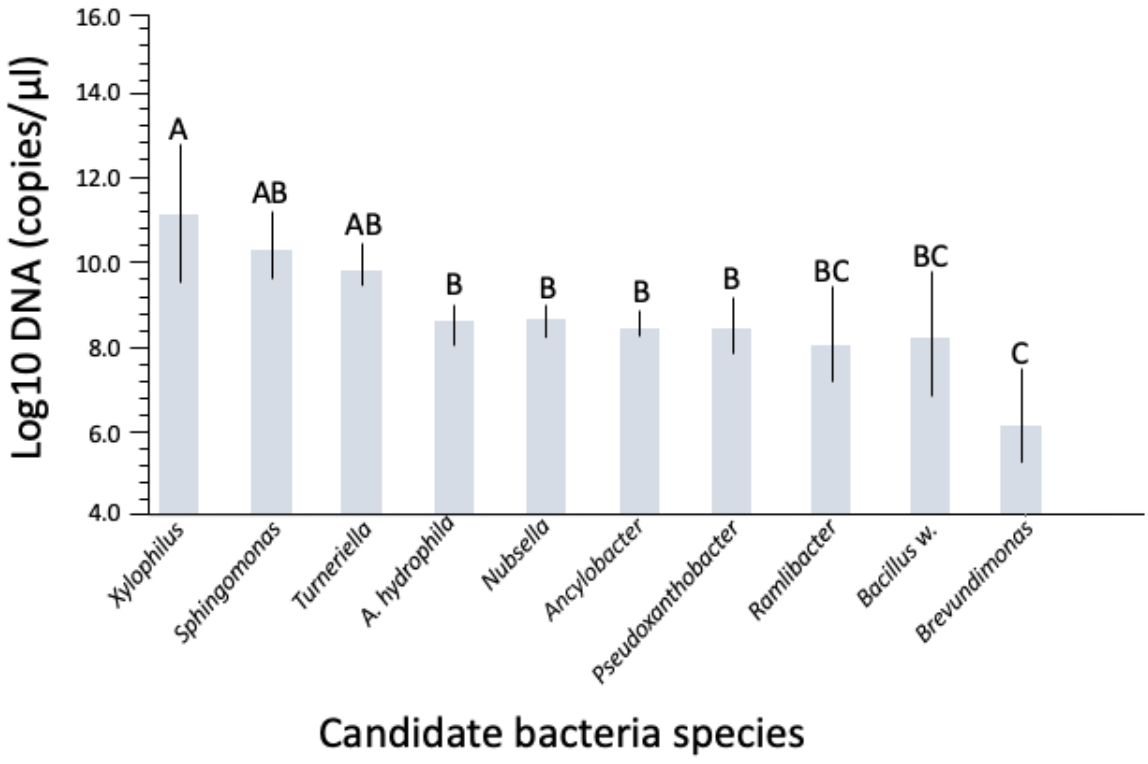

Figure 7.15: Log_{10} of bacteria DNA (copies/µl) of candidate bacteria species isolated from *An. coluzzii* larval trays. Light blue bars represent mean Log_{10} DNA abundance of individual bacteria species. Whiskers represent 95% confidence intervals. Bars with different letters are significantly different. Bars sharing any letter are not significantly different.

Table 7.10: Classification of the candidate bacteria species ranked by copy number abundance in larval trays.

Rank	Phylum	Class	Family	Genus/Species	Log_{10} DNA (Copies/µl)
1	Proteobacteria	Betaproteobacteria	Comamonadaceae	*Xylophilus* sp. PDD-37_7j_hv_b-3	10.92 (9.28 - 12.57)
2	Proteobacteria	Alphaproteobacteria	Sphingomonadaceae	*Sphingomonas wittichii*	10.31 (9.57 – 11.06)
3	Spirochaetae	Spirochaetes	Leptospiraceae	*Turneriella parva* DSM 21527	9.91 (9.39 – 10.43)
4	Bacteriodetes	Sphingobacteriia	Sphingobacteriaceae	*Nubsella* sp. EsD18	8.61 (8.25 – 8.96)
4	Proteobacteria	Gammaproteobacteria	Aeromonadaceae	*Aeromonas hydrophila*	8.61 (8.18 – 9.04)
5	Proteobacteria	Alphaproteobacteria	Xanthobacteraceae	*Ancylobater* sp. FA202	8.58 (8.30 – 8.86)
6	Proteobacteria	Alphaproteobacteria	Xanthobacteraceae	*Pseudoxanthobacter soli*- DSM 19599	8.52 (7.80 – 9.25)
7	Proteobacteria	Betaproteobacteria	Comamonadaceae	*Ramlibacter tataouinensis*	8.28 (7.17 – 9.38)
8	Firmicutes	Bacilli	Bacillaceae	*Bacillus weihenstephanensis*	8.27 (6.77 – 9.78)
9	Proteobacteria	Alphaproteobacteria	Caulobacteraceae	*Brevundimonas diminuta*	6.25 (5.05 -7.45)

Notes: P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant). Species in blue are candidate beneficial bacteria. Sample size = 64.

There was an overall impact of water replacement on total abundance among bacteria species in *An. coluzzii* larval trays (Table 7.12a). Larval trays with no water replacement (Control and Zeolite) contained significantly higher (P = 0.0002) numbers of candidate bacteria species compared to trays with water replacement (CWC, CWZ) (Table 7.12a). Similarly, larval trays treated with zeolite had significantly (P = 0.0044) more bacteria species compared to those without zeolite (Table 7.12a).

Table 7.11a: Test of effect (T-test) of the means of Log10 bacteria DNA (copies/µl) by species and between treatment groups.

Parameter	df	Mean Square	P-value
Species	9	108.589	<0.0001***
Treatment class 1 (NWR vs WR)	1	220.887	0.0002**
Treatment class 2 (NZT vs ZT)	1	131.897	0.0044*

Notes: P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant)

Candidate bacteria species significantly differed (P = 0.0002) in DNA abundance (copies/µl) between the subgroups in treatment class 1 (NWR vs WR). (Table 7.11a). One-tailed t-test revealed significant differences in abundance for *Ancylobacter* sp. FA202 (Xanthobacteraceae) at P = 0.0254, *Bacillus weihenstephanensis* (Bacillaceae) at P = 0.0215 and *Pseudoxanthobacter soli*-DSM 19599 (Xanthobacteraceae), P = 0.0056 and *Sphingomonas wittichii* (Sphingomonadaceae) at P = 0.0289 as significantly higher in NWR compared to WR. There were no significant differences in bacteria abundance observed among treatment class 1 for other candidate bacteria species (Table 7.11b).

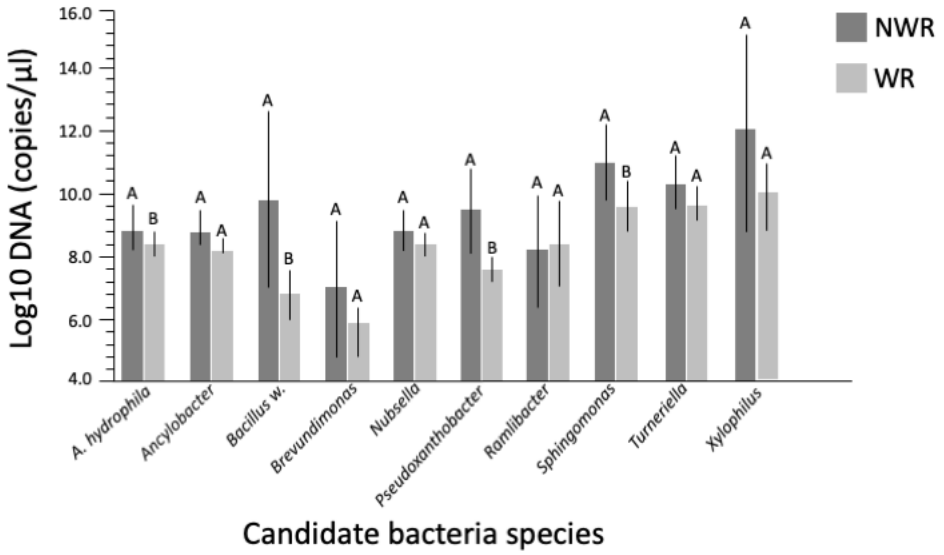

Figure 7.16: Log10 of bacteria DNA (copies/µl) of candidate bacteria species isolated from *An. coluzzii* larval trays. Dark grey bars represent the NWR (no-water-replacement) treatment level and light grey bars show WR (water-replacement) level within species. Whiskers represent 95% confidence intervals. Within species, bars with different letters are significantly different.

Table 7.11b: Test of effect (T-test) of the means of Log10 bacteria DNA (copies/µl) by species between treatment levels.

Parameter	Source (species)	df	Spearman's R	Spearman's P	P-value (2-way)	P-value (1-way)
Treatment class 1	*Aeromonas hydrophila* ^	1	-0.5786[2]	0.0010**	0.2374[ns]	0.1187 [ns]
(NWR vs WR)	***Ancylobacter* sp.^**	**1**	**0.7764[1]**	**<0.0001*** **	**0.0507[ns]**	**0.0254***
	***Bacillus weihenstephanensis* ^**	**1**	**0.5977[1]**	**0.0006*** **	**0.0430***	**0.0215***
	Brevundimonas diminuta ^	1	-0.4739[2]	0.0094**	0.2666[ns]	0.1333[ns]
	Nubsella sp.^	1	0.4889[1]	0.0071**	0.2374[ns]	0.1187[ns]
	***Pseudoxanthobacter soli* ^**	**1**	**0.5613[1]**	**0.0015** **	**0.0111***	**0.0056***
	Ramlibacter tataouinensis	1	0.6468[1]	0.0001***	0.8636[ns]	0.4318[ns]
	Xylophilus sp. PDD-37_7j_hv_b-3 ^	1	0.5971[1]	0.0006**	0.2058[ns]	0.1029[ns]
	***Sphingomonas wittichii* ^**	**1**	**0.5981[3]**	**0.0006*** **	**0.0578[ns]**	**0.0289***
	Turneriella parva DSM 21527^	1	-0.5715[4]	0.0012**	0.1668[ns]	0.0838[ns]
Treatment class 2	*Aeromonas hydrophila*	1	-0.5786[2]	0.0010**	0.1217[ns]	0.0608[ns]
(NZT vs ZT)	*Ancylobacter* sp.	1	0.7764[1]	<0.0001***	0.1850[ns]	0.0925[ns]
	Bacillus weihenstephanensis	1	0.5977[1]	0.0006***	0.2719[ns]	0.1358[ns]
	Brevundimonas diminuta	1	-0.4739[2]	0.0094**	0.1732[ns]	0.0866[ns]
	Nubsella sp.	1	0.4889[1]	0.0071**	0.3412[ns]	0.1706[ns]
	Pseudoxanthobacter soli	1	0.5613[1]	0.0015**	0.5555[ns]	0.2777[ns]
	Ramlibacter tataouinensis	1	0.6468[1]	0.0001***	0.3949[ns]	0.1975[ns]
	Xylophilus sp. PDD-37_7j_hv_b-3	1	0.5971[1]	0.0006**	0.2565[ns]	0.1282[ns]
	Sphingomonas wittichii	1	0.5981[3]	0.0006***	0.5252[ns]	0.2626[ns]
	Turneriella parva DSM 21527	1	-0.5715[4]	0.0012**	0.2272[ns]	0.1136[ns]

Notes: P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, [ns] > 0.05 (not significant). ^ Species followed the expected trend from the filters established by ultra-sequencing. Species in bold font were significantly different between treatment groups at one way T-test. Species in black ink were categorised from the filter tables as detrimental microbes (associated with higher ammonia content, lower nitrate content increased mortality) and with experimental trays labelled as NWR (Zeolite and Control). Species in blue ink were categorised as beneficial bacteria from the filtering system earlier described (lower ammonia content, higher nitrate conversion, reduced larval mortality, WR-CWC and CWZ).

Although there was an overall significant difference in the abundance of bacteria species between NZT and ZT, individual bacteria species did not significantly differ among the sub-treatment groups (Tables 7.11a, 7.11b).

Within treatment class 1 subgroups, there were significant differences (NWR-P = 0.0053, and WR- P <0.0001) in DNA abundance of bacteria species (Table 7.12). Post-hoc analysis revealed significant differences the two most abundant species (*Xylophilus* sp. PDD-37_7j_hv_b-3 and *Sphingomonas wittichii)* and the least abundant species (*Brevundimonas diminuta)* within the NWR sub-group (Figure 7.17, Appendix- I).

Table 7.12: Analysis of variance of the means of Log10 bacteria DNA (copies/µl) by species within treatment groups.

Parameter	Source	df	Mean Square	P-value
Species	NWR	9	66.4064	0.0053*
	WR	9	56.9570	<0.0001***
	NZT	9	59.9773	<0.0001***
	ZT	9	54.3614	0.0178**

Notes: P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ns > 0.05 (not significant)

The other 7 species in the median range of abundance did not differ significantly with the high abundance group or with the least abundant species in the subgroup (Figure 7.12, Appendix I). Likewise, in the WR sub-group, the three most abundant species (*Xylophilus* sp. PDD-37_7j_hv_b-3, *Sphingomonas wittichii* and *Turneriella parva* DSM 21527*)* differed significantly from the least abundant species (*Brevundimonas diminuta)* but not with the other six in the median range (Figure 7.17, Appendix I).

Within treatment class 2 subgroups, there were significant differences (NZT P < 0.0001, and ZT = 0.0178) in the DNA abundance of candidate bacteria species in *An. coluzzii* larval trays (Table 7.12, Figure 7.18). *Post-hoc* analysis revealed four levels of significance in bacteria abundance within the NZT treatment sub-group, with *Sphingomonas wittichii* and *Xylophilus* sp. PDD-37_7j_hv_b-3 being the most abundant and *Brevundimonas diminuta* as the least abundant bacteria species (Figure 7.18). Similarly, within the ZT treatment sub-group, 3 levels of significance were revealed

following *post-hoc* analysis. *Xylophilus* sp. PDD-37_7j_hv_b-3 was the most abundant and *Brevundimonas diminuta* was the least abundant species (Figure 7.18).

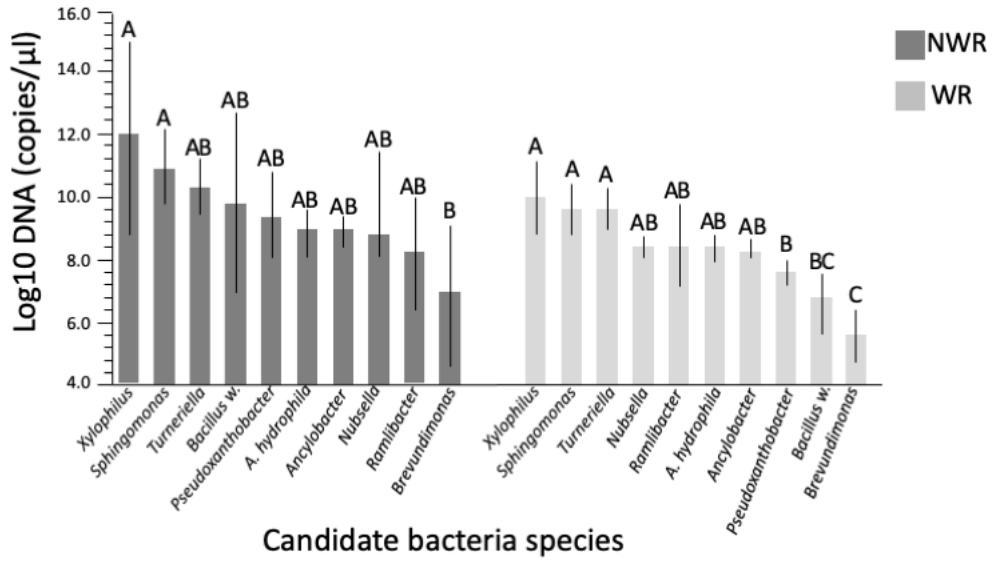

Figure 7.17: *Log*$_{10}$ of bacteria DNA (copies/µl) of candidate bacteria species isolated from *An. coluzzii* within treatment class 1 subgroups (NWR and WR). Dark grey bars represent the NWR (no-water-replacement) treatment level and light grey bars show WR (water-replacement) level within species. Whiskers represent 95% confidence intervals. Within treatment sub-groups (NWR and WR), bars with different letters are significantly different. Bars sharing any letter are not significantly different.

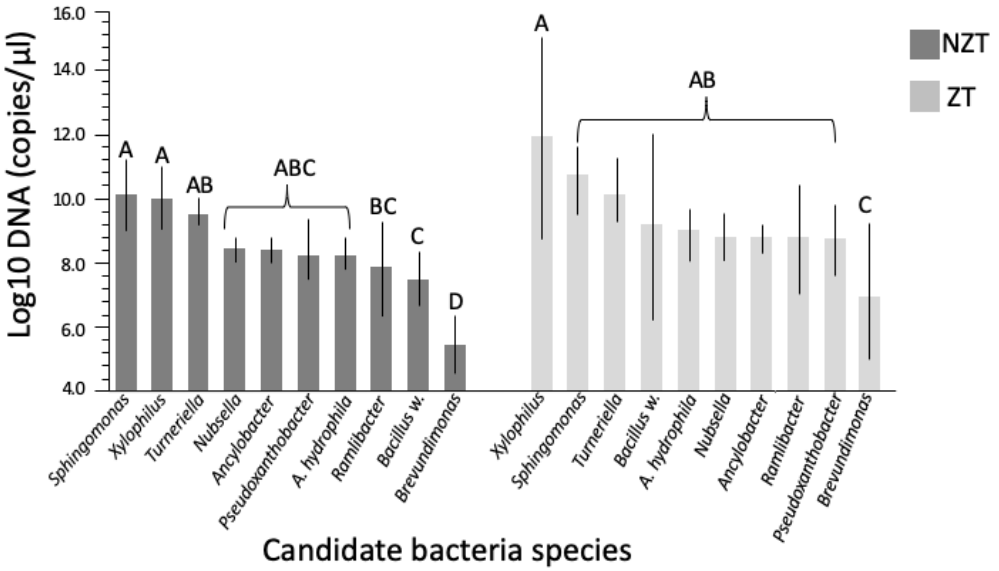

Figure 7.18: *Log*$_{10}$ of bacteria DNA (copies/µl) of candidate bacteria species isolated from *An. coluzzii* within treatment class 2 subgroups (NZT and ZT). Dark grey bars represent the NZT (none-zeolite-treatment) and light grey bars show ZT (zeolite-treatment) level within species. Whiskers represent 95% confidence intervals. Within treatment sub-groups (NZT and ZT), bars with different letters are significantly different. Bars sharing any letter are not significantly different.

7.4. Discussion

7.4.1. 16S rRNA gene amplicon sequencing of bacteria communities in *An. coluzzii* larval rearing trays by Illumina MiSeq system.

The most abundant bacteria phyla in the water samples from the *An. coluzzii* experimental trays were Proteobacteria (~60%), followed by Bacteriodetes (~25%), Firmicutes (~6%), Actinobactera (~4%), Deinococcus Thermus (~3%), Verrucomicrobia (~2%). These six phyla comprised of almost 100% of the bacteria phyla identified with - Armatimonadetes, Cyanobacteria, Planctomycetes and Gemmatimonadetes, occurring in less than 1% of the total relative bacteria abundance. Of the ten candidate species streamlined for further analysis and quantification by qPCR, seven fall under the Proteobacteria phylum and three of these are alphaproteobacteria. In a decreasing order of magnitude, families with highest OTU abundance among larval trays include Comamonadaceae, Cytophagaceae, Oxalobacteraceae, Aeromonadaceae, Sphingobacteriaceae, Clostridiaceae, Rhodocyclaceae, Micrococcaceae, Deinococcaceae, Chitinophagaceae. At species level, *Emticia* sp., *Pseudomonas* sp., *Pseudomonas putida*, and *Rathayibacter festuceae* were among the most abundant bacteria (OTU abundance) species within the larval trays. Several uncultured bacteria species were also represented. The range of bacteria species and phyla found in the experimental trays are similar to dominant bacteria species usually found in freshwater habitats (Zhou et al., 2009). The Micrococcacea family that is usually dominant in seawater was also isolated from the larval trays. A similar study that conducted microbiome analysis of *An. coluzzii* larval habitat reported similar phyla composition (Gimonneau et al., 2014).

The Simpson's index alpha diversity rarefaction results suggest that the number of species present in the samples were sufficient to resolve the microbial diversity within the experimental trays. The rarefaction curves showed maximum variability in bacterial

communities among samples from 25 -240 OTUs and this is consistent with the abiotic variation (nutrient content, ammonia, pH) among larval trays (Chapter 6). The full resolution of the curves is indicative of the robust depth of sampling and that the results obtained are reproducible and were sufficient to encapsulate all possible diversity (Gloor et al., 2010).

Species richness among treatment trays, evaluated by beta analysis showed that the microbiome composition of trays differed significantly. Qualitative (presence or absence of species) beta analysis based on phylogeny revealed differences in species composition between samples while quantitative beta analysis (using sequence abundance) not based on phylogeny, show an even spread of species composition within samples. The differences in species richness were significant between the subgroups in treatment class 1, those with water-replacement (WR - CWC & CWZ) had lower species richness compared to those without water-replacement (NWR- Control & Zeolite). Within these broad groups species richness did not differ significantly, thus indicating no significant difference between the subgroups in treatment class 2 (NZT- no zeolite treatment-CWC & Control and ZT – zeolite treatment- Zeolite & CWZ). The summary of the results from the experiment on the impact of zeolite treatment and water change (Chapter 6 of this thesis) showed that mosquitoes reared in the trays where water was continuously refreshed had significantly higher survival and adult quality compared to those reared in stagnant water. The results also reveal that zeolite treatment positively impacted mosquito survival and adult phenotypic quality at the lower rearing density.

The higher bacteria species richness and OTU abundance in NWR was expected, as nutrient-rich, stagnant aquatic pools support various bacteria species and abundance levels are higher in eutrophic/near eutrophic habitats compared to nutrient poor aquatic habitats (Gimonneau et al., 2014). In addition, the microbial content of the groups with

water change will have been diluted by the continuous water replacement, hence the reduced diversity and relative abundance. Mosquito survival and adult body size were maximised in this group (WR) partly due to the absence or minimal presence of toxic compounds such as ammonia (Hargreaves, 1998). Furthermore, the continuous water replacement replenished dissolved oxygen content in the WR group, and reduced the level of other pollutants such as ammonia, hence the higher mosquito productivity in these trays (Moniz, 2013). Organic and inorganic compounds (ammonia) similar to those described in the experiment in Chapter 6 of this thesis have been shown to favour the growth of the identified bacteria phyla (Liu et al., 2014). Furthermore, there is the possibility that the increased bacteria content in the NWR groups led to competition for available nutrients and dissolved oxygen in the larval trays and hence the death of mosquito larvae. Alternatively, it might have led to nutrient enrichment (bacteria serve as food for *Anopheles* larvae) which also results in the death of aquatic invertebrates (Mamai, et al., 2016).

Amongst the most abundant family identified, there were a range of beneficial bacteria including denitrifiers (Cytophagaceae), nitrate reducing and ammonium salt absorbing (Aeromonadaceae) (Appendix D). Other families have the potential to become pathogens when present in sufficient amounts, a few families are harmless (neutral) (Appendix D) (Zhou et al., 2009)

7.4.2. Quantification of candidate bacteria species by qPCR.

The overall direction of bacteria DNA abundance in the larval trays was higher in NWR compared to WR as predicted by the filter tables and Venn diagrams. Comparisons of DNA abundance of individual bacteria species between NWR and WR groups revealed that the qPCR validation step supported the OTU abundance data from 16S rRNA gene

sequencing. Further validating the 16S rRNA sequencing step, the qPCR quantification of candidate bacteria species revealed that larval trays with no-water-replacement (Control and Zeolite-NWR) contained significantly higher numbers of candidate bacteria species compared to trays with water-replacement (CWC, CWZ - WR). These results offer explanation for the lower mosquito survival and reduced adult quality reported from the NWR treatment class groups in the earlier chapter of this thesis (Chapter 6). Other laboratory-based study that focused on the reuse and recycling of water in a mosquito insectary for the purpose of mass rearing, show similar results with the dirty water groups having lower survival and adult quality (Mamai et al., 2017, 2016). Authors had speculated that their results could be a function of the reduction in the diversity of mosquito gut bacteria during development and hence, death as a result of reduced immunity. They also speculated that the lower mosquito survival and reduced adult quality, observed in the "dirty" trays could be as a result of the depletion of beneficial and nutritional microbes overtime through larval grazing, resulting in the dominance of less-nutritious or deleterious species (Mamai et al., 2017, 2016). They also argued that microbes might compete with mosquito larvae for nutritionally available nitrogen in a mosquito rearing tray and that any imbalance in the symbiotic relationship between microbiome and mosquito larvae might be toxic to mosquito larvae and subsequently negatively impact adult phenotype. The result from this study support these theories as it shows that microbial communities found in *An. coluzzii* rearing trays vary within larval treatment trays and impact on larval survival, development, adult emergence and the size of emerged adults.

Among the subgroups in treatment class 1 (NWR vs WR), significant differences in bacteria DNA abundance (copies/µl) were observed for *Bacillus weihenstephanensis* (Bacillaceae), *Pseudoxanthobacter soli*-DSM 19599 (Xanthobacteraceae),

Sphingomonas wittichii (Sphingomonadaceae) and *Ancylobacter* sp. FA202 (Xanthobacteraceae). Other bacteria species did not significantly differ in DNA abundance among this treatment subgroups.

Bacillus weihenstephanensis is a member of the *Bacillus cereus* group comprising of 6 species (*B. anthracis, B. cereus, C. mycoides, B. pseudomycoides, B. thuringiensis* and *B. weihenstephanensis*). Although, classical microbial taxonomy recognises them as distinct species, newer molecular phylogenies and comparative genome sequencing suggest they are a single species (Schmidt, et al., 2011). They are endospore forming organisms, with high resistance to heat, radiation, chemicals and drought, allowing them to survive adverse conditions for prolonged periods. Members of the *B. cereus* group are widely distributed in nature with habitats ranging from soil, sediment, air, freshwater, marine ecosystems and sludge. Some members of the group play key roles in nitrogen cycling such as denitrification, nitrogen fixation and mineralization (Mandic-Mulec, et al., 2015). The significantly higher abundance of *B. weihenstephanensis* in larval trays with no-water-replacement (NWR) is symptomatic of *B. weihenstephanensis'* natural ecology. Denitrification and mineralisation are majorly anaerobic processes, the presence of *B. weihenstephanensis* would indicate low levels of dissolved oxygen which resulted in increased larval mortality in NWR groups (Mandic-Mulec, et al., 2015).

Further, the relatively higher abundance of this denitrifying bacteria could have led to high nitrite concentrations in larval trays, a situation that has been demonstrated in various studies as a major cause of mortality in fish ponds (Amarasinghe & Weerakkodi, 2014; Tchigossou et al., 2018; Ward & Jensen, 2014). Nitrite (NO_2) toxicity in aquatic animals involves the conversion of the oxygen bearing pigment (haemoglobin/haemocyanin) into the inhibited form (methaemoglobin) which is incapable of oxygen transport (Amarasinghe & Weerakkodi, 2014; Tchigossou et al.,

2018). In fish, the nitrite turns the blood to brown colour (Brown blood disease) which results in gasping behaviour and subsequent suffocation despite adequate oxygen in the water (Amarasinghe & Weerakkodi, 2014). *Anopheles* larvae like fish embryos depend on cutaneous respiration and their surface area is limited; and although breathing tubes are present, they are rudimentary in the immature stages (Bardon-Albaret & Saillant, 2016). Many authors have demonstrated that the presence or absence, and the abundance of *Anopheles* larvae is positively associated with the concentration of DO in oviposition sites (Dejenie, et al., 2011; Oyewole et al., 2009).

There is also the possibility that *B. weihenstephanesis* might have served a beneficial role in reducing the ammonia and nitrite content in these lentic, nutrient rich habitats (larval trays). Likewise, of the three most abundant bacteria species in all larval trays (*Xylophilus* sp. PDD-37_7j_hv_b-3 (Comamonadaceae), *Sphingomonas wittichii* (Sphingomonadaceae), *Turneriella parva* DSM 21527), the last two were the suspect beneficial species identified by the filters and Venn diagrams implemented in this study. The relatively higher presence of these beneficial bacteria in the NWR subgroup may have resulted in improved mosquito survival compared to their absence (Chapter 6). Beneficial bacteria species have been used as probiotics in aquaculture systems to decompose organic matter to meet the growth requirements of cultured species, maintain the eco-equilibrium, inhibit the proliferation of harmful organisms and disintegrate harmful chemicals (such as unionised ammonia NH_3 and nitrites); whilst the pathogenic microbes cause diseases (Zhou et al., 2009). For example, photosynthetic bacteria and *Bacillus* spp. have been shown to improve the growth of white leg prawns with an increase in lipase and cellulase activity (Wang, 2007). Likewise, *Aeromonas hydrophila*, even though a proven fish pathogen (Hai, 2015), reduced the infection of *Aeromonas salmonicida* in rainbow trout when used as a probiotic (Irianto & Austin, 2002). To

maintain a healthy closed aquatic ecosystem, there has to be a balance of the microecology to ensure beneficial microbes are present in sufficient amounts to suppress the population and effect of pathogens. In the future, mosquito larval rearing trays could benefit from an inoculation of these denitrifying bacteria (as probiotics) to combat ammonia build-up in facilities where continuous water change is not feasible.

Pseudoxanthobacter soli-DSM 19599 (Xanthobacteraceae), is a gram-negative, aerobic nitrogen fixing bacteria, typically found in the soil. The higher abundance of this species in the NWR larval trays may have contributed to nutrient enrichment that usually result in larval death (Arun et al., 2008).

Sphingomonas wittichii is a known potential degrader of toxic dioxin pollutants that completely mineralizes the organic back-bone of the dibenzo-p-dioxin structure (Guo et al., 2010; Hong et al., 2002). These pollutants formed as a by-product of agricultural pesticides are of considerable environmental concern due to their persistence and toxicity, and they are ubiquitous in distribution (Nam et al., 2005). There is the possibility that they were introduced into the larval trays from the fish feed formulation used in the insectary or from other unknown sources. It is also possible that these compounds were not in the trays as they were not tested for. Whatever the case, *Sphingomonas wittichii'*s ability to degrade toxic compounds may have played a role in the degradation of other polluting chemical compounds in larval trays, resulting in improved mosquito development.

Interestingly, the results in this study show zeolite treatment increased the bacterial load in *An. coluzzii* larval trays. In the case where most where pathogenic, it may have impacted on the development of beneficial bacteria, hence circumventing the process of ammonia detoxification and reducing the beneficial impact of these species. Several studies have shown that biological nitrification process was greatly influenced

when zeolite was used to remove ammonia (Montalvo et al., 2014; Motesharezadeh et al., 2015; Yang, 1997). Zeolite was able to absorb ammonia more quickly than the nitrifying bacteria which was then unable to use NH_4^+ for their metabolic necessities (Montalvo et al., 2014). In a subsequent study, authors showed that zeolite effectively reduced ammonia and nitrite but negatively impacted the development of nitrifying bacteria in the biological filter of aquaculture systems (Skleničková et al., 2020). In the previous chapter of this thesis (Chapter 6), we demonstrated that zeolite was effective in reducing ammonia and improving mosquito yield where water change is not possible but not adult quality. This sub-optimal result may be because mosquitoes reared under these conditions were severely impacted by proliferation of pathogenic bacteria which thrive where the populations of beneficial species are reduced. Other ecosystem services rendered by beneficial bacteria (probiotics) such as the reduction in organic matter accumulation that usually results in hypoxia when allowed to accumulate, may have also reduced the overall impact of zeolite in improving water quality and thus mosquito yield and quality. There has to be a balance between zeolite application and the maintenance of healthy bacteria that are necessary for the growth and development of reared species, in this case *Anopheles* mosquitoes.

This study has been successful, firstly, in the characterisation of the bacterial communities present in *An. coluzzii* larval trays under standard insectary conditions. It has further provided information on the likely beneficial and detrimental species found under various rearing conditions. A more robust review of the 1031 species identified is needed to determine beneficial species that might serve as probiotics in the future or deployed to outcompete the detrimental bacteria species in facilities where water replacement is not feasible or unavailable. The information on *Anopheles* larval tray microbial communities can serve to improve mass rearing protocols as well as protocols

for small/medium insect cultures. This information on the bacterial communities present in *An. coluzzii* larval trays can also be applied in the vector control of this species as previous studies have suggested that mosquito immunity against malaria parasites is partly dependent on their microbiota which they utilise as antibacterial mechanism to limit *Plasmodium* infections (Dong et al., 2009). This knowledge can be manipulated for control and/or reduction of malaria transmission.

Secondly, the findings of this study corroborate other studies that have demonstrated that the bacteria community acquired at larval stage of *Anopheles* mosquitoes can impact adult survival and phenotypic quality (Coon et al., 2014; Gimonneau et al., 2014). Although microbiome analysis was not conducted for *An. coluzzii* in this study the impact of the presence and abundance of bacterial load in the larval trays impacted mosquito survival and adult phenotypic quality (Chapter 6). Microbial communities in *Anopheles* larval habitat are required for the growth and development of the mosquito until adult emergence whether in the field or laboratory (Coon et al., 2014). These microorganisms contribute to digestion, nutrition, reproduction and help maintain mosquito host immunity (Gimonneau et al., 2014). In that earlier study, the removal of the majority of *An. gambiae* s.l. midgut bacteria through antibiotic treatment resulted in increased susceptibility to *Plasmodium falciparum* (Dong et al., 2009). As a next step, future sampling should include larvae , pupae and adult midgut, for the microbiome analysis of *An. coluzzii* to validate the effect of the bacteria community in the larval trays on *An. coluzzii* development and adult phenotypic quality.

Further research is recommended to improve the understanding of the function/role of the characterised bacteria species within larval trays. The aim should be to maintain a balance of the bacterial communities in larval trays to ensure beneficial microbes are present in sufficient amounts to suppress the population and effect of

pathogens. These studies can involve the inoculation of pure cultures of bacteria species with known DNA concentration/copies into larval rearing trays before and after ammonia accumulation, to establish a direct link between bacteria species and mosquito survival and quality. Pure culture inoculation will further serve to mitigate the limitations associated with of the use of 16S rRNA gene sequencing for characterising bacterial communities in environmental samples. The 16S rRNA gene has poor discriminatory power at species level and even at the genus level for certain bacteria clades (Mignard & Flandrois, 2006; Větrovský & Baldrian, 2013; Winand et al., 2020). Systematic investigations have indicated that the use of 16S rRNA gene can reliably identify up to 90% (genus level) and 86% (species level) (Tremblay et al., 2015; Yang, et al., 2016). Further, especially in mixed or environmental samples, the copy number variation of the 16SrRNA gene presents complications for bacteria identification and quantification. There is the possibility that bacteria species with low copy numbers remain undetected and those with high copy numbers are overestimated (Bercovier et al., 1986; Rainey et al., 1996; Winand et al., 2020). Since the filters and Venn diagrams used to select the bacteria species for qPCR quantification were based on sequences generated by 16S rRNA sequences, we recommend the use of pure cultures to confirm the effect of these candidate bacteria on mosquito larvae development. The use of pure culture strains for the calibration of standard curves is also recommended as that will improve quantification by qPCR. The use of RNA for qPCR for microbial quantification is also recommended for future studies as the DNA template used for this study only indicates the presence or absence of the bacteria species and does not distinguish among viable and dead cells. Possible pathogens can then be isolated if found as viable within larval trays. (Kralik & Ricchi, 2017). Further, measurements of dissolved oxygen in larval trays as ammonia

content increases will improve the understanding of the impact of bacteria on mosquito development.

Finally, efficient water management in mosquito insectaries to minimise the presence of ammonia and nitrites as well as encourage the proliferation of beneficial microorganisms is recommended for achieving optimal rearing results both for small *An. coluzzii* cultures and for mass-rearing facilities.

Chapter 8

General discussion

As the malaria scourge continues to strike hardest against the most vulnerable people in our society, we must do more to protect children and pregnant women, against the world's deadliest mosquito borne disease that claims more that 400,000 lives every year (WHO, 2015; WHO, 2019). Innovative research is required to protect the gains of malaria control and elimination achieved in the last two decades via the use of long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) (WHO, 2015; WHO, 2019). In order to accomplish the bold vision of a malaria free world predicated on the ambition to reduce malaria burden by 90% in 2030 from the 2016 baseline, the WHO's Global Technical Strategy for Malaria 2016-2030 identifies innovative, basic and implementation vector control research as vital components (WHO, 2015). The emergence and continued spread of insecticide resistance are further complicated by additional biological challenges such as speciation in the *Anopheles gambiae* s.l. complex whose members are the major vectors of malaria in Africa (Benelli & Beier, 2017; Lanzaro & Lee, 2013). In many parts of Africa, current vector control tools cannot effectively protect against the disease, because of the diversity and distribution of these malaria vectors especially that of the sibling species *An. gambiae* s.s. and *An. coluzzii* (Hemingway et al., 2016a). Basic research is essential for a better understanding of the speciation process in the sibling species which impacts on their distribution, epidemiology and transmission potential (WHO, 2015). This understanding can both be beneficial for improving current vector control tools (LLINs, IRS, LSM) and for driving novel control tools (gene drive, SIT, Eave tube concept) (WHO, 2019-Vector control guidelines). In Chapters 4 and 5, we reveal interesting genotype × environment interactions of the sibling species to ammonia both as a single environmental stressor

(Chapter 4) and in a microcosm-setting (Chapter 5). *An. coluzzii* showed more tolerance for ammonia and thrived better in its preferred larval habitat, and the opposite was true for *An. gambiae* s.s. These results provide an explanation for the adaptive behaviour underlying the divergent larval preferences of the sibling species which has in part driven eco- speciation between these species (Dao et al., 2014; Diabaté et al., 2008; Gimonneau et al., 2012; Kamdem et al., 2012; Roux et al., 2014). Furthermore, the result aids the understanding of their distribution in West Africa around rice field domestication hot spots (Dolo et al., 2004; Epopa et al., 2017; Faye et al., 1995; Marrama et al., 2004; Mwangangi et al., 2010; Sawadogo et al., 2017). Understanding speciation both improves our knowledge of the biodiversity in our environment which is positive when considering environmental conservation but in the light of malaria epidemiology, speciation confounds an already complex, evolving disease and makes its control more challenging (Aboagye-Antwi et al., 2015; WHO, 2015). This study therefore provides valuable information on these malaria vectors that can be further manipulated for their control such as in the identification of larval habitats for larval source management (LSM), entomological surveillance, and for entomological mapping for mass release programmes.

In recent history, malaria endemic countries in sub-Saharan Africa are increasing efforts towards food security and economic independence through increased investment in irrigated agriculture particularly rice field (Sikirou et al., 2015). From an annual average growth rate of 1.76% between 1991 to 2001, rice production in Africa has doubled to 3.96%, between 2002 – 2013 (Sikirou et al., 2015). Although these efforts are commendable, these policies must be balanced with the knowledge of the impact of such developments on the disease cycle of malaria especially in sub-Saharan Africa which bears 93% of the global malaria burden (WHO, 2019). The availability of permanent

larval habitat for these disease vectors changes their epidemiology from seasonal rainfall peaks to all-year-disease-transmission (Tene Fossog et al., 2015). To further escalate the situation, there are several reports of increasing urban malaria as a result of *An. coluzzii*'s preference for nutrient rich permanent water bodies (Tene Fossog et al., 2013, 2015). The unplanned nature of many sub-Saharan African urban centres makes for the provision of multiple larval habitats for these disease vectors, such as stagnant water bodies, that are used as municipal waste dumping sites, inefficient drainage systems. These newly created habitats further expands the niche of this malaria vector and it is leading to a rise in urban malaria transmission (Ijumba & Lindsay, 2001; Ijumba et al., 2002; Tene Fossog et al., 2013).

The microcosm-model developed in this study (Chapter 5) provides an excellent study tool for further ecological studies on the sibling species such as oviposition site preference, the inheritance of plastic responses, longevity, male competitiveness, female fecundity of the sibling species, among other traits. It can also serve as a model mini-ecosystem for gene mapping and silencing studies to investigate if there is a link between ammonia tolerance and the 'island of speciation' within the genomes of these sibling species.

Sterile insect technique (SIT) and other innovative GMM-based (genetically modified mosquitoes) programmes rely on the economic and efficient mass production of mosquitoes for release in the field to suppress or replace wild populations (Bourtzis, et al., 2016; Burt, 2014; Lees, et al., 2015). Effective water quality management in the insectary is essential for the production of adult mosquitoes of desirable standard and quality that are able to compete favourably with wild populations (HDV, 2017; Mamai et al., 2017, 2016). In Chapter 3 of this thesis, we show that the use of mineral water in *Anopheles* larval trays improved overall mosquito yield and phenotypic quality of adult

mosquitoes (larger body size) in comparison to the commonly used deionised water. Body size in both male and female *Anopheles* mosquitoes is important for competitiveness, fecundity, successful mating, longevity and increased vectorial capacity (Aboagye-Antwi & Tripet, 2010; Diabate & Tripet, 2015; Takken et al., 2013). For the effective delivery of mass release vector control programmes, mosquito adults of standard size and phenotypic quality is essential (HDV, 2017; Mamai et al., 2017). In Chapter 4, the importance of mineral water in improving mosquito rearing outcomes was further validated, as its use served to buffer ammonia toxicity in larval habitats. These results obtained in Chapters 3 and 4 can be used to improve rearing protocols for mass release programmes.

Although widely applied in fish and crustacean aquaculture this is the first time ammonia absorbing zeolite has been evaluated for application in mosquito rearing for malaria control. In Chapter 6 of this thesis we demonstrated that use of zeolite can improve water quality in *Anopheles gambiae* s.l. larval rearing trays subsequently resulting in optimal mosquito yield of adult mosquitoes with desirable phenotypic quality (large adult size). This result makes for improved mosquito rearing protocols and provides a cheaper, eco-friendly approach to rearing millions of mosquitoes for mass release programmes. Considering that large amounts of water is required for mass rearing (approximately 100,000L for 10,000,000 sterile males per week), and the water conservation issues associated with arid regions were some of these mass release programmes will be conducted, the use of zeolite to treat mosquito rearing water for reuse becomes an excellent tool for water management in SIT and GMM facilities (Mamai et al., 2017). A Hungarian based private investment project had demonstrated that the use of zeolite in a water-purification system is 40% cheaper to install and saves 20% in

operational costs in comparison with the current system that employs reverse osmosis and ultra-filtration systems (StradiSound Strategy, 2020).

We also elucidated in this thesis how the nitrogen conversion dynamics in a mosquito larval tray impacts on mosquito development and phenotypic quality. We demonstrated in Chapter 6, how the efficient management of ammonia build-up in mosquito larval trays using zeolite and the optimal feed type results in improved rearing results. To further understand the factors inhibiting larval growth and development in *Anopheles* larval trays, we characterised the bacterial communities in the trays to identify bacteria species that are present in the trays. This is the first study on the identification of bacteria communities in *Anopheles* larval trays in a mosquito insectary using 16S rRNA gene sequencing. Further analyses employing a system of filters and Venn diagrams resulted in the identification of 10 candidate bacteria species that could be important either as beneficial microorganisms in larval trays or as pathogens. We moved a step further to quantify the bacteria DNA of these candidate bacteria species using qPCR, and the results both validates the 16S step and opens up possibilities of answering other questions surrounding the microbial dynamics in the *Anopheles gambiae* s.l. larval tray. The characteristics of candidate bacteria species that were significantly higher in all experimental trays indicates that they could serve as probiotics to improve water quality in the trays by decomposing organic matter, converting toxic ammonia to nitrate, boosting the immunity of mosquitoes, outcompeting pathogens and also serve as larval food (Gimonneau et al., 2014).

Interestingly, we identified a gap in the use of zeolite for ammonia absorption in these larval trays. Zeolite could absorb ammonia faster than the bacteria can convert it to nitrate, making it unavailable for the bacteria species to utilise, subsequently leading to a decline in beneficial bacteria (Montalvo et al., 2014; Motesharezadeh et al., 2015; Yang,

1997). This finding will serve to inform the efficient balancing of both resources (zeolites and denitrifying bacteria) for overall improvement of mosquito yield. We were limited in the interpretation of our results as the study was mainly correlational. We did not manipulate the bacteria content of the trays to determine direct impact. For further study on these bacteria species, we recommend the inoculation of pure culture to ascertain the direct effects of individual bacteria species on mosquito development. The use of DNA for qPCR quantification also limited our ability to infer on the pathogenicity of the bacteria species as the use of DNA only shows presence or absence and not viability. The use of RNA for future qPCR quantification is recommended.

Future direction

This study has provided insight into the divergent larval preferences of *An. coluzzii* and *An. gambiae* s.l. under insectary conditions. The phenotypic plastic responses elucidated in this study reveal a set of reaction norms developed by *An. coluzzii* in response to anthropogenic modifications (ammonia and minerals in this case) of their ecosystem via rice domestication. It has thus opened up discussions and research on other factors that surround this evolutionary event that is driving the speciation of the sibling species. We only investigated tolerance to mineral water and ammonia, other aspects/factors characterising the new niche created by rice cultivation can be investigated to improve our understanding of this evolutionary process. The microcosm-model simulating divergent larval habitat preferences of the sibling species can be used for different types of ecological and translational research surrounding the speciation or control of these malaria vectors.

Although, we show that zeolite is effective for improving water quality in *An. gambiae* s.l. insectary, further research on its application, delivery and dosage is required

to maximise the biological and economic potential of its use. Studies involving direct bacteria inoculation to determine the direct impact of the candidate bacteria species characterised in this study is required to further benefit from the findings in chapter 7 of this study. The use of RNA for bacteria quantification is recommended to enable the identification of viable species that may be pathogenic or beneficial. Further, the physicochemical chemical parameters essential for mosquito development such as dissolved oxygen content, pH, temperature, ammonia, nitrate will need to assessed extensively in conjunction with pure culture inoculation to provide a wholistic understanding of the impact of the microbial communities present in mosquito larval trays.

In conclusion, malaria is a treatable and preventable disease and should not be allowed to continue claiming the lives of vulnerable people in impoverished societies. Innovation in vector control research and development is key to protect the gains of the past two decades and continue the progress towards elimination. The challenge is enormous, but the vision of a malaria free world is achievable through the concerted efforts of all stakeholders.

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Appendices

Appendix A: Akpodiete *et al.*, Parasites & Vectors, (2019)12:210

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Parasites & Vectors

RESEARCH Open Access

Effect of water source and feed regime on development and phenotypic quality in *Anopheles gambiae* (*s.l.*): prospects for improved mass-rearing techniques towards release programmes

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Abstract

Background: In many malaria-endemic sub-Saharan countries, insecticide resistance poses a threat to existing mosquito control measures, underscoring the need for complementary control methods such as sterile and/or genetically-modified mosquito release programmes. The sibling species *Anopheles gambiae* and *An. coluzzii* are responsible for malaria transmission in most of this region. In their natural habitat, these species generally breed in clean, soft water and it is believed that divergent preference in their larval breeding sites have played a role in their speciation process. Mosquito release programmes rely on the rearing of mosquitoes at high larval densities. Current rearing protocols often make use of deionised water regardless of the strain reared. They also depend on a delicate balance between the need for adequate feeding and the negative effect of toxic ammonia and food waste build-up on mosquito development, making managing and improving water quality in the insectary imperative.

Methods: Here, we investigated the impact of water source and feed regimes on emergence rate and phenotypic quality of mosquitoes in the insectary. First-instar larvae of *An. gambiae* (Kisumu strain) and *An. coluzzii* (Mopti and VK3 strains) were reared in three water sources with varying degrees of hardness (deionised, mineral and a mix of the two), with a daily water change. Larvae were fed daily using two standardised feeding regimes, solution and powder feed.

Results: Water source had a significant impact on mosquito size and development time for all strains. Earlier emergence of significantly larger mosquitoes was observed in mineral water with the smallest mosquitoes developing later from deionised water. Wing-length was significantly longer in mineral, mixed water and in powder feed, irrespective of sex, strains or water types. Deionised water was the least favourable for mosquito quality across all strains.

Conclusions: Mineral water and powder feed should be used in rearing protocols to improve mosquito quality where the optimal quality of mosquitoes is desired. Although results obtained were not significant for improved mosquito numbers, the phenotypic quality of mosquitoes reared was significantly improved in mineral water and mix water. Further studies are recommended on the impact mineral water has on other fitness traits such as longevity, fecundity and mating competitiveness.

Keywords: Water hardness, Feed, *Anopheles coluzzii*, *Anopheles gambiae*, Mass rearing, Insectary, Kisumu, Mopti, VK3

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Appendix B: (Chapter 6) - Mean nitrates and ammonia values in larval trays

Treatment	Larval density	Feed	Day	Nitrate (NO_3^-) (mg/L)	Ammonia (NH_3) (mg/L)
Control	200	Solution	4	18.75 (-5.88 – 43.38)	1.98 (-0.57 – 4.52)
			6	2.50 (-2.09 – 7.09)	4.25 (2.65 – 5.85)
			8	0	13.85 (10.92 – 16.78)
			10	0	12.57 (2.46 – 22.67) *3*
		Powder	4	13.75 (-14.84 – 42.34)	1.13 (-0.68 – 2.93)
			6	27.5 (3.63 – 51.37)	4.25 (1.84 – 6.66)
			8	7.50 (-6.28 – 21.28)	12.43 (6.26 – 18.59)
			10	0	12.27 (7.79 – 16.75)
	400	Solution	4	26.25 (-0.83 – 53.34)	0.73 (-0.13 – 1.58)
			6	7.50 (-0.46 – 15.46)	5.23 (0.27 – 10.18)
			8	1.25 (-2.73 – 5.23)	12.70 (12.01 – 13.39)
			10	0	15.38 (4.82 – 25.93)
		Powder	4	16.25 (-9.22 – 41.72)	1.08 (0.28 – 1.87)
			6	36.25 (-15.46 – 87.96)	6.20 (0.64 – 11.76)
			8	35.00 (-19.35 – 89.35)	13.91 (11.37 – 16.44)
			10	0	15.43 (4.66 – 26.19)
CWC	200	Solution	4	10.00 (-1.25 – 21.25)	1.38 (-0.69 – 3.44)
			6	8.75 (4.77 – 12.73)	2.13 (0.87 – 3.38)
			8	10.00 (-2.99 – 22.99)	4.4 (3.49 – 5.30)
			10	0	-
		Powder	4	10.00 (-1.25 – 21.25)	1.45 (-0.08 – 2.98)
			6	26.25 (-0.83 – 53.33)	2.45 (-0.65 – 5.55)
			8	40.00 (-5.01 – 85.01)	3.65 (0.36 – 6.94)
			10	0	0.30 (-3.51 – 4.11) *2*
	400	Solution	4	10.00 (-1.25 – 21.25)	1.45 (0.11 – 2.79)
			6	8.75 (4.77 – 12.73)	2.65 (1.60 – 3.70)
			8	32.50 (-18.44 – 83.44)	3.13 (1.00 – 5.25)
			10	2.50 (-2.09 – 7.09)	1.10 (0.12 – 2.08)
		Powder	4	10.00 (-1.25 – 21.25)	1.43 (0.08 – 2.77)
			6	45.00 (4.96 – 85.04)	2.58 (1.20 – 3.95)
			8	42.50 (-3.20 – 88.20)	3.35 (0.54 – 6.16)
			10	3.75 (-3.87 – 11.37)	1.45 (0.73 – 2.17)
CWZ	200	Solution	4	18.75 (-5.88 – 43.38)	1.23 (-0.54 – 2.99)
			6	8.75 (4.77 – 12.73)	1.1 (-0.88 – 3.08)
			8	12.50 (4.54 – 20.46)	3.25 (2.08 – 4.42)
			10	0	0.1 (-0.33 – 0.53) *3*
		Powder	4	16.25 (-9.22 – 41.72)	1.03 (-0.30 – 2.35)
			6	35.00 (19.09 – 50.91)	1.38 (-0.22 – 2.97)
			8	60.00 (23.25 – 96.75)	3.40 (0.94 – 5.86)
			10	5.00 (-7.42 – 17.42)	0.23 (-0.77 – 1.24) *3*
	400	Solution	4	17.50 (-8.89 – 43.89)	0.45 (-0.58 – 1.48)
			6	20.00 (-2.50 – 42.50)	0.75 (-0.16 – 1.66)
			8	16.25 (-9.22 – 41.72)	3.68 (2.55 – 4.80)
			10	1.25 (-2.73 – 5.23)	0.40 (-0.37 – 1.17)

Treatment	Larval density	Feed	Day	Nitrate (NO_3^-) (mg/L)	Ammonia (NH_3) (mg/L)
		Powder	4	26.25 (-0.83 – 53.33)	1.05 (0.31 – 1.79)
			6	45.00 (4.96 – 85.04)	1.28 (0.48 – 2.07)
			8	60.00 (23.25 – 96.75)	3.45 (1.27 – 5.63)
			10	25.00 (-35.25 – 85.24)	0.57 (-1.26 – 2.40) *3*
Zeolite	200	Solution	4	18.75 (-5.88 – 43.38)	1.6 (-0.31 – 3.51)
			6	18.75 (-5.88 – 43.38)	2.25 (0.47 – 4.03)
			8	2.50 (-5.46 – 10.46)	8.53 (5.73 – 11.32)
			10	0	6.73 (0.42 – 13.05) *3*
		Powder	4	11.25 (1.24 – 21.26)	1.03 (-0.24 – 2.29)
			6	35.00 (19.09 – 50.91)	2.28 (0.05 – 4.50)
			8	15.00 (-11.78 – 41.78)	7.80 (5.94 – 9.66)
			10	0	8.03 (6.34 – 9.72) *3*
	400	Solution	4	26.25 (-0.83 – 53.33)	1.28 (-0.47 – 3.02)
			6	6.25 (-1.37 – 13.87)	2.28 (-1.66 – 6.21)
			8	0	8.13 (5.46 – 10.79)
			10	0	8.55 (-2.54 – 19.64)
		Powder	4	23.75 (-6.28 – 53.78)	1.13 (-0.05 – 2.30)
			6	51.25 (-6.26 – 108.76)	2.60 (-0.34 – 5.54)
			8	25.00 (-35.24 – 85.24)	8.05 (6.16 – 9.94)
			10	0	10.45 (0.94 – 19.96)

Notes: Sample size is 4 except in cases where pupation is completed in trays, then italicised. Ninety-five percent confidence intervals are in brackets.

Appendix C: (Chapter 7)- PCR reverse primer list for sequence library preparation

S/N	Sample description	Reverse primers
1	200/Control/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATTACGAGCCCTAAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
2	200/Control/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATAGTCGTGCACATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
3	200/Control/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATCAGCTCATCAGCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
4	200/Control/D10/Solution/R3	5'-CAAGCAGAAGACGGCATACGAGATACCGGTATGTACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
5	400/Control/D8/Powder/R3	5'-CAAGCAGAAGACGGCATACGAGATAATTGTGTCGGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
6	400/Control/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATATCCTTTGGTTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
7	400/Control/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATTAGGAACTGGCCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
8	400/Control/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATAATCAGTCTCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
9	200/Zeolite/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATACGCGCAGATACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
10	200/Zeolite/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATCAGCGGTGACATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
11	200/Zeolite/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATCAGGCGTATTGGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
12	200/Zeolite/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGTATGCGCTGTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
13	400/Zeolite/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATAGGCTACACGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
14	400/Zeolite/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATACGCCACGAATGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
15	400/Zeolite/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATCACGCCATAATGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')

S/N	Sample description	Reverse primers
16	400/Zeolite/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATCTATTTGCGACAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
17	200/CWZ/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATAAGAGATGTCGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
18	200/CWZ/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGACTTTCCCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
19	200/CWZ/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATCCTGAACTAGTTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
20	200/CWZ/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATCGGAGCTATGGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
21*	400/CWZ/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATTACTACGTGGCCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
22*	400/CWZ/D8/Solution/R3	(5'- CAAGCAGAAGACGGCATACGAGATCTCACAACCGTGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
23*	400/CWZ/D10/Powder/R3	(5'- CAAGCAGAAGACGGCATACGAGATCAACTCCCGTGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
24	400/CWZ/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGCACGACAACACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
25	200/CWC/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATGGATCGCAGATCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
26	200/CWC/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGTCAATTGACCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
27	200/CWC/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATGTTGGTCAATCTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
28	200/CWC/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATTCGAGGACTGCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
29	400/CWC/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATATCCCGAATTTGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
30	400/CWC/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGAGGCTCATCATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
31	400/CWC/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATATCGGCGTTACA

S/N	Sample description	Reverse primers
		AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
32	400/CWC/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATAATGCCTCAACT AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
33	200/Control/D8/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATAGCTGTTGTTTG AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
34	200/Control/D8/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATAGCTGGAAGTCC AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
35	200/Control/D10/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATCGATCCGTATTA AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
36	200/Control/D10/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATGAATAGAGCCAA AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
37	400/Control/D8/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATCTAGCGAACATC AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
38	400/Control/D8/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATGGAAACCACCAC AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
39	400/Control/D10/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATGCACACACGTTA AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
40	400/Control/D10/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATCAAGCATGCCTA AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
41	200/Zeolite/D8/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATGTCTAATTCCGA AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
42	200/Zeolite/D8/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATTAGGATTGCTCG AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
43	200/Zeolite/D10/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATCAGTGCATATGC AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
44	200/Zeolite/D10/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATACGGGACATGCT AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
45	400/Zeolite/D8/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATTCTAGCGTAGTG AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
46	400/Zeolite/D8/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATTAGCTCGTAACT AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')

S/N	Sample description	Reverse primers
47	400/Zeolite/D10/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATTCCTCTGTCGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
48	400/Zeolite/D10/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATCTGCTAACGCAAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
49	200/CWZ/D8/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATGAAGAAGCGGTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
50	200/CWZ/D8/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATTTACTGTGCGATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
51	200/CWZ/D10/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATTACAGATGGCTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
52	200/CWZ/D10/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATGAGTGGTAGAGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
53	400/CWZ/D8/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATTGAGTCACTGGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
54	400/CWZ/D8/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATACACCTGGTGATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
55	400/CWZ/D10/Powder/R4	(5'-CAAGCAGAAGACGGCATACGAGATAGGCATCTTACGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
56	400/CWZ/D10/Solution/R4	(5'-CAAGCAGAAGACGGCATACGAGATCCAGTGTATGCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
57	200/CWC/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATCGAGAAGAGAACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
58	200/CWC/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGAATCTTCGAGCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
59	200/CWC/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATTCCAAAGTGTTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
60	200/CWC/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGAACTAGTCACCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
61	400/CWC/D8/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATAAGGAGCGCCTTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
62	400/CWC/D8/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATTCCGAATTCACA

S/N	Sample description	Reverse primers
		AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
63	400/CWC/D10/Powder/R3	(5'-CAAGCAGAAGACGGCATACGAGATACATTCAGCGCA AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')
64*	400/CWC/D10/Solution/R3	(5'-CAAGCAGAAGACGGCATACGAGATGCGATATATCGC AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3')

Notes: * Samples with low DNA concentration and did not progress to subsequent analysis.

Appendix D: (Chapter 7) Filter 1-Biological characteristics of bacteria families by OTU abundance

S/N	Bacteria family	Criteria	OTU abundance in Treatment groups			
			Control	Zeolite	CWC	CWZ
1	Cytophagaceae (Cytophagales)	Found in organically rich material, also adapt to low nutrient, gram negative, may be aerobic, microaerophilic, capnophilic (CO2 requiring) or facultatively anaerobic are organotrophs, able to degrade biomacromolecules like proteins, chitin, pectin, agar, starch (McBride, et al., 2014).	198,501	136,604	97,857	65,058
2	Comamonadaceae (Burkholderiales)	Include plant and human pathogen. Order is phenotypically, metabolically & ecologically diverse. Includes strictly aerobic & facultatively anaerobic chemoorganotrophs. Include obligate & facultative chemolithotrophs, nitrogen fixing organisms (Garrity, et al., 2005).	165,623	287,350	186,086	198,021
3	Oxalobacteriaceae (Burkholderiales)	Mostly aerobic, microaerobic to facultatively anaerobic. Found in diverse environmental habitats like water, soil, plant associated. Some species are mild plant pathogens, some are opportunistic human pathogens. Gram-negative. Mesophilic, with some psychrophilic (Baldani et al., 2014)	85,999	61,010	114,740	163,769
4	Burkholderiaceae *Pseudomonas* sp.	Several species are pathogenic for humans, other warm blooded animals, fish, eels as well as other vertebrates & invertebrates such as leeches (Garrity, et al., 2005)	56,863	18,120	14,349	3,110
5	Sphingobacteriaceae	Most species occur in freshwater, soil & compost, but some occur in clinical specimens. Gram-negative. No flagella but may exhibit sliding mobility. Chemoorganotrophs without specialized growth factor requirements (Yabuuchi et al., 1983).	49,641	27,098	23,997	4,881
6	Aeromonadaceae (Aeromonadales) *Pseudomonas putida*	Gram-negative, straight, rigid, facultatively anaerobic & chemoorganotrophic fermentative or respiratory with oxygen as a universal electron acceptor reduce nitrates but do not denitrify. Ammonium salts utilized by most isolates as a sole source of nitrogen primarily aquatic, mostly isolated from fresh & estuarine waters & in association with aquatic animals also found in sewage, surface waters, sediments & biofilms (Garrity, et al., 2005)	42,369	33455	42,540	69,753
7	Clostridiaceae (Clostridiales)	Gram-positive. Pathogenic produces toxins in water (in eutrophic conditions) that are deadly to birds and mammals includes several human pathogens (causative agent of botulism & an important cause of	37,412	64,290	18,651	16,142

S/N	Bacteria family	Criteria	OTU abundance in Treatment groups			
			Control	Zeolite	CWC	CWZ
		diarrhoea) obligate anaerobes has an associated foul smell due to butyric acid & in some cases hydrogen sulphide (Atarashi et al., 2011)				
8	Chitinophagaceae	Isolated from water samples, gram-negative, strictly aerobic, non-sporulating, grows optimally at 30°C & pH 7. Family of 7 genera -Balneola, Filimonas, Flavisolibacter, Gracilimonas, Lacibacter, Niastella, Terrimonas & Chitinophaga (Lim et al., 2009)	31,423	41,635	20,907	21,080
9	Rhodospirallaceae	Phototrophic. Fixes molecular nitrogen. Hydrogen metabolism (Shoreit et al., 1992)	26,496	3,729	2,159	2,802
10	Rhizobiaceae	Includes bacteria capable of fixing nitrogen when in symbiosis with leguminous plants. Obligate & facultative intracellular bacteria, include animal & plant pathogens (Breedveld & Miller, 1994)	24,394	16,990	21,160	21,525
11	Rhodocyclaceae (Rhodocyclales)	Members display different modes of living: anoxygenic photoheterotrophs, plant associated nitrogen fixing, aerobes, species that degrade a wide range of carbon sources, anaerobes that perform propionic acid fermentation. Isolated from diverse environments: soil, sewage treatment plants, polluted and unpolluted pond water, rivers, aquifers and plant roots (Garrity, et al., 2005)	22,482	25,710	47,885	27,188
12	Micrococcaceea (Micrococcales)	Gram-positive. Soil-borne bacterium Plant-pathogenic, causes gumming disease characterised by yellow bacterial slime on seed-heads, stems & leaves of the plant host (Schumann et al., 2009)	21,950	78,335	13,521	6,901
13	Verrucomicrobiaceae	Isolated from freshwater & marine water ecosystems & from animal feaces. Gram-negative. Chemoorganotrophic. Includes obligate aerobes, facultative anaerobes & strict anaerobes (Rivas-Marín et al., 2016; Wertz et al., 2012)	18,115	29,924	4,163	4,978
14	Hydrogenophilaceae (Hydrogenophilales)	Gram negative. Non-sporulating. Chemolithotrophic. Either mesophilic or thermophilic. No report of any pathogens. Isolated from various environments – freshwater, sludge treatments and hot springs (Garrity et al., 2015)	15,575	21,837	0	35,275
15	Flavobacteriales	Gram- negative. Flavobacteriaceae & Cryomorphaceae consist of strictly aerobic & facultatively anaerobic chemo-organotrophs with respiratory metabolism. Non-motile or motile by gliding, with yellow or orange colonies due to	15,351	18,818	26,589	4,491

S/N	Bacteria family	Criteria	OTU abundance in Treatment groups			
			Control	Zeolite	CWC	CWZ
		production of carotenoid &/or flexirubin type pigments. Some members of Flavobacteriaceae are pathogenic for humans, fish or amphibians. Include chemo-organotrophs (Bernardet & Bowman, 2006)				
16	Deinococcaceae (Deinococcales)	Gram-positive, most radiation resistant vegetative cell, chemo-organotrophic. Aerobic, highly resistant to environmental hazards. Non-pathogenic (Rosenberg, 2014)	13,370	58,811	35,842	8,177
17	Sphingobacteriaceae	cells are rod-shaped & non-motile, aerobic or facultatively anaerobic, limited fermentative abilities are observed in some members, free living & saprophytic, some species are opportunistic pathogens. Gram-negative (Kampfer, 2011).	10,312	11,910	109,086	14,092

Appendix E : (Chapter 7) Filter 2Ai-OTU abundance vs emergence @ Spearman's correlation -30.

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	FR667304.1.1357	*Roseomonas* (UB-Acetobacteraceae)	207	-0.6636	<.0001****
2	EU773989.1.1373	Clostridiaceae1 (UB)*	15	-0.5918	0.0007***
3	KC432217.1.1330	*Clostridium* sensu stricto 3 (UB-Clostridiaceae1)*	7,024	-0.5909	0.0007***
4	HM778794.1.1387	Gammaproteobacteria-aaa34a10-UB	448	-0.5905	0.0007***
5	HM778860.1.1375	*Aeromonas* (uncultured Aeromonadaceae)*	80	-0.5813	0.0009***
6	DQ166946.1.1431	*Aeromonas hydrophila** (Aeromonadaceae)*	112	-0.5786	0.0010**
7	AB487832.1.1332	*Clostridium* sensu stricto 10*(Clostridiaceae1)	19	-0.5762	0.0011**
8	HM779015.1.1417	*Shewanella* (UB- Shewanellaceae)	228	-0.5507	0.0020**
9	JF808900.1.1502	*Tolumonas* sp. (UB- Aeromonadaceae)*	7,676	-0.5408	0.0025**
10	FJ205850.1.1269	*Clostridium* sensu stricto 1* (Clostridiaceae1)	27	-0.5089	0.0048**
11	JF775632.1.1472*	*Clostridium* sensu stricto 9* (UB-Clostridiaceae1)	308	-0.5069	0.0050**
12	EU801494.1.1452*	Polynucleobacter (UB-Burkholderiaceae)*	126	-0.5040	0.0053**
13	KF835793.1.1402	*Aeromonas hydrophila**	152	-0.5037	0.0053**
14	FM213077.1.1512*	*Tahibacter* (UB- Xanthomonadaceae)*	51	-0.5022	0.0055**
15	GU356337.1.1344	*Aeromonas* sp. (UB-JI49D030)	76	-0.4951	0.0063**
16	AB845279.1.1381	*Enterobacter* sp. Bdr5 (Enterobacteriaceae)*	544	-0.4917	0.0067**
17	EF111197.1.1232*	*Aeromonas* (uncultured Aeromonadaceae)*	1,513	-0.4796	0.0085**
18	EU434572.1.1389	*Brevundimonas diminuta* (Caulobacteraceae)	60	-0.4739	0.0094**
19	JN379402.1.1475	*Clostridium* sensu stricto 12 (UB-Clostridiaceae1)*	23	-0.4688	0.0103*
20	AB486966.1.1314	*Clostridium* sensu stricto 8* (Clostridiaceae1)	218	-0.4681	0.0104*
21	KF381405.1.1505	*Enterobacter* (Enterobacteriaceae)*	172	-0.4613	0.0118*
22	CU922145.1.1372	*Hydrogenophaga* (UB)* (Comamonadaceae)*	32	-0.4594	0.0122*
23	CU923555.1.1349	*Hydrogenophaga* (UB)*(Comamonadaceae)*	39	-0.4585	0.0124*
24	HM069053.1.1447	Rhizobiales- MNG7-UB	5,036	-0.4518	0.0139*
25	DQ814980.1.1413	*Aeromonas* (UB)* (Aeromonadaceae)*	148	-0.4501	0.0143*
26	FJ494907.1.1294	*Aeromonas* sp. 08006 (Aeromonadaceae)*	92	-0.4494	0.0145*
27	AB114256.1.1414	*Clostridium* sp. Uslt101-1* (Clostridiaceae1)	26,792	-0.4443	0.0158*
28	FJ896014.1.1453	*Aeromonas* sp. kumar (Aeromonadaceae)*	33	-0.4414	0.0165*
29	EF205513.1.1470	*Aeromonas* (uncultured Aeromonadaceae)*	110	-0.4402	0.0169*
30	FR853451.1.1447	*Phenylobacterium* (UB-Caulobacteraceae)*	28	-0.4368	0.0178*
31	EU800506.1.1501	Comamonadaceae (UB)*	103	-0.4352	0.0183*
32	HQ178724.1.1455	*Simplicispira* (uncultured Comamonadaceae)*	34	-0.4342	0.0186*
33	JF703410.1.1375	*Sphingomonas* (UB-Sphingomonadaceae)*	11	-0.4332	0.0189*
34	EF205504.1.1436	*Clostridium* sensu stricto 8* (UB-Clostridiaceae)	203	-0.4318	0.0193*
35	FM213035.1.1498	*Thiobacillus* (Hydrogenophilaceae)	131	-0.4289	0.0203*
36	JN863503.1.1293	*Leucobacter* sp. LC390 (Microbacteriaceae)*	10	-0.4230	0.0222*
37	JX223620.1.1368	*Clostridium* sensu stricto 12 (UB-Clostridiaceae1)*	816	-0.4166	0.0246*
38	EF010981.1.1437	*Pseudomonas putida* (Aeromonadaceae)*	157,079	-0.4135	0.0258*
39	HM277678.1.1359	*Lampropedia* (UB-Comamonadaceae)*	34	-0.4131	0.0259*
40	JN391577.1.1483	*Clostridium* sensu stricto 1 (UB-Clostridiaceae1)*	29	-0.4061	0.0288*
41	HM779400.1.1470	*Aeromonas* (uncultured Aeromonadaceae)*	493	-0.4015	0.0309*
42	JX120471.1.1504	*Acidovorax* (UB- Comamonadaceae)*	13	-0.4014	0.0309*
43	FQ658643.1.1351	Comamonadaceae- UB-Soil*	17	-0.3975	0.0327*
44	JX222293.1.1480	*Clostridium* sensu stricto 1 (UB-Clostridiaceae1)*	877	-0.3945	0.0342*

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
45	EF446897.1.1305	*Aeromonas* (uncultured Aeromonadaceae)*	63	-0.3937	0.0346*
46	EU771671.1.1398	*Enterobacter* (Enterobacteriaceae)*	26	-0.3915	0.0357*
47	DQ835530.1.1454	*Klebsiella oxytoca* (Enterobacteriaceae)*	644	-0.3917	0.0356*
48	AB486626.1.1336	*Clostridium* sensu stricto 10* (Clostridiaceae1)	52	-0.3903	0.0363*
49	AB355053.1.1493	*Aeromonas* (UB-Aeromonadaceae)*	176	-0.3889	0.0371*
50	JQ599074.1.1333	*Aeromonas* sp. 416 (Aeromonadaceae)*	328	-0.3885	0.0373*
51	EU801655.1.1498	*Variovorax* (uncultured Comamonadaceae)*	12	-0.3884	0.0374*
52	EF679188.1.1481	*Arcobacter* (Campylobacteraceae)	97	-0.3852	0.0391*
53	FJ825549.1.1400	*Variovorax* (uncultured Comamonadaceae)*	50	-0.3838	0.0399*
54	AB487891.1.1338	*Clostridium* sensu stricto 10* (Clostridiaceae1)	550	-0.3832	0.0402*
55	JX222454.1.1499	Moraxellaceae-UB	73	-0.3806	0.0417*
56	JF222314.1.1357	*Haloferula* (UB-Verrucomicrobiaceae)	12,885	-0.3794	0.0423*
57	FM213027.1.1507	UB- Xanthomonadaceae*	458	-0.3774	0.0436*
58	DQ294627.1.1336	*Ensifer adhaerens* (Rhizobiaceae)*	185	-0.3747	0.0452*
59	CU922621.1.1367	*Azospira* (UB- Rhodocyclaceae)	12	-0.3742	0.0455*
60	JQ624342.1.1501	Comamonadaceae – UB*	37	-0.3739	0.0457*
61	EU801259.1.1424	Comamonadaceae (UB)*	1,284	-0.3724	0.0466*
62	EU790196.1.1206	*Cupriavidus* (Burkholderiaceae)*	96	-0.3713	0.0474*
63	JX223231.1.1393	*Acidovorax* (UB- Comamonadaceae)*	40	-0.3711	0.0475*
64	FM213017.1.1496	*Dokdonella* (UB- Xanthomonadaceae)*	54	-0.3710	0.0476*
65	EU850462.1.1456	*Enterobacter* (UB-Enterobacteriaceae)*	13	-0.3707	0.0477*
66	HM780351.1.1517	*Aeromonas* (uncultured Aeromonadaceae)*	229	-0.3694	0.0486*
67	JN120259.1.1367	*Aeromonas sobria* (Aeromonadaceae)*	107	-0.3652	0.0514 ns
68	FQ659536.2.1358	*Tahibacter* (UB- Xanthomonadaceae)*	19	-0.3631	0.0529 ns
69	KF039904.1.1408	*Budvicia* sp. S1-5 (Enterobacteriaceae)*	27	-0.3621	0.0535 ns
70	JN868977.1.1523	Comamonadaceae - UB	48	-0.3574	0.0570 ns
71	FM213031.1.1507	*Rhodanobacter* (UB- Xanthomonadaceae)*	62	-0.3573	0.0570 ns
71	JQ291601.1.1466	*Siphonobacter aquaeclarae* (Cytophagaceae)	153	-0.3555	0.0584 ns
72	JX094902.1.1456	*Staphylococcus* sp. UAs Du23(Staphylococcaceae)*	82	-0.3539	0.0597 ns
73	JX233484.1.1404	*Enterobacter* sp. ATA2 (Enterobacteriaceae)*	20	-0.3518	0.0612 ns
74	AY370187.1.1454	Uncultured *Bartonella* sp. (Bartonellaceae)	12	-0.3512	0.0617 ns
75	JF830188.1.1510	*Aeromonas* (uncultured Aeromonadaceae)*	124	-0.3502	0.0626 ns
76	KC749105.1.1485	Rhizobiales-MNG7-UB*	12	-0.3472	0.0650 ns
77	HE681227.1.1529	*Leucobacter* (UB- Microbacteriaceae)*	52	-0.3454	0.0665 ns
78	AF144383.1.1437	*Ramlibacter tataouinensis* (Comamonadaceae)*	489	-0.3437	0.0680 ns
79	JF183865.1.1324	*Clostridium* sensu stricto 10* (UB-Clostridiaceae1)	34,300	-0.3409	0.0703 ns
80	GQ158268.1.1407	Lampropedia (UB-Comamonadaceae)*	154	-0.3312	0.0792 ns
81	DQ816568.1.1407	*Aeromonas* (UB)* (Aeromonadaceae)*	81	-0.3232	0.0872 ns
82	JF830173.1.1510	*Aeromonas* (uncultured Aeromonadaceae)*	671	-0.3231	0.0873 ns
83	FJ660541.1.1496	*Variovorax* (uncultured Comamonadaceae)*	16	-0.3192	0.0914 ns
84	JQ599381.1.1405	*Aeromonas* hydrophila (Aeromonadaceae)*	287	-0.3170	0.0938 ns
85	DQ303126.1.1253	*Aeromonas* sp. HM-6* (Aeromonadaceae)*	143	-0.3169	0.0939 ns
86	JX489918.1.1504	Gammaproteobacteria-NKB5-UB-Soil	737	-0.3163	0.0945 ns
87	FJ626628.1.1404	*Brachybacterium* sp. MH133 (Dermabacteraceae)	30	-0.3163	0.0945 ns
88	KC001353.1.1278	*Clostridium* sensu stricto 10 (UB-Clostridiaceae1)*	47	-0.3146	0.0964 ns
89	EF515224.1.1409	Aeromonas (uncultured Aeromonadaceae)*	606	-0.3117	0.0998 ns
90	JQ793488.1.1491	*Pseudorhodoferax* sp. (UB- Comamonadaceae)*	16	-0.3115	0.1000 ns
91	FM178827.1.1375	*Ramlibacter* (UB- Comamonadaceae)*	13	-0.3107	0.1009 ns
92	HQ592576.1.1490	Massilia (UB-Oxalobacteraceae)	14	-0.3104	0.1013 ns
93	AF004845.1.1337	*Methylorhabdus multivorans* (Hyphomicrobiaceae)	12	-0.3091	0.1027 ns

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
94	GQ379542.1.1200	*Curvibacter* (UB- Comamonadaceae)*	524	-0.3091	0.1027 ns
95	AB240488.1.1494	Comamonadaceae (UB)*	18	-0.3073	0.1048ns
96	JQ769899.1.1508	*Dokdonella* (UB- Xanthomonadaceae)*	34	-0.3031	0.1099 ns
97	CU921876.1.1304	Beijerinckiaceae (UB)	10	-0.3031	0.1099 ns
98	KF465099.1.1216	*Acidovorax* (UB- Comamonadaceae)*	1,828	-0.2999	0.1140 ns
99	FN658985.1.1339	*Aureimonas altamirensis* (Aurantimonadaceaea)	39	-0.2967	0.1181 ns
100	JN392782.1.1395	*Staphylococcus* (Staphylococcaceae)*	12	-0.2937	0.1219 ns
101	EF205512.1.1462	*Aeromonas* (uncultured Aeromonadaceae)*	301	-0.2913	0.1253 ns
102	ARCM01000002.993920.995384	Ancylobacter sp. FA202(Xanthobacteriaceae)	14,777	-0.2835	0.1362 ns
103	KF037462.1.1463	*Sphingomonas* (UB- Sphingomonadaceae)*	35	-0.2807	0.1403 ns
104	U87772.1.1455	*Afipia* genosp. 6 (Beijerinckiaceae)*	25	-0.2736	0.1509 ns
105	GQ249371.1.1503	*Zoogloea* sp. (UB-Rhodocyclaceae)	3,302	-0.2730	0.1519 ns
106	KF803323.1.1432	*Bosea* sp. FZ88 (Bradyrhizobiaceae)	34	-0.2717	0.1539 ns
107	EF173349.1.1511	*Variovorax* (uncultured Comamonadaceae)*	49	-0.2701	0.1565 ns
108	JX515499.1.1498	*Variovorax* (UB- Comamonadaceae)*	75	-0.2686	0.1588 ns
109	EA273524.82.1536	*Luteimonas* (unidentified-Xanthomonadaceae)*	55,395	-0.2669	0.1616 ns
110	JF504704.1.1498	*Clostridium* sp. SN-1 (Clostridiaceae1)*	88	-0.2655	0.1639 ns
111	JN650265.1.1438	*Clostridium* sensu stricto 1 (UB-Clostridiaceae1)*	496	-0.2606	0.1722 ns
112	JX105681.1.1378	*Mycobacterium* (UB-Mycobacteriaceae)	4,552	-0.2525	0.1864 ns
113	GQ205102.1.1311	*Delftia* sp. RF-83 (Comamonadaceae)*	2,033	-0.2185	0.2547 ns
114	KF037812.1.1478	*Optitutus* (UB-Opitutaceae)	368	-0.1737	0.3674 ns
115	AB594686.1.1432	*Clostridium* sensu stricto 1 (UB-Clostridiaceae1)*	64	-0.1592	0.4095 ns
116	AJ295350.1.1354	Rape rhizosphere bacterium cse003 (Comamonadaceae)*	51	-0.1515	0.4326 ns
117	FR853515.1.1500	*Aeromonas* (uncultured Aeromonadaceae)*	160	-0.1473	0.4457 ns
118	AF423291.1.1411	*Sphingobium* (UB- soil) (Sphingomonadaceae)*	1,655	-0.1469	0.4470 ns
119	CU920570.1.1311	*Arthrobacter* (UB- Micrococcaceae)	303	-0.1443	0.4550 ns
120	KF464379.1.1285	*Comamonas* (UB-Comamonadaceae)*	68	-0.1429	0.4595 ns
121	CU918968.1.1340	*Shinella* (UB) (Rhizobiaceae)*	3,557	-0.1417	0.4635 ns
122	HQ121144.1.1504	*Variovorax* (uncultured Comamonadaceae)*	398	-0.1278	0.5090 ns
123	CU919367.1.1325	SHA-109 (UB) (Unidentified family)	597	-0.1141	0.5555 ns
124	JF421145.1.1509	*Ramlibacter* (UB- Comamonadaceae)*	404	-0.0816	0.6737 ns
125	JF508892.1.1421	*Sphingomonas* sp. SJ-1 (Sphingomonadaceae)*	62	0.0747	0.7003 ns
126	FJ375453.1.1424	*Phenylobacterium* (UB-Caulobacteraceae)*	1,408	-0.0608	0.7541ns
127	AJ318158.1.1485	*Leucobacter* (UB-Microbacteriaceae)*	1,539	-0.0409	0.8331 ns
128	CU926479.1.1339	Actinobacteria PeM15 (UB- Unidentified family)	250	-0.0336	0.8625 ns
129	JX515472.1.1471	*Acidovorax* (UB- Comamonadaceae)*	250	0.0194	0.9202 ns
130	JF706531.1.1366	*Xylophilus* sp. PDD-37_7j_hv_b-3 (Comamonadaceae)*	55,011	-0.0005	0.9980 ns

UB-Uncultured bacteria; *Name: recurring species/family/groups

Appendix E2: (Chapter 7) Filter 2Aii- OTU abundance vs adult emergence @ Spearman's positive correlation +30

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
1	AB255079.1.1492	*Variovorax* (UB- Comamonadaceae)*	63	0.6933	<.0001****
2	KF037634.1.1519	*Phaselicystis* (UB-Phaselicystidaceae)	2,586	0.6345	0.0002***
3	EU730907.1.1387	*Sphingomonas wittichii* (Sphingomonadaceae)*	56	0.5981	0.0006***
4	FJ374243.1.1485	*Comamonas* (UB- Comamonadaceae)*	73	0.5844	0.0009***
5	KF010745.1.1485	Comamonadaceae-UB*	85,589	0.5736	0.0011**
6	AY695728.1.1430	*Nitratireductor* (UB-Phyllobacteriaceae)*	47	0.5831	0.0009***
7	EU704796.1.1279	*Aquabacterium* sp. (UB-Comamonadaceae)*	4,706	0.5727	0.0012**
8	HM277954.1.1356	Chitinophagaceae -UB*	10,053	0.5714	0.0012**
9	HQ166654.1.1466	*Leptothrix* (UB-Comamonadaceae)*	1,162	0.5591	0.0016**
10	AB539840.1.1439	*Spirosoma* sp. APU1a (Cytophagaceae)	241	0.5555	0.0018**
11	JN113079.1.1443	*Altererythrobacter* (UB-Erythrobacteraceae)	462	0.5346	0.0028**
12	ARBA01000003.218623.220140	*Methylotenera mobilis* 13 (Methylophilaceae)*	386	0.5195	0.0039**
13	DQ232437.1.1280	(Comamonadaceae- UB)*	276	0.5191	0.0039**
14	EU704736.1.1232	*Sphingomonas* sp. (UB-Sphingomonadaceae)*	331	0.5163	0.0041**
15	KF381493.1.1596	*Acinetobacter indicus* (Moraxellaceae)*	58	0.5108	0.0046**
16	FJ562145.1.1221	*Rhizobium* (UB-Rhizobiaceae)*	1,019	0.5098	0.0047**
17	KF385053.1.1517	*Pseudomonas* sp. (UB-Comamonadaceae)*	206,335	0.5049	0.0052**
18	JF225921.1.1338	*Blastocatella* (UB-Acidobacteria)*	49	0.4996	0.0058**
19	AB559013.1.1413	*Sideroxydans* (UB-Gallionellaceae)	20	0.4979	0.0060**
20	AY922120.1.1402	Verrucomicrobiaceae-UB*	27	0.4900	0.0070**
21	HF544322.1.1424	*Hymenobacter* sp. KBP- 30 (Cytophagaceae)*	5,240	0.4819	0.0081**
22	AY792290.1.1396	*Novosphingobium* (UB-Sphingomonadaceae)	31,696	0.4780	0.0087**
23	GU208407.1.1430	Comamonadaceae-UB*	1,491	0.4766	0.0089**
24	FJ960270.1.1374	*Candidatus captivus* (UB-Rickettsiales Incertae Sedis)*	211	0.4763	0.0090**
25	AB614560.1.1419	*Azohydromonas* (UB-Comamonadaceae)*	141	0.4754	0.0092**
26	AF468333.1.1426	Comamonadaceae-UB	24	0.4696	0.0102*
27	AB722229.1.1347	*Paucibacter* (UB-Comamonadaceae)*	105	0.4679	0.0105*
28	EU536498.1.1387	*Aquabacterium* (UB-Comamonadaceae)*	28,506	0.4647	0.0111*
29	JF167662.1.1343	*Gemmatimonas* (UB-Gemmatimonadaceae)	44	0.4545	0.0133*
30	AB608673.1.1453	Comamonadaceae-UB	239,153	0.4536	0.0135*
31	CP002959.3819378.3820883	*Turneriella parva* DSM 21527 (Leptospiraceae)	17	0.4515	0.0140*
32	AB220090.1.1368	*Methylobacterium* sp.PB145 (Methylobactericeae)	39	0.4496	0.0144*
33	AB255118.1.1496	Rhodocyclaceae-UB	12	0.4476	0.0149*
34	HM251127.1.1306	*Sphingomonas* (UB- Sphingomonadaceae)*	52	0.4464	0.0152*
35	EU705018.1.1281	*Novosphingobium* sp. (UB-Sphingomonadaceae)*	187	0.4464	0.0152*
36	FJ546409.1.1222	*Prosthecobacter* (UB-Verrumicrobiaceae)*	144	0.4444	0.0157*
37	HM099645.1.1450	*Sphingomonas* sp. oral taxon F71 (Sphingomonadaceae)*	27	0.4400	0.0169*
38	EU705344.1.1299	*Acinetobacter* sp. (Moraxellaceae)	23	0.4398	0.0170*
39	FJ529982.1.1445	*Phenylobacterium* (UB-Caulobacteraceae)*	1,722	0.4360	0.0181*
40	AZND01000010.5793.7314	*Methylibium* sp. T29 (UB- Comamonadaceae)*	427	0.4331	0.0189*
41	DQ125688.1.1407	*Rhizobium* (UB-Rhizobiaceae)*	50	0.4291	0.0202*

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
42	FJ375464.1.1465	*Aquabacterium* (UB- Comamonadaceae)*	9,540	0.4284	0.0204*
43	JQ684312.1.1480	*Sediminibacterium* (UB-Chitinophagaceae)*	3,480	0.4282	0.0205*
44	L79964.1.1326	*Sphaerotilus*- Eikelbloom type 1701 (Comamonadaceae)*	22	0.4242	0.0218*
45	KC464858.1.1441	Roseateles-T30 (Comamonadaceae)*	41	0.4178	0.0241*
46	AB476287.1.1464	*Methylotenera* (UB-Methylophilaceae)	14	0.4149	0.0252*
47	HQ860616.1.1449	*Limnohabitans* (UB-Comamonadaceae)*	219	0.4082	0.0279*
48	Y18838.1.1485	*Hymenobacter ocellatus* (Cytophagaceae)	416	0.4070	0.0284*
49	FJ152778.1.1486	Comamonadaceae-UB	580	0.4046	0.0295*
50	JF808978.1.1497	*Dechloromonas* sp. (UB- Pseudomonadaceae)*	6,179	0.4034	0.0300*
51	JQ769534.1.1454	7B-8-Sphingomonadales-UB	12	0.4019	0.0307*
52	JX431978.1.1436	*Variovorax* (UB-Comamonadaceae)*	162	0.3901	0.0365*
53	EU790404.1.1211	*Pelomonas* (UB-Comamonadaceae)*	13	0.3867	0.0382*
54	JF167832.1.1330	Armatimonadetes-UB*	1,380	0.3855	0.0389*
55	EU133861.1.1359	*Azoarcus* (UB-Rhodocyclaceae)*	43	0.3771	0.0437*
56	KC994861.1.1473	Armatimonadetes-UB*	5,674	0.3750	0.0450*
57	CU926220.1.1354	*Simplicispir*a (UB- Comamonadaceae)*	19	0.3704	0.0480*
58	FJ382809.1.1403	*Methylophilus* (UB-Methylophilaceae)*	89	0.3687	0.0490*
59	FJ946583.1.1290	*Mesorhizobium* sp. (Phyllobacteriaceae)	265	0.3639	0.0523 ns
60	EU133425.1.1263	*Ensifer* (UB- Rhizobiaceae)*	68	0.3562	0.0579 ns
61	JF176913.1.1349	Env.OPS 17 -Sphingobacteriales*	5,461	0.3553	0.0586 ns
62	JN679119.1.1503	*Variovorax* sp. (UB-Comamonadaceae)*	80	0.3549	0.0589 ns
63	JX105554.1.1489	*Undibacterium* (UB-Oxalobacteraceae)	167,323	0.3530	0.0603 ns
64	JF176780.1.1348	Env.OPS 17 -Sphingobacteriales*	14	0.3518	0.0613 ns
65	AY792258.1.1537	*Variovorax* (UB- Comamonadaceae)*	19	0.3501	0.0626 ns
66	FM164634.1.1279	*Novosphingobium mathurense* (Sphingomonadaceae)*	368	0.3501	0.0627 ns
67	JF222253.1.1350	*Leptothrix* (UB-Comamonadaceae)*	86	0.3418	0.0695 ns
68	KC620963.1.1509	*Prosthecobacter* (UB-Verrucomicrobiaceae)*	9,086	0.3368	0.0740 ns
69	EU881220.1.1494	Comamonadaceae-UB*	26	0.3314	0.0791 ns
70	AM936716.1.1318	*Mesorhizobium* sp. (UB-Phyllobacteriaceae)	73	0.3310	0.0795 ns
71	JX458449.1.1232	*Moraxellaceae bacterium* W2.09-231 (Moraxellaceae)	56	0.3309	0.0795 ns
72	AB167232.1.1352	*Aminobacter aminovorans* (Phyllobacteriaceae)	361	0.3306	0.0799ns
73	HM845855.1.1338	*Siphonobacter* (UB- Cytophagaceae)*	25	0.3297	0.0807 ns
74	GU179688.1.1345	*Novosphingobium* (UB- Sphingomonadaceae)*	252	0.3234	0.0870 ns
75	EF654712.1.1493	Comamonadaceae- UB*	64	0.3233	0.0871 ns
76	JF925036.1.1500	Uncultured marine bacterium (Methylophilaceae)*	370	0.3164	0.0945 ns
78	DQ836748.1.1400	Rhododcyclaceae-UB	24	0.3130	0.0983 ns
79	JX224080.1.1489	*Polaromonas* (UB-Comamonadaceae)	31	0.3101	0.1016 ns
80	EU803334.1.1400	*Sediminibacterium* (UB-Chitinophagaceae)*	64	0.3004	0.1133 ns
81	FM872911.1.1473	*Pseudoclavibacter* (UB-Microbacteriaceae)	86	0.3002	0.1136 ns
82	KC358498.1.1263	Rhodocyclaceae-UB	99	0.2882	0.1294 ns
83	FJ612260.1.1479	Comamonadaceae-UB*	18	0.2866	0.1318 ns
84	EU703461.1.1354	*Rhodobacter* sp. (UB-Rhodobacteraceae)	10	0.2814	0.1391 ns
85	EF018596.1.1401	*Acidovorax* (Comamonadaceae-UB)*	334	0.2813	0.1393 ns
86	EF020225.1.1333	Caulobacteraceae-UB	357	0.2804	0.1407 ns
87	JX644252.1.1459	*Xylophilus* (UB-Comamonadaceae)*	281	0.2692	0.1579 ns
88	DQ664244.1.1470	*Piscinibacter aquaticus* (Comamonadaceae)	352	0.2692	0.1580 ns
89	EF018778.1.1384	*Variovorax* (UB- Comamonadaceae)*	38	0.2688	0.1585 ns

S/N	Species number	Name	OTU Abundance	Spearman's R	P-value
90	EU801622.1.1502	LD28 freshwater group (UB- Methylophilaceae)*	2969	0.2560	0.1801 ns
91	KC994702.1.1531	Uncultured Verrucomicrobia bacteria*	9,829	0.2551	0.1817 ns
92	HE798200.1.1359	*Methyloversatilis* sp. (UB-Rhodocyclaceae)*	38,277	0.2551	0.1817 ns
93	AB487141.1.1377	*Paucimonas* (UB- Oxalobacteriaceae)	188	0.2530	0.1855 ns
94	GU295961.1.1444	*Oxalicibacterium* sp. JC-21 (Oxalobacteraceae)*	24	0.2528	0.1858 ns
95	EU000442.1.1295	*Novosphingobium* (UB-Sphingomonadaceae)*	28	0.2502	0.1905 ns
96	EF516172.1.1475	*Opitutus* (UB-Opitutaceae)	119	0.2364	0.2170 ns
97	FN984864.1.1396	Hydrotaleae (UB-Chitinophagaceae)*	33	0.2355	0.2188 ns
98	HQ752265.1.1428	*Sediminibacterium* (UB-Chitinophagaceae)*	245	0.2303	0.2293 ns
99	JQ684468.1.1422	*Chitinophaga* sp. (UB-Chitinophagaceae)*	51,840	0.2229	0.2452 ns
100	FJ562149.1.1286	Isosphaera (UB-Planctomycetaceae)	17	0.2192	0.2533 ns
101	EU801174.1.1476	OM43 clade (UB-Methylophilaceae)*	217	0.2181	0.2558 ns
102	JF135969.1.1355	Cellvibrio (UB-Pseudomonadaceae)*	5,263	0.2037	0.2893 ns
103	FJ936734.1.1481	Cytophagaceae-UB*	160	0.1976	0.3041 ns
104	JF049431.1.1349	Cytophagaceae -UB*	28	0.1957	0.3090 ns
105	JX224874.1.1304	OM43 clade- Methylophilaceae-UB	103	0.1912	0.3205 ns
106	FJ535545.1.1510	*Opitutus* (UB-Opitutaceae)	71	0.1578	0.4135 ns
107	EU132256.1.1324	*Candidatus solibacter* (UB-Acidobacteria)	368	0.1375	0.4769 ns
108	FJ439850.1.1462	*Candidatus captivus* (UB-Rickettsiales Incertae Sedis)*	737	0.1066	0.3059 ns
109	CU920376.2.1285	*Rhodobacter* (UB-Rhodobacteraceae)	3,056	0.0960	0.6203 ns
110	FJ432478.1.1245	Rhodospirillaceae-UB*	479	0.0910	0.6388 ns
111	FJ946599.1.1290	*Rhodobacter* (UB-Rhodobacteraceae)*	254	0.0804	0.6783 ns
112	EF516040.1.1394	*Bryobacter* (UB- Acidobacteria)	11	0.0701	0.7179 ns

P-value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria. *Name: recurring species/family/groups

Appendix F: (Chapter 7) Filter 2Bi- OTU abundance vs NH_3 @ Spearman's correlation +30 (positive correlation)

S/N	Species number	Name	Abundance	Spearman's R	P-value
1	ARCM01000002.993920.995384	*Ancylobacter* sp. FA202 (Xanthobacteriaceae)	14,777	0.7764	<.0001****
2	KC254734.1.1308	*Kaistia hirudinis* (Rhizobiaceae)	961	0.7210	<.0001****
3	HG529104.1.1303	*Prosthecobacter* (UB-Verrucomicrobiaceae)*	16,796	0.6787	<.0001****
4	GQ263646.1.1467	*Pedobacter* (UB-Sphingobacteriaceae)*	234	0.6616	<.0001****
5	HM274274.1.1354	Chitinophagaceae-UB*	1,421	0.6606	<.0001****
6	AF144383.1.1437*	*Ramlibacter tataouinensis* (Comamonadaceae)*	489	0.6468	0.0001***
7	HM341157.1.1356	*Variovorax* (uncultured Comamonadaceae)*	41	0.6303	0.0002***
8	GQ158268.1.1407*	*Lampropedia* (UB-Comamonadaceae)*	154	0.6272	0.0003***
9	JN868977.1.1523*	Comamonadaceae - UB	48	0.6190	0.0003***
10	KF150693.1.1413	*Pedobacter* sp. THG-G12 (Sphingobacteriaceae)	245	0.6157	0.0004***
11	AJ867899.1.1483	*Polynucleobacter* (UB-Burkholderiaceae)*	56	0.6120	0.0004***
12	JX223230.1.1329	Caulobacteraceae-UB	42	0.6102	0.0004***
13	EU104166.1.1477	*Prosthecobacter* (UB-Verrucomicrobiaceae)*	623	0.6041	0.0005***
14	FJ390462.1.1455	*Bacillus weihenstephanensis* (Bacillaceae)	13	0.5977	0.0006***
15	JF706531.1.1366	*Xylophilus* sp. PDD-37_7j_hv_b-3 (Comamonadaceae)*	55,011	0.5971	0.0006***
16	EU801259.1.1424*	Comamonadaceae (UB)*	1,284	0.5842	0.0009***
17	HM438647.1.1489	*Variovorax* sp. (uncultured Comamonadaceae)*	142	0.5761	0.0011**
18	HQ178724.1.1455	*Simplicispira* (uncultured Comamonadaceae)*	34	0.5710	0.0012**
19	JF715447.1.1366	*Acidovorax* sp. IMCC12689 (Comamonadaceae)*	160	0.5707	0.0012**
20	GQ487901.1.1491	Comamonadaceae- UB*	693	0.5706	0.0012**
21	GQ379542.1.1200	*Curvibacter* (UB- Comamonadaceae)*	524	0.5627	0.0015**
22	EF465533.1.1436	*Pseudoxanthobacter* soli-DSM 19599 (Xanthobacteraceae)	296	0.5613	0.0015**
23	JF421145.1.1509	*Ramlibacter* (UB- Comamonadaceae)*	404	0.5506	0.0020**
24	EU801494.1.1452	Polynucleobacter (UB-Burkholderiaceae)*	126	0.5492	0.0020**
25	JX971539.1.1361	*Pedobacter boryungensis* (Sphingobacteriaceae)*	175	0.5360	0.0027**
26	CU923555.1.1349	Hydrogenophaga (UB)* (Comamonadaceae)*	39	0.5348	0.0028*
27	EU801655.1.1498	*Variovorax* (uncultured Comamonadaceae)*	12	0.5295	0.0031**
28	JF222314.1.1357	*Haloferula* (UB-Verrucomicrobiaceae)	12,885	0.5275	0.0033**
29	FQ658643.1.1351	Comamonadaceae- UB-Soil*	17	0.5274	0.0033**

S/N	Species number	Name	Abundance	Spearman's R	P-value
30	CU922145.1.1372	Hydrogenophaga (UB)* (Comamonadaceae)*	32	0.5273	0.0033**
31	JX646636.1.1421	*Sphingobacterium thalpophilum* (Sphingobacteriaceae)	685	0.5247	0.0035**
32	FJ230903.1.1506	*Polynucleobacter* (UB-Burkholderiaceae)*	2,535	0.5202	0.0038**
33	EU800506.1.1501	Comamonadaceae (UB)*	103	0.5190	0.0039**
34	HE589817.1.1428	Dyadobacter (UB-Cytophagaceae)*	831	0.5089	0.0048**
35	DQ248235.1.1498	*Variovorax* (UB- Comamonadaceae)*	15	0.5030	0.0054**
36	GU980236.1.1327	*Sphingomonas* sp. CCGE4131 (Sphingomonadaceae)	19	0.5006	0.0057**
37	EU850462.1.1456	*Enterobacter* (UB-Enterobacteriaceae)*	13	0.4987	0.0059**
38	JX879739.1.1388	*Nubsella* sp. EsD18 (Sphingobacteriaceae)*	4,977	0.4889	0.0071**
39	EU802044.1.1501	*Polynucleobacter* (UB-Burkholderiaceae)*	8,310	0.4884	0.0072**
40	GU731291.1.1336	*Devosia* (bacteria enrichment culture clone heteroB30_4W)	24	0.4879	0.0072**
41	EU801830.1.1499	*Variovorax* (uncultured Comamonadaceae)*	1,614	0.4868	0.0074**
42	JX949945.1.1399	*Cryobacterium* sp. TMS1-11-A (Microbacteriaceae)	50	0.4855	0.0076**
43	EU801186.1.1501	*Polynucleobacter* (UB-Burkholderiaceae)*	362	0.4829	0.0080**
44	FM178827.1.1375	*Ramlibacter* (UB- Comamonadaceae)*	13	0.4813	0.0082**
45	FJ825549.1.1400	*Variovorax* (uncultured Comamonadaceae)*	50	0.4811	0.0082**
46	JQ793488.1.1491	*Pseudorhodoferax* sp. (UB-Comamonadaceae)*	16	0.4778	0.0088**
47	HE589840.1.1456	*Acidovorax* (UB-Comamonadaceae)*	1,280	0.4768	0.0089**
48	AF177943.1.1493	*Tepidimonas ignava* (Comamonadaceae)*	132	0.4759	0.0091**
49	AB240488.1.1494	Comamonadaceae (UB)*	18	0.4720	0.0097*
51	FN658985.1.1339	*Aureimonas altamirensis* (Aurantimonadaceaea)	39	0.4696	0.0102*
52	DQ450743.1.1359	Chitinophagaceae-UB*	32	0.4611	0.0118*
53	HM069053.1.1447	Rhizobiales- MNG7-UB	5,036	0.4610	0.0118*
54	JN113079.1.1443	*Altererythrobacter* (UB-Erythrobacteraceae)*	462	0.4534	0.0135*
55	JQ977543.1.1446	*Polaromonas* sp. Ala11 (Comamonadaceae)*	61	0.4431	0.0161*
56	HM274183.1.1350	*Pedobacter* (UB-Sphingobacteriaceae)*	17	0.4412	0.0166*
57	DQ675026.1.1502	*Sphingobacterium* (UB-Sphingobacteriaceae)*	49	0.4395	0.0171*
58	JQ769899.1.1508	*Dokdonella* (UB- Xanthomonadaceae)*	34	0.4390	0.0172*
59	CU921876.1.1304	Beijerinckiaceae (UB)	10	0.4390	0.0172*
60	KC749105.1.1485	Rhizobiales-MNG7-UB*	12	0.4263	0.0211*
61	AR381566.2.1448	*Lysinimonas* (unidentified Microbacteriaceae)*	35	0.4241	0.0219*
62	HQ860602.1.1449	Comamonadaceae-UB*	67	0.4223	0.0225*
63	FM213035.1.1498	*Thiobacillus* (Hydrogenophilaceae-UB)	131	0.4223	0.0225*
64	JF775632.1.1472	*Clostridium* sensu stricto 9* (UB-Clostridiaceae1)	308	0.4201	0.0233**
65	DQ988309.1.1323	*Bacteriovorax* (UB-Bacteriovoracaceae)	992	0.4177	0.0242*
66	JQ769816.1.1484	Env.OPS17-Sphingobacteriales	16	0.4148	0.0253*
67	EU305572.1.1463	*Nocardia* sp. (UB-Micrococcaceae)*	21	0.4115	0.0266*
68	JX647712.1.1500	Comamonadaceae-UB*	10	0.4099	0.0272*
69	CU923553.1.1353	*Acidovorax* (UB- Comamonadaceae)*	19	0.4081	0.0280*

S/N	Species number	Name	Abundance	Spearman's R	P-value
70	HE681227.1.1529	*Leucobacter* (UB- Microbacteriaceae)*	52	0.4077	0.0282*
71	KF464379.1.1285	*Comamonas* (UB-Comamonadaceae)*	68	0.4012	0.0310*
72	AB511012.1.1469	0319-6G20 (UB-Myxococcales)	622	0.4010	0.0311*
73	FR853451.1.1447	*Phenylobacterium* (UB-Caulobacteraceae)*	28	0.3963	0.0333*
74	EF204468.1.1373	*Pedobacter* sp. H37 (Sphingobacteriaceae)*	26,294	0.3946	0.0342*
75	CU926479.1.1339	Actinobacteria PeM15 (UB- Unidentified family)	250	0.3940	0.0345*
76	AY921814.1.1392	Comamonadaceae-UB*	19	0.3889	0.0371*
77	EU861941.1.1499	Microbacteriaceae-UB*	42	0.3880	0.0375*
78	GQ113033.1.1323	*Bdellovibrio* (UB-Bdellovibrionaceae)	2,465	0.3873	0.0379*
79	EA273524.82.1536	*Luteimonas* (unidentified-Xanthomonadaceae)*	55,395	0.3865	0.0383*
80	AY792238.1.1529	*Polynucleobacter* (UB-Burkholderiaceae)*	18	0.3853	0.0390*
81	DQ294627.1.1336	*Ensifer adhaerens* (Rhizobiaceae)*	185	0.3844	0.0395*
82	AF423291.1.1411	*Sphingobium* (UB- soil) (Sphingomonadaceae)*	1,655	0.3805	0.0417*
83	AB486966.1.1314	*Clostridium* sensu stricto 8*(Clostridiaceae1)	218	0.3792	0.0425*
84	AB637065.1.1480	*Pedobacter* (UB-Sphingobacteriaceae)*	227	0.3784	0.0430*
85	JF217076.1.1347	*Perlucidibaca* (UB-Moraxellaceae)*	6,700	0.3772	0.0437*
86	FJ167458.1.1488	Hot Creek 32 -UB	30	0.3768	0.0439*
87	JN713172.1.1520	*Ottowia* sp. canine oral taxon 014 (Comamonadaceae)*	46	0.3763	0.0442*
88	CU918968.1.1340	*Shinella* (UB) (Rhizobiaceae)*	3,557	0.3740	0.0457*
89	AJ296565.1.1297	*Aeromonas*-GR-WP33-14 (UB-Aeromonadaceae)*	10	0.3666	0.0505 ns
90	CU919622.1.1305	*Devosia* (UB-Hyphomicrobiaceae)	11,883	0.3640	0.0522 ns
91	HM277678.1.1359	*Lampropedia* (UB-Comamonadaceae)*	34	0.3614	0.0541 ns
92	FN436173.1.1478	*Noviherbaspirillum* (UB-Oxalobacteraceae)*	932	0.3551	0.0587 ns
93	FJ812377.1.1410	*Pedobacter* sp. AR-138 (Sphingobacteriaceae)*	18	0.3469	0.0653 ns
94	KF411733.1.1307	*Novosphingobium* (UB-Sphingomonadaceae)*	121	0.3450	0.0668 ns
95	AB637048.1.1477	*Taibaiella* (UB-Chitinophagaceae)*	34	0.3449	0.0669 ns
96	EU083480.1.1470	*Azoarcus* (UB-Rhodocyclaceae)*	15	0.3393	0.0718 ns
97	JX105681.1.1378	*Mycobacterium* (UB-Mycobacteriaceae)	4,552	0.3380	0.0729 ns
98	HM558928.1.1327	*Devosia* (UB-Hyphomicrobiaceae)*	35	0.3313	0.0791 ns
99	KF804094.1.1319	*Pseudoxanthobacter* sp. S50 (Rhizobiales)	112	0.3300	0.0805 ns
100	KC967412.1.1394	*Clostridium* sp. LAM1030 (Clostridiaceae)	63,906	0.3209	0.0896 ns
101	JF703441.1.1378	*Ochrobactrum* (UB-Brucellaceae)	19	0.3197	0.0909 ns
102	GU731323.1.1328	Caulobacteraceae (bacteria enrichment culture clone auto83_4W)	86	0.3185	0.0922 ns
103	GQ389171.1.1497	Comamonadaceae- UB*	10	0.3177	0.0931 ns
104	FJ375445.1.1562	*Diaphorobacter* (UB- Comamonadaceae)*	56	0.3168	0.0940 ns
105	AF255632.1.1380	*Gemmatimonas* (UB-Gemmatomonadaceae)*	550	0.3163	0.0946 ns
106	KF881976.1.1419	*Microbacteriaceae* sp. b180 (Microbacteriaceae)*	22	0.3142	0.0969 ns
107	FM213077.1.1512	*Tahibacter* (UB- Xanthomonadaceae)*	51	0.3065	0.1058 ns
108	FN668067.1.1483	*Sphingobacterium* sp. (NS11-12 marine group)-UB	7031	0.2991	0.1150 ns

S/N	Species number	Name	Abundance	Spearman's R	P-value
109	AB666454.1.1503	*Pedobacter* sp. Mal11-5 (Sphingobacteriaceae)*	30	0.2972	0.1175 ns
110	HM266914.1.1335	Armatimonadetes-UB*	36	0.2950	0.1203 ns
111	JX233484.1.1404	Enterobacter sp. ATA2 (Enterobacteriaceae)*	20	0.2900	0.1271 ns
112	JN379402.1.1475	*Clostridium* sensu stricto 12 (UB-Clostridiaceae1)*	23	0.2837	0.1358 ns
113	KF037462.1.1463	*Sphingomonas* (UB-Sphingomonadaceae)*	35	0.2790	0.1427 ns
114	JX489898.1.1487	NS11-12 marine group-Sphingobacteriales	251	0.2776	0.1448 ns
115	JF095776.1.1306	Sphingomonas (UB-Sphingomodaceae)*	84	0.2747	0.1492 ns
116	JX489918.1.1504	Gammaproteobacteria-NKB5-UB-Soil	737	0.2723	0.1530 ns
117	FJ626628.1.1404	*Brachybacterium* sp. MH133 (Dermabacteraceae)	30	0.2723	0.1530 ns
118	FJ382209.1.1356	*Mycobacterium* (UB-Mycobacteriaceae)*	13	0.2673	0.1609 ns
119	JQ977359.1.1399	*Devosia* sp. Bzb6 (Hyphomicrobiaceae)*	19	0.2624	0.1691 ns
120	FM213027.1.1507	UB- Xanthomonadaceae*	458	0.2622	0.1694 ns
121	EF103202.1.1400	*Rhodococcus* sp. MSCB-5 (Microbacteriaceae)*	107	0.2616	0.1705 ns
122	EU440723.1.1414	*Kaistobacter* sp. (UB-Sphingomonadaceae)	339	0.2544	0.1829 ns
123	AJ318158.1.1485	*Leucobacter* (UB-Microbacteriaceae)*	1,539	0.2536	0.1844 ns
124	FM213017.1.1496	Dokdonella (UB- Xanthomonadaceae)*	54	0.2530	0.1854 ns
125	JQ977465.1.1431	Pedobacter sp. Zs28 (Sphingobacteriaceae)*	18	0.2451	0.2000 ns
126	EU434572.1.1389	*Brevundimonas diminuta* (Caulobacteraceae)	60	0.2390	0.2118 ns
127	HM838886.1.1303	*Devosia* (UB-Hyphomicrobiaceae)*	18	0.2378	0.2141 ns
128	EU289427.1.1447	*Devosia* (UB-Hyphomicrobiaceae)	12	0.2340	0.2218 ns
129	EF679188.1.1481	*Arcobacter* (Campylobacteraceae)	97	0.2255	0.2395 ns
130	KF381405.1.1505	*Enterobacter* (Enterobacteriaceae)*	172	0.2253	0.2399 ns
131	FM213031.1.1507	*Rhodanobacter* (UB- Xanthomonadaceae)*	62	0.2243	0.2421 ns
132	KC749203.1.1504	*Acidovorax* (UB- Comamonadaceae)*	58	0.2206	0.2502 ns
133	JX223620.1.1368	*Clostridium* sensu stricto 12 (UB-Clostridiaceae1)*	816	0.2148	0.2631 ns
134	JX647782.1.1507	*Methylibium* sp. (UB-Comamonadaceae)*	10	0.2137	0.2657 ns
135	JF830173.1.1510	*Aeromonas* (uncultured Aeromonadaceae)*	671	0.2096	0.2752 ns
136	KF560339.1.1348	*Martelella* sp. BM5-7 (Aurantimonadaceae)	15	0.2082	0.2784 ns
137	AB682425.1.1453	*Niabella soli* (Chitinophagaceae)*	2,340	0.2035	0.2897 ns
138	EF111197.1.1232	*Aeromonas* (uncultured Aeromonadaceae)*	1,513	0.2008	0.2962 ns
139	AY370187.1.1454	Uncultured *Bartonella* sp. (Bartonellaceae)	12	0.1990	0.3008 ns
140	EU488023.1.1501	Comamonadaceae (UB)*	30	0.1915	0.3196 ns
141	JF703410.1.1375	*Sphingomonas* (UB-Sphingomonadaceae)*	11	0.1835	0.3406 ns
142	EU771671.1.1398	*Enterobacter* (Enterobacteriaceae)*	26	0.1789	0.3530 ns
143	CU920570.1.1311	*Arthrobacter* (UB- Micrococcaceae)	303	0.1778	0.3562 ns
144	FJ375453.1.1424	*Phenylobacterium* (UB-Caulobacteraceae)*	1,408	0.1743	0.3658 ns
145	AAAA02020732.6.1490	*Oryza sativa* Indica group	30	0.1430	0.4592 ns
146	JX134453.1.1497	*Photobacterium* sp. B2-19-1 (Vibronaceae)	39	0.1355	0.4833 ns
147	HM587924.1.1418	*Agromyces* sp. m7-5 (Microbacteriaceae)*	199	0.1296	0.5030 ns

S/N	Species number	Name	Abundance	Spearman's R	P-value
148	DQ248285.1.1502	*Escherichia*-Shigella (UB- Enterobacteriaceae)*	11	0.1273	0.5106 ns
149	JF497830.1.1490	*Dechloromonas* (UB-Rhodocyclaceae)*	322	0.0985	0.6113 ns
150	JF508892.1.1421	*Sphingomonas* sp. SJ-1(Sphingomonadaceae)*	62	0.0965	0.6186 ns
151	KC551718.1.1496	*Rhodocyclaceae*-UB*	103	0.0892	0.6455 ns
152	DQ450189.1.1495	BAL58 marine group (Comamonadaceae)*	68	0.0655	0.7356 ns
153	KC211823.1.1410	*Sphingopyxis* (UB-Sphingomonadaceae)	38	0.0552	0.7762 ns
154	JN023744.1.1442	*Sphingomonas* (UB-Sphingomonadaceae)	147	0.0535	0.7828 ns
155	JN391735.1.1497	Chitinophagaceae-UB	29,677	0.0247	0.8986 ns

P-value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species/family/groups

Appendix F2: (Chapter 7) Filter 2Bii- OTU abundance vs NH_3 @ Spearman's correlation -30 (negative correlation)

S/N	Species number	Name	Abundance	Spearman's R	P-value
1	FN563004.1.1426	*Noviherbaspirillum* (photoautotrophic bacterium GMMC _photoauto_1) Oxalobacteraceae	49	-0.6787	<.0001****
2	FJ802311.1.1212	Comamonadaceae (iron reducing enrichment culture clone FEA_2_E4)	74	-0.6528	0.0001***
3	JF429369.1.1488	*Acidovorax* (Comamonadaceae-UB)*	6,575	-0.6194	0.0003***
4	JF222253.1.1350	*Leptothrix* (UB-Comamonadaceae)*	86	-0.6089	0.0005***
5	EF018753.1.1401	*Acidovorax* (Comamonadaceae-UB)*	119	-0.5944	0.0007***
6	AB599879.1.1412	Comamonadaceae-USH1-UB*	100	-0.5835	0.0009***
7	DQ664240.1.1431	*Aquabacterium*-IMCC1721 (Comamonadaceae)*	42	-0.5759	0.0011**
8	JN217068.1.1494	*Acidovorax* sp. (Tepidicella-Comoamonadaceae)*	218	-0.5744	0.0011**
9	CP002959.3819378.3820883	*Turneriella parva* DSM 21527 (Leptospiraceae)	17	-0.5715	0.0012**
10	AY792258.1.1537	*Variovorax* (UB- Comamonadaceae)*	19	-0.5693	0.0013**
11	JQ684472.1.1537	*Janthinobacterium* sp. (UB-Oxalobacteraceae)*	150	-0.5690	0.0013**
12	AB378588.1.1486	*Massilia* (UB-Oxalobacteraceae)*	130	-0.5642	0.0014**
13	AB487141.1.1377	*Paucimonas* (UB- Oxalobacteriaceae)*	188	-0.5640	0.0014**
14	AB074612.1.1302	Cytophagaceae-UB*	31,202	-0.5608	0.0016**
15	GU940705.1.1318	*Massilia* (UB-Oxalobacteraceae)*	89,727	-0.5523	0.0019**
16	GU295961.1.1444	*Oxalicibacterium* sp. JC-21 (Oxalobacteraceae)*	24	-0.5389	0.0026**
17	FJ960270.1.1374	*Candidatus captivus* (UB-Rickettsiales Incertae Sedis)*	211	-0.5357	0.0027**
18	AY838519.1.1507	*Massilia* (UB-Oxalobacteraceae)*	1561	-0.5275	0.0033**
19	GU208407.1.1430	Comamonadaceae-UB*	1,491	-0.5250	0.0035**
20	JQ278772.1.1501	*Candidatus nitrotoga* (UB-Gallionellaceae)	21	-0.5191	0.0039**
21	JX223693.1.1490	*Acidovorax* (UB-Comamonadaceae)*	149	-0.5189	0.0039**
22	JN869122.1.1525	*Massilia* (UB-Oxalobacteraceae)*	6,371	-0.5186	0.0039**

S/N	Species number	Name	Abundance	Spearman's R	P-value
23	X85209.1.1487	*Runella* (unidentified Cytophagaceae)*	272	-0.5184	0.0040**
24	DQ824754.1.1391	*Acidovorax* (UB- Comamonadaceae)*	329	-0.5158	0.0042**
25	FJ230930.1.1500	*Variovorax* (UB- Comamonadaceae)*	372	-0.5140	0.0043**
26	HQ592584.1.1491	*Variovorax* (UB- Comamonadaceae)*	72	-0.5106	0.0047**
27	EF516660.1.1284	Oxalobacteraceae-UB*	29	-0.5076	0.0049**
28	CU922352.1.1348	Comamonadaceae-UB*	39	-0.5075	0.0050**
29	EU801159.1.1495	*Limnohabitans* (UB- Comamonadaceae)*	27	-0.5043	0.0053**
30	JX105683.1.1389	*Undibacterium* (Oxalobacteraceae)*	44	-0.5023	0.0055**
31	EU800167.1.1396	*Variovorax* (UB- Comamonadaceae)*	38	-0.5004	0.0057**
32	AB220090.1.1368	*Methylobacterium* sp.PB145 (Methylobactericeae)*	39	-0.4954	0.0063**
33	KC505151.1.1394	*Aquabacterium* sp. JJ2211 (Comamonadaceae)*	9,925	-0.4929	0.0066**
34	EF018847.1.1380	Rhodocyclaceae-UB*	150	-0.4904	0.0069**
35	DQ675026.1.1502	*Sphingobacterium* (UB-Sphingobacteriaceae)*	49	-0.4866	0.0074**
36	JN596640.1.1414	Undibacterium (UB-Oxalobacteraceae)*	55	-0.4788	0.0086**
37	DQ316803.1.1405	Coxiellaceae-UB*	29	-0.4756	0.0091**
38	EU037350.1.1446	*Azospira* (UB- Rhodocyclaceae)*	8325	-0.4679	0.0105*
39	AB614560.1.1419	*Azohydromonas* (UB-Comamonadaceae)*	141	-0.4660	0.0108*
40	EU801055.1.1492	*Limnohabitans* (UB- Comamonadaceae)*	7,397	-0.4610	0.0118*
41	JF917188.1.1444	Comamonadaceae-UB*	386	-0.4606	0.0119*
42	JQ029110.1.1422	*Undibacterium* sp. CMJ-9 (Oxalobacteraceae)*	231	-0.4598	0.0121*
43	EU133861.1.1359	*Azoarcus* (UB-Rhodocyclaceae)*	43	-0.4564	0.0128*
44	KF010745.1.1485	Comamonadaceae-UB*	85,589	-0.4486	0.0147*
45	JN391900.1.1499	Comamonadaceae-UB*	178	-0.4470	0.0151*
46	KF385135.1.1392	*Undibacterium* sp. (UB-Oxalobacteraceae)*	246	-0.4413	0.0165*
47	GQ379585.1.1216	*Massilia* (UB-Oxalobacteraceae)*	103	-0.4407	0.0167*
48	GQ079259.1.1354	*Ramlibacter* (UB- Comamonadaceae)*	86	-0.4392	0.0171*
49	JN679119.1.1503	*Variovorax* sp. (UB-Comamonadaceae)*	80	-0.4355	0.0182*
50	AY662049.1.1519	*Undibacterium* (UB-Oxalobacteracea)*	69	-0.4330	0.0190*
51	JN020169.1.1341	Comamonadaceae-UB*	222	-0.4320	0.0193*
52	EU801679.1.1492	*Variovorax* (UB- Comamonadaceae)*	66	-0.4314	0.0195*
53	FJ152778.1.1486	Comamonadaceae-UB*	580	-0.4312	0.0195*
54	FJ793166.1.1501	*Polaromonas* (UB- Comamonadaceae)*	103	-0.4289	0.0203*
55	L79964.1.1326	*Sphaerotilus*- Eikelbloom type 1701 (Comamonadaceae)*	22	-0.4228	0.0233*
56	KC747739.1.1382	*Vibrio cholera* (Vibrionaceae)*	2,122	-0.4155	0.0250*
57	EF018476.1.1391	*Variovorax* (UB- Comamonadaceae)*	49	-0.4142	0.0255*
58	GQ472940.1.1224	*Comamonas* (UB- Comamonadaceae)*	41	-0.4084	0.0279*
59	EU133888.1.1352	UCT N117-UB	95	-0.4061	0.0288*
60	HM159974.1.1513	*Klebsiella* sp. (UB-Enterobacteriaceae)*	433	-0.4041	0.0297*
61	AY082472.1.1432	*Candidatus accumulibacter* (UB-Rhodocyclaceae)*	122	-0.4005	0.0313*
62	JN868886.1.1527	*Undibacterium* (UB-Oxalobacteraceae)*	1,716	-0.3978	0.0326*
63	GQ141052.1.1319	*Fibrella aestuarina* (Cytophagaceae)*	1,997	-0.3972	0.0329*
64	HQ860586.1.1449	*Brachymonas* (UB- Comamonadaceae)*	239	-0.3950	0.0339*
65	HQ121144.1.1504	*Variovorax* (UB- Comamonadaceae)*	398	-0.3887	0.0372*
66	JX223166.1.1492	*Undibacterium* (Oxalobacteraceae)*	51	-0.3871	0.0380*
67	JX105682.1.1391	*Undibacterium* (Oxalobacteraceae)*	147,248	-0.3804	0.0418*

S/N	Species number	Name	Abundance	Spearman's R	P-value
68	HE576067.1.1475	MOB164-Lactobacillales-UB	99,422	-0.3734	0.0460*
69	JF925020.1.1487	*Methyloversatilis* (UB-Rhodocyclaceae)	754	-0.3730	0.0463*
70	HQ755856.1.1434	*Pseudorhodoferax* (UB- Comamonadaceae)*	74	-0.3725	0.0466*
71	JQ191135.1.1356	Comamonadaceae-UB*	3,210	-0.3689	0.0489*
72	EU809285.1.1289	*Bdellovibrio* (UB-Bdellovibrionaceae)*	103	-0.3660	0.0509 ns
73	JX431991.1.1428	*Leptothrix* (UB- Comamonadaceae)*	71	-0.3639	0.0523 ns
74	AB672298.1.1460	Zoogloea (UB-Rhodocyclaceae)*	1,036	-0.3576	0.0568 ns
75	HQ856420.1.1500	*Methyloversatilis* (UB-Rhodocyclaceae)	2694	-0.3574	0.0570 ns
76	GQ001587.1.1339	*Runella* (UB-Cytophagaceae)*	122,959	-0.3541	0.0595 ns
77	FJ517705.1.1480	Env.OPS17-Sphingobacteriales-UB	85	-0.3515	0.0615 ns
78	CU926220.1.1354	*Simplicispira* (UB- Comamonadaceae)*	19	-0.3510	0.0619 ns
79	HQ178855.1.1461	*Variovorax* (UB- Comamonadaceae)*	18	-0.3457	0.0662 ns
80	FQ659997.1.1362	*Candidatus accumilibacter* (Rhodocyclaceae)	130	-0.3412	0.0701 ns
81	HE798191.1.1300	*Caldimonas hydrothermale* (Comamonadaceae)*	276	-0.3336	0.0770ns
82	JQ818823.1.1265	*Comamonas testoteroni* (Comamonadaceae)*	150	-0.3305	0.0799 ns
83	EF018596.1.1401	Acidovorax (Comamonadaceae-UB)*	334	-0.3159	0.0951 ns
84	FJ193907.1.1439	Comamonadaceae-UB*	20	-0.2986	0.1156 ns
85	JN391862.1.1500	Zoogloea (UB-Rhodocyclaceae)*	61,734	-0.2932	0.1227 ns
86	FN667146.1.1464	*Xenophilus* (UB-compost-Comamonadaceae)*	20	-0.2930	0.1230 ns
87	AB608673.1.1453	Comamonadaceae-UB	239,153	-0.2838	0.1357ns
88	CU925315.1.1355	*Variovorax* (UB- Comamonadaceae)*	19	-0.2591	0.1747 ns
89	HQ132422.1.1460	Endosymbiont of *Acanthamoeba* sp. (Rickettsiales)	168	-0.2463	0.1977 ns
90	DQ232437.1.1280	(Comamonadaceae- UB)*	276	-0.2444	0.2014 ns
91	HM445088.1.1309	*Sphingomonas* (UB-Sphingomonadaceae)*	788	-0.2024	0.2924 ns
92	JQ608102.1.1436	*Aeromonas* NLAE-zl-C396 (Aeromonadaceae)	24	-0.1457	0.4507 ns
93	AF245350.1.1489	*Dechloromonas*-SA35 (UB-Rhodocyclaceae)	32	-0.0154	0.9369 ns
94	DQ248264.1.1506	*Enterobacter* (Enterobacteriaceae)*	57	-0.0131	0.9462 ns
95	FJ439844.1.1439	UCT N117-UB	259	-0.0129	0.9471 ns
96	JF697561.1.1506	Hot Creek 32-UB	4,770	-0.0039	0.9839 ns

P-value: *** < 0.0001, ** < 0.005, * < 0.05, ns > 0.05. UB-Uncultured bacteria; *Name: recurring species/family/groups

Appendix G: (Chapter 7) Filter 2C- OTU abundance vs Nitrate @ Spearman's positive correlation +30.

S/N	Species number	Name	Abundance	Spearman's R	P-value
1	KC747739.1.1382	*Vibrio cholerae* (Vibrionaceae)*	2,122	0.6312	0.0002***
2	EU537205.1.1383	*Comamonas* (UB-Comamonadaceae)*	57	0.6162	0.0004***
3	HM779429.1.1454	MB19-Aeromonadales-UB	20	0.5952	0.0007***
4	FJ823923.1.1498	*Acidovorax* (UB-Comamonadaceae)*	936	0.5807	0.0010**
5	FJ347719.1.1277	Comamonadaceae-UB*	85	0.5704	0.0012**
6	FJ562171.1.1242	*Nitrobacter* (UB-Bradyrhizobiaceae)*	177	0.5672	0.0013**
7	JX521628.1.1499	*Acidovorax* (UB-Comamonadaceae)*	123	0.5635	0.0015**
8	EF555457.1.1469	*Achromobacter xylosoxidans* (Alcaligenaceae)	172	0.5606	0.0016**
9	FJ193907.1.1439	Comamonadaceae-UB*	20	0.5463	0.0022**
10	EF019908.1.1335	*Bradyrhizobium* (UB-Bradyrhizobiaceae)*	10	0.5425	0.0024**
11	GQ141052.1.1319	*Fibrella aestuarina* (Cytophagaceae)*	1,997	0.5301	0.0031**
12	FN667146.1.1464	*Xenophilus* (UB-compost-Comamonadaceae)*	20	0.5302	0.0031**
13	EU790196.1.1206	*Cupriavidus* (Burkholderiaceae)*	96	0.5275	0.0033**
14	GQ472940.1.1224	*Comamonas* (UB-Comamonadaceae)*	41	0.5208	0.0038**
15	DQ824754.1.1391	*Acidovorax* (UB-Comamonadaceae)*	329	0.5206	0.0038**
16	FM200992.1.1338	*Variovorax* (UB-Comamonadaceae)*	355	0.5181	0.0040**
17	JF071610.1.1354	*Comamonas* (UB-Comamonadaceae)*	33	0.5180	0.0040**
18	FJ823942.1.1499	*Cupriavidus* (UB-Burkholderiaceae)*	215	0.5045	0.0053**
19	HJ352584.1.1451	*Blastocatella* (UB-Acidobacteria)	40	0.5015	0.0056**
20	JF204571.1.1353	*Neisseriaceae* -UB	3,558	0.4996	0.0058**
21	AY945883.1.1500	*Variovorax* (UB-Comamonadaceae)*	91	0.4851	0.0077**
22	GU356337.1.1344	*Aeromonas* sp. (UB-JI49D030)	76	0.4841	0.0078**
23	AMQL01000001.12248.13793	(Enterobacteriaceae)*	99,658	0.4828	0.0080**
24	DQ017708.1.1459	*Deinococcus aqauticus* (Deinococcaceae)	50	0.4796	0.0085**
25	EU345000.1.1528	*Lactobacillus uvarum* (Lactobacillaceae)*	11	0.4777	0.0088**
26	EF428991.1.1402	*Serratia* sp. GIST-WP3w2 (Enterobacteriaceae)*	91	0.4777	0.0088**
27	JQ8181823.1.1265	*Comamonas testoteroni* (Comamonadaceae)*	150	0.4674	0.0106*

S/N	Species number	Name	Abundance	Spearman's R	P-value
28	FQ659997.1.1362	*Candidatus accumilibacter* (Rhodocyclaceae)	130	0.4596	0.0121*
29	KF465820.1.1231	*Rahnella* sp. BSP16 (Enterobacteriaceae)*	31	0.4584	0.0124*
30	KC357993.1.1269	*Dechloromonas* (UB-Rhodocyclaceae)*	158	0.4495	0.0144*
31	HM445088.1.1309	*Sphingomonas* (UB-Sphingomonadaceae)*	788	0.4485	0.0147*
32	HM779297.1.1455	*Plesiomonas* (UB-Enterobacteriaceae)*	45	0.4432	0.0160*
33	KF835793.1.1402	*Aeromonas hydrophila* (Aeromonadaceae)*	152	0.4378	0.0175*
34	HQ132422.1.1460	Endosymbiont of *Acanthamoeba* sp. (Rickettsiales)	168	0.4281	0.0205*
35	GU454953.1.1495	*Simplicispira* (UB-Comamonadaceae)*	30	0.4248	0.0216*
36	HM780351.1.1517	*Aeromonas* (uncultured Aeromonadaceae)*	229	0.4223	0.0225*
37	AY599666.1.1420	Env. OPS-Sphingobacteriales	15	0.4222	0.0225*
38	HM817497.1.1355	*Variovorax* (UB-Comamonadaceae)*	11	0.4179	0.0241*
39	AF143840.1.1341	*Extensismonas* (UB-Comamonadaceae)*	596	0.4148	0.0253*
40	HE576067.1.1475	MOB164-Lactobacillales-UB	99,422	0.4133	0.0258*
41	FJ849086.1.1446	Env. OPS-Sphingobacteriales-UB	52	0.4130	0.0260*
42	HQ219947.1.1457	*Erwinia persicina* (Enterobacteriaceae)*	15	0.4117	0.0265*
43	EU464873.1.1360	*Comamonas* (UB-Comamonadaceae)*	132	0.4094	0.0274*
44	EF438210.1.1467	(UB- Enterobacteriaceae)*	33	0.4091	0.0276*
45	AY770428.1.1413	*Chryseobacterium* SV70AB1-7 (Flavobacteriaceae)*	6,306	0.3937	0.0346*
46	AF307869.1.1218	*Pseudomonas putida* (Shewanellaceae)	327	0.3897	0.0366*
47	HM779467.1.1455	*Aeromonas* (UB-Aeromonadaceae)*	65	0.3870	0.0381*
48	AB066235.1.1271	*Comamonas* sp. PJ111 (Comamonadaceae)*	10	0.3860	0.0386*
49	KC775436.1.1234	*Rhizobium* (UB-Rhizobiaceae)*	33	0.3858	0.0387*
50	EU037350.1.1446	*Azospira* (UB- Rhodocyclaceae)*	8,325	0.3816	0.0411*
51	U20275.1.1508	*Serratia* (UB-Enterobacteriaceae)*	10	0.3682	0.0494*
52	CU921399.1.1357	*Uruburuella* (UB-Neiserriaceae)	21	0.3662	0.0508 ns
53	JN207176.1.1264	*Reyranella* (UB-Rhodospirillales Incertae Sedis)	50	0.3574	0.0570 ns
54	JT845935.1.1202	*Cenchrus americanus* (Moraxellaceae)*	21	0.3518	0.0612 ns
55	GU454983.1.1494	*Comamonas* (UB-Comamonadaceae)*	764	0.3462	0.0658 ns
56	GQ264256.1.1252	AKIW852-Sphingomonadales-UB	32	0.3461	0.0659 ns

S/N	Species number	Name	Abundance	Spearman's R	P-value
57	KC734322.1.1350	*Chryseobacterium* W19(2013) (Flavobacteriaceae)*	13,446	0.3361	0.0747 ns
58	FJ975867.1.1366	*Citrobacter* (UB-Enterobacteriaceae)*	712	0.3357	0.0750 ns
59	HM779400.1.1470	*Aeromonas* (uncultured Aeromonadaceae)*	493	0.3323	0.0782 ns
60	JF830187.1.1508	*Acinetobacter* (UB-Moraxellaceae)*	47	0.3280	0.0824 ns
61	KF039904.1.1408	*Budvicia* sp. S1-5 (Enterobacteriaceae)*	27	0.3216	0.0889 ns
62	JQ599074.1.1333	*Aeromonas* sp. 416 (Aeromonadaceae)*	328	0.3186	0.0921 ns
63	AY379977.1.1431	*Acidovorax* sp. AHL 5 (Comamonadaceae)*	357	0.3116	0.0999 ns
64	EF205512.1.1462	*Aeromonas* (UB-Aeromonadaceae)*	301	0.3089	0.1030 ns
65	EF018476.1.1391	*Variovorax* (UB-Comamonadaceae)*	49	0.3086	0.1034 ns
66	HM778860.1.1375	*Aeromonas* (uncultured Aeromonadaceae)*	80	0.2968	0.1180 ns
67	FN689611.1.1298	*Devosia* sp. (UB-Hyphomicrobiaceae)	84	0.2798	0.1416 ns
68	DD216085.5.1553	*Lactobacillus* (unidentified Lactobacillaceae)*	56	0.2795	0.1420 ns
69	KF698724.1.1398	*Cupriavidus basilensis* (Burkholderiaceae)	42	0.2770	0.1457 ns
70	EU704957.1.1244	*Rhodopseudomonas* sp. (UB-Bradyrhizobiaceae)*	120	0.2724	0.1529 ns
71	JQ924018.1.1444	*Sphingomonas* (UB-Sphingomonadaceae)*	18	0.2721	0.1534 ns
72	JF915349.1.1403	*Buttiauxella* sp. NW51 (Enterobacteriaceae)*	84	0.2708	0.1554 ns
73	HM756489.1.1293	*Serratia liquefaciens* (Enterobacteriaceae)*	108	0.2637	0.1669 ns
74	EU706241.1.1234	*Delftia* sp. (UB-Comamonadaceae)*	236	0.2572	0.1781 ns
75	AB546235.1.1369	*Rhodobacter* sp. INCT285 (Rhodobacteraceae)*	18,723	0.2479	0.1948ns
76	FJ901027.1.1324	*Azospira* (UB- Rhodocyclaceae)*	39	0.2397	0.2104 ns
77	EF010981.1.1437	*Pseudomonas putida* (Aeromonadaceae)*	157,079	0.2111	0.2717 ns
78	JF808900.1.1502	*Tolumonas* sp. (UB-Aeromonadaceae)*	7,676	0.2065	0.2825 ns
79	HM778671.1.1451	*Aeromonas* (UB-Aeromonadaceae)*	134	0.2064	0.2828 ns
80	EU283364.1.1448	Env. OPS-Sphingobacteriales-UB	119,871	0.1881	0.3285 ns
81	JN869165.1.1459	*Rhodobacter* (Rhodobacteraceae)*	44	0.1870	0.3313 ns
82	CU922480.1.1353	*Simplicispira* (UB-Comamonadaceae)*	17	0.1772	0.3577 ns

S/N	Species number	Name	Abundance	Spearman's R	P-value
83	JN120259.1.1367	*Aeromonas sobria* (Aeromonadaceae)*	107	0.1547	0.4230 ^{ns}
84	HM778865.1.1456	Aaa34a10-gammaproteobacteria-UB	18	0.1506	0.4355 ^{ns}
85	DQ813307.1.1471	*Pseudomonas* sp. IBUN MAR1 (Pseudomonadaceae)	1,042	0.1483	0.4426 ^{ns}
86	AB845279.1.1381	*Enterobacter* sp. Bdr5 (Enterobacteriaceae)*	544	0.1306	0.4994 ^{ns}
87	JX222143.1.1320	*Rhizobium* (UB-Rhizobiaceae)*	29	0.1259	0.5152 ^{ns}
88	DQ835530.1.1454	*Klebsiella oxytoca*(Enterobacteriaceae)*	644	0.0992	0.6087 ^{ns}
89	DI206702.1.1362	*Rhodobacter* sp. (Rhodobacteraceae)*	39	0.0960	0.6204 ^{ns}
90	EF153298.1.1421	*Acinetobacter* (UB-Moraxellaceae)*	340	0.0860	0.6574 ^{ns}
91	DQ069193.1.1519	B38-UB-Gammaproteobacteria	32	0.0708	0.7150 ^{ns}
92	AM179916.1.1363	*Plesiomonas* (UB-Enterobacteriaceae)*	92	0.0661	0.7333 ^{ns}
93	GU826686.1.1256	*Salmonella* enterica subsp. enterica serovar Typhi (Enterobacteriaceae)*	113	0.0057	0.9767 ^{ns}
94	GU356216.1.1374	*Aeromonas* sp. (UB-Aeromonadaceae)*	30	-0.0065	0.9733 ^{ns}

P-value: *** < 0.0001, ** < 0.005, * < 0.05, ^{ns} > 0.05. UB-Uncultured bacteria; *Name: recurring species.

Appendix H: (Chapter 7) Filter 3A: Highest bacteria abundance Log_{10} ratio between Control vs CWC & Control vs Zeolite at 200 larval density

S/N	Species number	Name	OTU abundance
1	FN436071.1.1449	*Ochrobactrum*- UB (Brucellaceae)	288
2	FJ375362.1.1346	*Azospirillum*- UB (Rhodospirillaceae)	21,218
3	FJ393109.1.1505	*Variovorax*- UB (Comamonadaceae)	437
4	KC633571.1.1346	*Bergeyella*- UB (Flavobacteriaceae)	171
5	FJ375487.1.1458	*Cloacibacterium*- UB (Flavobacteriaceae)	6,048
6	FJ418700.1.1325	*Sinorhizobium* sp. CCBAU 51063 (Rhizobiaceae)	52
7	APMI01000355.1.1237	Wastewater metagenome -UB (Chitinophagaceae)	1,966
8	HE583162.1.1377	*Delftia*-UB (Comamonadaceae)	6,019
9	AM403225.1.1369	Myroides (Flavobacteriaceae bacterium D11-24b1)	2,699
10	ARCM01000002.993920.995384	*Ancylobacter* sp. FA202 (Xanthobacteraceae)	14,777
11	AF050533.1.1409	Rhodovarius -Uncultured Eubacterium WCHB1-87 (Acetobacteraceae)	559
12	FN436173.1.1478	*Noviherbaspirillum*-UB (Oxalobacteraceae)	932
13	HE589822.1.1405	*Rhizobium*-UB (Rhizobiaceae)	3,289
14	JF706531.1.1366	*Xylophilus* sp. PDD-37_7j_hv_b-3 (Comamonadaceae)	55,011
15	HE589840.1.1456	*Acidovorax*-UB (Comamonadaceae)	1,280
16	AY316683.1.1416	*Reichenowia parasiticae (*Rhizobiaceae)	78
17	JQ359107.1.1375	*Rhizobium* sp. DoB64 (Rhizobiaceae)	303
18	KC424772.1.1493	*Comamonas*-UB (Comamonadaceae)	340
19	AB487891.1.1338	*Clostridium* sensu stricto 10-UB (Clostridiaceae)	550

Appendix I (Chapter 7) Log_{10} DNA (copies/µl) of candidate bacteria species across *An. coluzzii* larval treatment trays.

S/N	Species	Treatment	Larval density	Feed	Log10 DNA (copies/µl)
1.	*Aeromonas hydrophila* (Aeromonadaceae)	Control	200	Powder	8.88 (4.74 – 13.02)
				Solution	8.15 (6.13 – 10.17)
			400	Powder	8.68 (5.93 – 11.43)
				Solution	8.75 (6.59 – 10.91)
		CWC	200	Powder	8.55 (7.44 – 9.65)
				Solution	8.05 (7.13 – 8.96)
			400	Powder	7.73 (5.19 – 10.28)
				Solution	7.44 (5.62 – 9.25)
		CWZ	200	Powder	7.65 (6.23 – 9.08)
				Solution	8.01 (7.25 – 8.76)
			400	Powder	9.86 (8.81 – 10.91)
				Solution	9.56 (7.41 – 11.71)
		Zeolite	200	Powder	7.80 (5.47 – 10.14)
				Solution	9.41 (5.19 – 13.63)
			400	Powder	9.97 (5.20 – 14.73)
				Solution	9.30 (4.45 – 14.14)
2	*Ancylobacter sp.* FA202 (Xanthobacteraceae)	Control	200	Powder	8.40 (6.43 – 10.36)
				Solution	8.58 (6.71 – 10.46)
			400	Powder	9.33 (7.10 – 11.55)
				Solution	8.43 (6.60 – 10.26)
		CWC	200	Powder	8.01 (7.67 – 8.35)
				Solution	8.43 (7.57 – 9.28)
			400	Powder	8.21 (6.39 – 10.03)
				Solution	7.76 (6.79 – 8.73)
		CWZ	200	Powder	8.08 (6.28 – 9.88)
				Solution	8.31 (7.98 – 8.64)
			400	Powder	9.01 (7.69 – 10.33)
				Solution	8.63 (7.78 – 9.49)
		Zeolite	200	Powder	8.85 (7.31 – 10.40)
				Solution	9.09 (5.87 – 12.32)
			400	Powder	8.88 (5.70 – 12.06)
				Solution	9.27 (6.70 – 11.84)
3	*Bacillus weihenstephanensis* (Bacillaceae)	Control	200	Powder	7.17 (0.98 – 13.37)
				Solution	9.08 (6.74 – 11.41)
			400	Powder	9.15 (7.65 – 10.65)
				Solution	7.28 (3.98 – 10.58)
		CWC	200	Powder	6.67 (3.08 – 10.26)
				Solution	7.63 (4.26 – 10.99)
			400	Powder	6.73 (3.98 – 9.48)
				Solution	5.77 (1.70 – 9.84)
		CWZ	200	Powder	5.93 (1.60 – 10.27)
				Solution	6.94 (2.10 – 11.78)
			400	Powder	7.29 (2.58 – 12.00)
				Solution	7.09 (3.36 – 10.82)
		Zeolite	200	Powder	7.51 (2.05 – 12.96)
				Solution	15.31 (-9.23 – 39.86)

S/N	Species	Treatment	Larval density	Feed	Log10 DNA (copies/µl)
			400	Powder	14.47 (-12.34 – 41.29)
				Solution	8.34 (5.14 – 11.54)
4	*Brevundimonas diminuta* (Caulobacteraceae)	Control	200	Powder	4.14 (-2.34 – 10.62)
				Solution	7.05 (1.12 – 12.97)
			400	Powder	5.64 (1.94 – 9.34)
				Solution	4.63 (0.55 – 8.71)
		CWC	200	Powder	5.71 (1.89 – 9.52)
				Solution	6.94 (4.42 – 9.47)
			400	Powder	5.03 (2.01 – 8.05)
				Solution	4.30 (0.49 – 8.10)
		CWZ	200	Powder	4.76 (-0.26 – 9.79)
				Solution	6.16 (1.62 – 10.70)
			400	Powder	6.54 (1.55 – 11.54)
				Solution	5.19 (1.45 – 8.93)
		Zeolite	200	Powder	5.05 (0.40 – 9.70)
				Solution	6.41 (-0.06 – 12.88)
			400	Powder	7.20 (-2.23 – 16.64)
				Solution	15.26 (-7.03 – 37.56)
5	*Nubsella sp.* EsD18 (Sphingobacteriaceae)	Control	200	Powder	8.38 (7.16 – 9.61)
				Solution	9.35 (7.12 – 11.59)
			400	Powder	8.55 (6.97 – 10.12)
				Solution	8.14 (6.54 – 9.74)
		CWC	200	Powder	8.58 (7.03 – 10.14)
				Solution	8.65 (7.20 – 10.10)
			400	Powder	8.09 (7.28 – 8.91)
				Solution	7.75 (6.18 – 9.32)
		CWZ	200	Powder	8.21 (6.63 – 9.78)
				Solution	8.69 (6.99 – 10.38)
			400	Powder	8.61 (7.15 – 10.07)
				Solution	8.58 (7.61 – 9.56)
		Zeolite	200	Powder	8.22 (6.63 – 9.80)
				Solution	8.62 (5.28 – 11.96)
			400	Powder	9.11 (5.22 – 13.01)
				Solution	10.19 (4.31 – 16.07)
6	*Pseudoxanthobacter soli*-DSM 19599 (Xanthobacteraceae)	Control	200	Powder	7.62 (5.35 – 9.90)
				Solution	8.19 (5.28 – 11.11)
			400	Powder	12.06 (2.82 – 21.30)
				Solution	8.68 (5.24 – 12.12)
		CWC	200	Powder	8.47 (7.83 – 9.11)
				Solution	7.98 (6.38 – 9.58)
			400	Powder	6.60 (5.51 – 7.69)
				Solution	6.84 (5.75 - 7.94)
		CWZ	200	Powder	7.00 (5.05 – 8.96)
				Solution	7.83 (6.20 – 9.46)
			400	Powder	8.64 (6.06 – 11.22)
				Solution	7.40 (6.16 – 8.64)
		Zeolite	200	Powder	7.54 (6.15 – 8.92)
				Solution	10.31 (1.41 – 19.22)
			400	Powder	9.85 (3.82 – 15.87)

S/N	Species	Treatment	Larval density	Feed	Log10 DNA (copies/µl)
				Solution	11.35 (2.85 – 19.85)
7	*Ramlibacter tataouinensis* (Comamonadaceae)	Control	200	Powder	7.33 (2.94 – 11.72)
				Solution	7.36 (-0.25 – 14.98)
			400	Powder	8.80 (1.52 – 16.07)
				Solution	6.57 (-2.12 – 15.25)
		CWC	200	Powder	9.63 (5.83 – 13.43)
				Solution	8.30 (0.40 – 16.21)
			400	Powder	7.76 (-0.49 – 16.01)
				Solution	6.64 (-3.17 – 16.46)
		CWZ	200	Powder	7.70 (1.95 – 13.46)
				Solution	8.84 (1.88 – 15.80)
			400	Powder	9.40 (6.20 – 12.59)
				Solution	8.70 (3.53 – 13.87)
		Zeolite	200	Powder	7.23 (1.65 – 12.80)
				Solution	10.44 (-4.39 – 25.28)
			400	Powder	7.92 (-4.56 – 20.39)
				Solution	9.79 (7.27 – 12.30)
8	*Sphingomonas wittichii* (Sphingomonadaceae)	Control	200	Powder	9.66 (8.59 – 10.74)
				Solution	10.98 (2.63 – 19.34)
			400	Powder	12.37 (7.22 – 17.51)
				Solution	9.87 (6.52 – 13.23)
		CWC	200	Powder	8.96 (5.67 – 12.25)
				Solution	10.21 (3.04 – 17.38)
			400	Powder	9.65 (4.88 – 14.42)
				Solution	8.90 (6.12 – 11.69)
		CWZ	200	Powder	8.94 (6.54 – 11.33)
				Solution	9.07 (5.64 – 12.51)
			400	Powder	11.04 (9.01 – 13.07)
				Solution	10.13 (7.73 – 12.52)
		Zeolite	200	Powder	9.23 (6.91 – 11.55)
				Solution	10.86 (2.69 – 19.04)
			400	Powder	12.07 (6.57 – 17.57)
				Solution	13.09 (5.96 - 20.21)
9	*Turneriella parva* DSM 21527 (Leptospiraceae)	Control	200	Powder	9.65 (8.85 – 10.44)
				Solution	9.54 (7.77 – 11.32)
			400	Powder	10.17 (8.11 – 12.23)
				Solution	9.67 (6.98 – 12.37)
		CWC	200	Powder	9.73 (7.05 – 12.42)
				Solution	9.75 (6.85 – 12.66)
			400	Powder	9.16 (6.43 – 11.90)
				Solution	9.04 (6.60 – 11.49)
		CWZ	200	Powder	9.09 (7.37 – 10.81)
				Solution	9.64 (6.87 – 12.40)
			400	Powder	9.97 (7.68 – 12.26)
				Solution	9.97 (7.98 – 11.96)
		Zeolite	200	Powder	9.17 (6.72 – 11.61)
				Solution	11.03 (4.22 – 17.84)
			400	Powder	10.61 (5.87 – 15.35)
				Solution	12.32 (4.82 – 19.82)
10		Control	200	Powder	10.74 (7.06 – 14.43)

S/N	Species	Treatment	Larval density	Feed	Log10 DNA (copies/µl)
	Xylophilus sp. PDD-37_7j_hv_b-3 (Comamonadaceae)			Solution	9.59 (6.59 – 12.60)
			400	Powder	10.62 (8.16 – 13.09)
				Solution	9.38 (5.95 – 12.82)
		CWC	200	Powder	9.62 (5.17 – 14.07)
				Solution	10.21 (3.73 – 16.69)
			400	Powder	10.71 (1.75 – 19.67)
				Solution	8.98 (4.33 – 13.64)
		CWZ	200	Powder	9.25 (6.98 – 11.52)
				Solution	9.47 (5.16 – 13.79)
			400	Powder	10.78 (6.83 – 14.72)
				Solution	9.99 (5.80 – 14.18)
		Zeolite	200	Powder	9.20 (6.39 – 12.00)
				Solution	10.27 (2.91 – 17.64)
			400	Powder	15.24 (-0.39 – 30.87)
				Solution	20.72 (-15.15 – 56.60)

Notes: 95% Confidence intervals are in parenthesis and sample size = 4.

Appendix J: KAPABIOSYSTEMS SYBR FAST qPCR protocol link

KAPABIOSYSTEMS

Technical Data Sheet

KAPA SYBR® FAST qPCR Master Mix (2X) Kit

KR0389 – v10.16

Product Description

KAPA SYBR FAST qPCR Master Mix (2X) is designed for high-performance real-time PCR. The kit contains a novel DNA polymerase—engineered via a process of molecular evolution—resulting in a unique enzyme specifically designed for real-time quantitative PCR (qPCR) using SYBR Green I dye chemistry.

KAPA SYBR FAST DNA Polymerase has been engineered to perform optimally in stringent qPCR reaction conditions, exhibiting dramatic improvements in signal-to-noise ratio (fluorescence), quantification cycle (Cq), linearity, and sensitivity. The KAPA SYBR FAST DNA Polymerase and proprietary buffer system improves the amplification efficiency of difficult targets, including both GC- and AT-rich templates.

KAPA SYBR FAST qPCR Master Mix (2X) Kits are a ready-to-use cocktail containing all components (except primers and template) for the amplification and detection of DNA in qPCR. The KAPA SYBR FAST qPCR Kit is supplied as a 2X master mix with integrated antibody-mediated hot start, SYBR Green I fluorescent dye, $MgCl_2$, dNTPs, and stabilizers.

Ensure that the correct KAPA SYBR FAST qPCR Master Mix (2X) is used in accordance with the reference dye requirements (if any) of the qPCR instrument (Table 1).

Product Applications

KAPA SYBR FAST qPCR Kits are ideally suited for:

- gene expression analysis;
- gene knockdown validation;
- microarray validation;
- low copy gene detection; and
- absolute quantification of NGS libraries (when sold as part of the KAPA Library Quantification Kit).

Kapa/*Roche* Kit Codes and Components	
KAPA SYBR FAST qPCR Master Mix (2X) Universal qPCR Master Mix (2X) ROX High Reference Dye (50X) ROX Low Reference Dye (50X)	**KK4600** – *07959362001* (1 mL; 100 x 20 µL rxn)
	KK4601 – *07959389001* (5 mL; 500 x 20 µL rxn)
	KK4602 – *07959397001* (10 mL; 1000 x 20 µL rxn)
	KK4618 – *07959567001* (50 mL; 5000 x 20 µL rxn)
KAPA SYBR FAST qPCR Master Mix (2X) ABI Prism™ qPCR Master Mix (2X) with ROX High incorporated	**KK4603** – *07959419001* (1 mL; 100 x 20 µL rxn)
	KK4604 – *07959427001* (5 mL; 500 x 20 µL rxn)
	KK4605 – *07959435001* (10 mL; 1000 x 20 µL rxn)
	KK4617 – *07959559001* (50 mL; 5000 x 20 µL rxn)
KAPA SYBR FAST qPCR Master Mix (2X) optimized for LightCycler® 480 qPCR Master Mix (2X) with no passive reference dye	**KK4609** – *07959478001* (1 mL; 100 x 20 µL rxn)
	KK4610 – *07959486001* (5 mL; 500 x 20 µL rxn)
	KK4611 – *07959494001* (10 mL; 1000 x 20 µL rxn)
KAPA SYBR FAST qPCR Master Mix (2X) ROX Low qPCR Master Mix (2X) with ROX Low incorporated	**KK4619** – *07959575001* (1 mL; 100 x 20 µL rxn)
	KK4620 – *07959583001* (5 mL; 500 x 20 µL rxn)
	KK4621 – *07959591001* (10 mL; 1000 x 20 µL rxn)
	KK4622 – *07959605001* (50 mL; 5000 x 20 µL rxn)
KAPA SYBR FAST qPCR Master Mix (2X) Bio-Rad iCycler qPCR Master Mix (2X) with fluorescein incorporated	**KK4606** – *07959443001* (1 mL; 100 x 20 µL rxn)
	KK4607 – *07959451001* (5 mL; 500 x 20 µL rxn)
	KK4608 – *07959460001* (10 mL; 1000 x 20 µL rxn)

Quick Notes

- This kit contains an engineered enzyme optimized for qPCR using SYBR Green I dye chemistry.
- The 2X master mix contains a proprietary buffer. Together with the novel enzyme, this improves amplification efficiency of both GC- and AT-rich targets.
- 20 sec initial denaturation at 95°C is sufficient for enzyme activation. When working with complex templates, an initial denaturation of 3 min is recommended.
- For 3-step cycling, use 20 sec for primer annealing and 1 sec for extension/data acquisition at 72°C.
- Do not exceed 25 µL reaction volumes.

Appendix K: 16S rRNA metagenomic sequencing library preparation workflow protocol link

16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System

Part # 15044223 Rev. B

Appendix L: KAPABIOSYSTEMS sequence library quantification kit protocol link

KAPABIOSYSTEMS

Technical Data Sheet

KAPA Library Quantification Kit
Illumina® Platforms

KR0405 – v8.17

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Library Quantification Kits for Illumina platforms.

This document applies to KAPA Library Quantification Kits for Illumina platforms (07960166001, 07960140001, 07960204001, 07960255001, 07960336001 and 07960298001), KAPA Library Quantification Primer and PCR mix Kits for Illumina platforms (07960441001, 07960484001, 07960522001, 07960727001 and 07960573001), KAPA Library Quantification Standards and Primer Kit for Illumina platforms (07960085001), KAPA Library Quantification Standards Kits for Illumina platforms (07960409001, and 07960409001), KAPA Library Quantification Dilution Control Kit for Illumina platforms (07960417001), and KAPA Library Quantification Primer Kit for Illumina platforms (07960093001).

Contents

Kapa/*Roche* Kit Codes and Components	
Complete Kit with: DNA Standards 1 – 6 (80 µL each) Primer Mix (0.2 mL) KAPA SYBR® FAST qPCR Master Mix (1 mL)	**KK4828** – *07960166001* Universal qPCR Master Mix 200 µL ROX High (50X) and ROX Low (50X) supplied separately
Complete Kits with: DNA Standards 1 – 6 (80 µL each) Primer Mix (1 mL) KAPA SYBR® FAST qPCR Master Mix (5 mL)	**KK4824** – *07960140001* Universal qPCR Master Mix 200 µL ROX High (50X) and ROX Low (50X) supplied separately
	KK4835 – *07960204001* ABI Prism™ qPCR Master Mix
	KK4844 – *07960255001* Bio-Rad iCycler™ qPCR Master Mix
	KK4873 – *07960336001* ROX Low qPCR Master Mix
	KK4854 – *07960298001* qPCR Master Mix optimized for LightCycler® 480
Kits with: Primer Mix (1 mL) KAPA SYBR FAST qPCR Master Mix (5 mL)	**KK4923** – *07960441001* Universal qPCR Master Mix 200 µL ROX High (50X) and ROX Low (50X) supplied separately
	KK4933 – *07960484001* ABI Prism qPCR Master Mix
	KK4943 – *07960522001* Bio-Rad iCycler qPCR Master Mix
	KK4973 – *07960727001* ROX Low qPCR Master Mix
	KK4953 – *07960573001* qPCR Master Mix optimized for LightCycler 480
Kit with: Primer Mix (1 mL) DNA Standards (80 µL)	**KK4808** – *07960085001* DNA Standards 1 – 6
Kits with: DNA Standards (80 µL)	**KK4903** – *07960387001* DNA Standards 1 – 6 **KK4905** – *07960409001* DNA Standards 0 – 6
Kit with: Dilution Control (80 µL)	**KK4906** – *07960417001* DNA Standard 0
Kit with: Primer Mix (1 mL)	**KK4809** – *07960093001*

Quick Notes

- The DNA Standards provided in the kit represent a 10-fold dilution series (20 pM to 0.0002 pM).
- Ensure that the libraries to be quantified are compatible with the qPCR quantification primer sequences given on the next page.
- Select the correct version of KAPA SYBR FAST qPCR Master Mix for the qPCR instrument to be used.
- Refer to the **KAPA Library Quantification Technical Guide** for a more in-depth discussion of the various factors affecting accurate library quantification.

Effective date: January 2017

Appendix M: Sequence data clustering and secondary analyses (CLC) protocol link

Tutorial

OTU Clustering Step by Step

March 2, 2017

Sample to Insight

QIAGEN Aarhus · Silkeborgvej 2 · Prismet · 8000 Aarhus C · Denmark
Telephone: +45 70 22 32 44 · www.qiagenbioinformatics.com ·
AdvancedGenomicsSupport@qiagen.com

Appendix N: Qiagen Blood and tissue DNA extraction protocol link.

July 2006

DNeasy® Blood & Tissue Handbook

DNeasy Blood & Tissue Kit
DNeasy 96 Blood & Tissue Kit

For purification of total DNA from
- animal blood
- animal tissue
- rodent tails
- ear punches
- cultured cells
- fixed tissue
- bacteria
- insects

QIAGEN

WWW.QIAGEN.COM

Appendix O: Agencourt Ampure PCR purification protocol link

AGENCOURT® AMPURE®
PCR PURIFICATION

Please refer to http://www.agencourt.com/technical/ for updated protocols and refer to MSDS instructions when handling or shipping any chemical hazards.
AGENCOURT AMPURE is a registered trademark of Agencourt Bioscience and is for laboratory use only.

Agencourt AMPure PCR Purification Table of Contents

Introduction

The Agencourt AMPure PCR[1] Purification system utilizes Agencourt's solid-phase paramagnetic bead technology for high-throughput purification of PCR amplicons. Agencourt AMPure utilizes an optimized buffer to selectively bind PCR amplicons 100bp and larger to paramagnetic beads. Excess oligos, nucleotides, salts, and enzymes can be removed using a simple washing procedure. The resulting purified PCR product is essentially free of contaminants and can be used in the following applications:

- Fluorescent DNA sequencing, including capillary electrophoresis
- Microarray spotting[2]
- Cloning[2]
- Primer extension genotyping

The purification procedure is highly amenable to a variety of automation platforms because it utilizes magnetic separation and requires no centrifugation or vacuum filtration. More

[1] *The PCR process is covered by patents owned by Roche Molecular Systems, Inc., and F. Hoffman-La Roche, Ltd.*

[2] Please e-mail or call Agencourt support for beta protocols (support@agencourt.com; 1-800-773-9186)