KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNNOLOGY

COLLEGE OF SCIENCE

AN INTEGRATED APPROACH TO BURULI ULCER TRANSMISSION STUDIES: FROM AQUATIC ENVIRONMENTS TO HUMAN INFECTION

BY

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(MPhil. Applied Parasitology) (PG 4998210)

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DECLARATION

I hereby declare that this research thesis submitted to the Department of Theoretical and Applied Biology is the original work towards my PhD degree and that, it has not been presented anywhere for award of a degree and contains no materials previously published by another person nor material except for references to other peoples' work which have been duly acknowledgement in the text.

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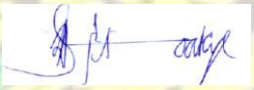

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DEDICATION

To My Family:

Both the one I have and the Ones I belong to.

ABSTRACT

Buruli ulcer (BU) is a debilitating skin infection usually of impoverished tropical rural populations. BU is caused by *Mycobacterium ulcerans* (MU), a pathogen described as a non-tuberculous mycobacteria (NTM). The exact mode of transmission of BU is not known and involvement of insect vectors including mosquitoes, animal and small mammal reservoirs and aerosols in transmission and dispersal has been proposed. A holistic approach to studies that involve NTM is proposed especially in transmission and control studies. This study related human MU infections to MU types in identified risk areas and establishing sources of human infections. Water body type preferences of members of the two most implicated insect families in BU transmission, Family Belostomatidae (Hemiptera: Heteroptera) and Family Naucoridae (Hemiptera: Heteroptera) collected from water bodies in Ghana and Cote d'Ivoire were also related to their probability of being involved in transmission of BU. Four BU endemic communities (Bepotenten, Sukuumu, Monia-Gyaman and Wromanso) in the Amansie Central District of the Ashanti Region of Ghana were selected for the study. Two hundred and twenty-four (224) questionnaires administered proportionally to population were used to elicit responses to water use and areas of contact, BU knowledge, animal contact and infection status. Ground truthing of the four communities and the capturing of geographical information allowed the reconciliation of questionnaire water contact points and the subsequent mapping to reveal all surface water contact points. *M. ulcerans* from environmental sources was detected using 16S rRNA and then *IS2404* detections. *M. ulcerans* genetic material from both human BU cases and identified environmental sources were VNTR (viable number tandem repeats)

typed at four loci (VNTR targets: MIRU1, Locus 6, ST1 and Locus 19. Swimming and bathing (p=0.04) in risk environments and the capture of squirrels (p=0.04) were associated with an increased in the risk of acquiring BU in the study communities (OR= 3.28 and OR=5.85 respectively). Rigorous activity in infected water was found to increase the risk of infection laying credence on a direct trauma infection route for *M. ulcerans*. VNTR typing of MU from human BU disease and environmental samples revealed localized infection with inhabitants mostly being infected from community associated water bodies. Four main VNTR types were detected in human disease MU based on number of copies of four loci: MIRU1, Locus 6, ST1 and Locus 19. These were 'W' (1,1,2,1), 'X' (1.1.2.2), 'Y' (1,2,2,1) and 'Z' (1,2,2,2) in the copy number order MIRU1, Locus 6, ST1, Locus 19. The 'Y' VNTR type was common to all communities being detected in human as well as environmental samples and is therefore the profile causing the most infections in the study communities. A total of 125 and 321 individual Naucoridae (Hemiptera: Heteroptera) and Belostomatidae (Hemiptera: Heteroptera) respectively were classified to at least genus level. Eight species of the Naucoridae were identified; *Ctenopocoris africanus* (Leach, 1815), *Aneorocoris insolitus* (Montandon, 1897), *Laccocoris limicola* (Stal, 1855), *Laccocoris spurcus congoensis* (Poisson, 1949), *Neomacrocoris bondelaufa* (Sites, 2015), *Neomacrocoris vuga* (Sites, 2015), *Naucoris obscuratus* (Montandon, 1913) and *Neomacrocoris usambaricus* (Montandon, 1913). Differences in habitat preference of the Naucoridae were observed. *Naucoris* spp and *Laccocoris* spp showed the least preference for lotic water bodies ($p<0.05$). Four genera of the family Belostomatidae; *Abedus*,

Diplonychus, *Lethocerus* and *Benacus*, were identified in collections from 82 water bodies in Ghana and 7 water bodies in Cote d'Ivoire. *Abedus* spp were the most abundant and most widely distributed. Belostomatidae showed no preference to type of water flow (p=0.2068). The integrated approach of the study offered complementary information, observations and deductions which together enabled verifiable conclusions to be drawn on the transmission of the environmental mycobacterium. The use of few communities facilitated a more concentrated search for risk areas and their link to BU infections in the study communities. A study involving a higher number of communities in a similar geographical range is recommended. In insect transmission studies, individual species competence should be considered and generalizations minimized as species preferences to habitat are important especially in studies involving NTMs.

LIST OF PUBLICATIONS/CONFERENCES

List of Publications

Quaye C, Mosi L, Narh AC, Dassi C, Konan DO, Twumasi-Mensah T, ObiriDanso K, Boakye DA, Bonfoh B. (2017). *Importance of Mapping in Risk Determination for Buruli ulcer Transmission Studies*. Submitted to EcoHealth (Under editorial review).

Narh CA, Mosi L, **Quaye C**, Dassi C, Konan DO, Tay SCK, de Souza D, Boakye DA, Bonfoh B. (2015). *Source Tracking Mycobacterium ulcerans Infections in the Ashanti Region,* Ghana. PLoS Neglected Tropical Diseases 9(1)

Dassi C, Mosi L, Akpatou B, Narh CA, **Quaye C**, Konan DO, Djaman JA, Bonfoh B. (2015). *Detection of Mycobacterium ulcerans in Mastomys natalensis and Potential Transmission in Buruli ulcer Endemic Areas in Côte d'Ivoire.* Mycobacterial Diseases 5:184.

Conference Proceedings/Abstracts

Oral Presentation: One Health EcoHealth Conference, 3-7 December 2016, Melbourne, Australia - *Deciphering Transmission of an Environmental Mycobacterial Infection: Is an EcoHealth approach plausible?*

Oral Presentation: African Meeting on EcoHealth, 1-4 October 2014, Grand Bassam, Cote d'Ivoire. Title: *Identifying risk environments and behaviours for Buruli ulcer infection: The use of maps and a simple questionnaire.*

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LIST OF ABBREVIATIONS AND ACRONYMS

NTM	Non-tuberculous mycobacteria
BU	Buruli ulcer
MUD	*Mycobacterium ulcerans* disease
WHO	World Health Organization
PCR	Polymerase Chain Reaction
MPM	Mycolactone-Producing-Mycobacteria
MAC	*Mycobacterium avium* complex
NZD	Neglected Zoonotic disease

VNTR	Variable Number Tandem Repeats
NTD	Neglected Tropical Disease
ARB	Amoeba Resistant Bacteria
LLAPs	Legionella-like amoebal pathogens
IS2404 / *IS2606*	Insertion sequence 2404 / Insertion Sequence 2606
ER	Enoyl Reductase
KR	Keto Reductase
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotype Polymorphisms
DNA	Deoxyribonucleic Acid
ST1	Sequence typing number 1
MIRU1	Mycobacterial interspersed repetitive unit 1
ACDHD	Amansie Central District Health Directorate
FNA	Fine Needle Aspirate
PBS	Phosphate Buffered Saline
RCF	Relative Centrifugal Force
EDTA	Ethylenediamine tetraacetic acid
NaCl	Sodium Chloride
SDS	Sodium dodecyl sulfate
RNase	Ribonuclease
RNA	Ribonucleic Acid

v/v	volume/volume percent
AMI	Aquatic Macro Invertebrate
mm	millimeter
m	meter
km	kilometer
g	grams
ml	milliliter
Inch	Inches
µm	micrometer
OR	Odds Ratio
CI	Confidence Interval
GPS	Geographical Positioning System

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Environmental mycobacteria, which are generally free-living saprophytes, have been detected in or isolated from a wide variety of environments including water, soil, dust and aerosols (Falkinham, 1996; Falkinham, 2011). Some of these environmental mycobacteria are described as emerging pathogens, causing opportunistic infections in humans and animals. These environmental emerging pathogens include the non-tuberculous mycobacteria (NTM) (Wolinsky, 1979). The health and economic impacts of human-environmental mycobacterial interactions are complex and likely much broader than is currently known (Tortoli *et al.*, 2001; Primm *et al.*, 2004). Reviews of the epidemiology of NTM describe *Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium avium complex, Mycobacterium paratuberculosis* and *Mycobacterium ulcerans*, as examples of over 30 common NTM found in various environments that may cause human and animal infections (Falkinham, 1996; Katoch, 2004).

Buruli ulcer (BU), also referred to as *Mycobacterium ulcerans* disease (MUD), is a necrotizing skin infection of humans reported mainly in rural communities of the tropical and subtropical regions of the world (WHO, 2014a). The infection, caused by *M. ulcerans*, has been reported in over 32 countries worldwide (Figure 1.1). Buruli ulcer typically begins as a nodule or papule which often ulcerates within a few weeks or as an extensive oedema which also ulcerates into some of the most extensive forms of the infection (Figure 1.2).

The geographical distribution and focal nature of the occurrence of BU clearly suggest that environmental factors and local ecology play major roles in the epidemiology of the infection. The exact prevalence of the infection even in recognized endemic areas is however still not certain as the reporting of new infections appear to be on a steady rise (WHO, 2014a). Earlier epidemiological studies found the disease biased toward age and gender with most infections in children under 15 years and in females (Debacker *et al.*, 2004), however, more recent reports describe infection not to be gender or age correlated (Bratschi *et al.*, 2013). Until recently, the only form of treatment was the excision of infected tissue (surgery) followed by grafting of infected area with skin tissue from other parts of the body. This left sufferers with extensive scaring and deformities (Adu *et al.*, 2011). The exclusive use of antibiotics for the treatment of the disease, a recommendation by the WHO in 2004, has been reported successful in many cases but the development of resistance to effective antibiotics and long therapeutic duration remains a major concern (Sarfo *et al.*, 2010; Sarfo *et al.*, 2013; Yeboah-Manu *et al.*, 2013; Converse *et al.*, 2015).

The challenges of understanding, diagnosing and treating infections caused by NTM are also characteristic to *M. ulcerans* (Katoch, 2004). The precise mode of transmission of BU is still not clearly understood although an enormous amount of information is available on the biology, pathogenesis and the genetics of the pathogen (Stinear *et al.*, 2004; Yip *et al.*, 2007; En *et al.*, 2008; Merritt *et al.*, 2010; En *et al.*, 2011; Roltgen *et al.*, 2012; Trubiano *et al.*, 2013).

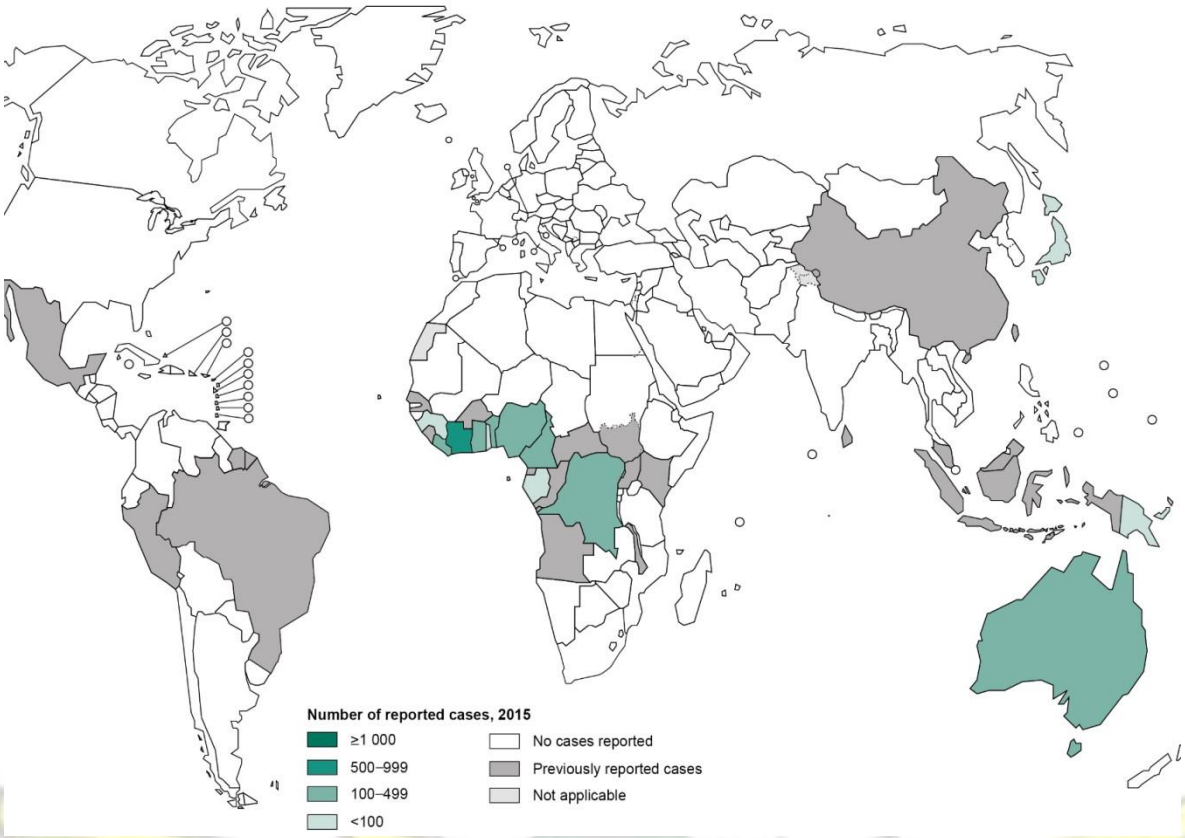


Figure 1.1 Global distributions of reported cases of Buruli ulcer in 2015. West Africa (Ghana, Cote d'Ivoire and Benin) are the most affected (WHO, 2015).

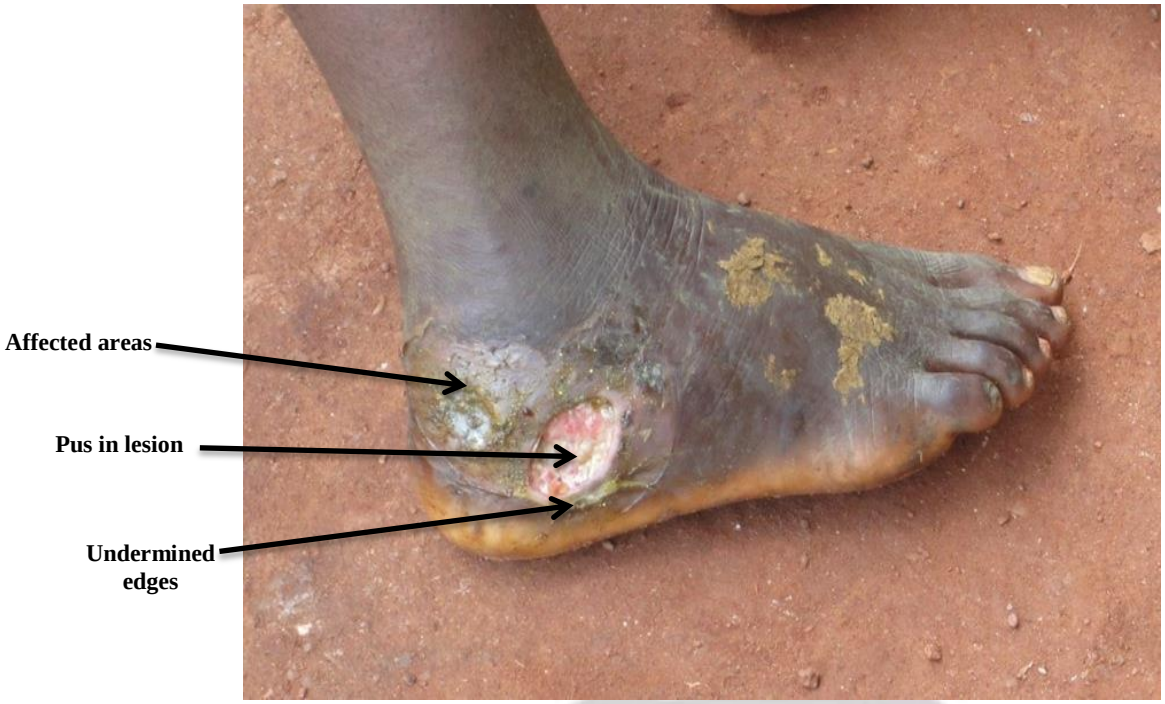


Figure 1.2 A typical case of Buruli ulcer on the foot, observed in the Zaibo community in Cote d'Ivoire.

Lesion shows undermined edges. Case had not undergone any hospital treatment regimen. Permission to use this photo was by kind courtesy of Dr. Christelle Dassi of the Centre Suisse de Recherches Scientifiques en Cote d'Ivoire, Abidjan, Cote d'Ivoire.

Studies have demonstrated that the use of routine methods in culturing *M. ulcerans* from environmental samples have not been successful due to contamination from rapidly growing bacteria (Merritt *et al.*, 2010). Hence ecological studies and the general detection of the pathogen especially in environmental samples has relied on the detection of a variety of *M. ulcerans* genetic markers by polymerase chain reaction (Ross *et al.*, 1997; Stinear *et al.*,

1999; Williamson *et al.*, 2008). A viable strain of the pathogen has only once been successfully isolated, through very complex procedures, from an environmental source. This was from a hemipteran of the Family Gerridae. This single study established a risk of viable human infections occurring from environmental sources (Portaels *et al.*, 2008). The molecular technique used for detection of *M. ulcerans* in environmental samples and clinical sample confirmation has undergone several modifications increasing sensitivity and specificity (Stinear *et al.*, 1999; Narh *et al.*, 2014). However, the erroneous detection of other closely related mycolactone-producing-mycobacteria (MPM) with comparable PCR targets has been a challenge (Ablordey, Hilty, *et al.*, 2005; Stragier *et al.*, 2005; Williamson *et al.*, 2008). Targets located on the 174 kbp plasmid found in *M. ulcerans* but absent in other related mycobacteria, have been successfully used for detection (Stinear *et al.*, 2004; Fyfe *et al.*, 2007; Nakanaga *et al.*, 2013).

The wide distribution of environmental mycobacteria has led to the assumption of several possible sources and modes of transmission from their natural environments to intermediate hosts. Insects, widely known not to be actively involved in the transmission of mycobacterial infections have been described to be vectors of some mycobacterial infections. For instance, beetles in the transmission of *Mycobacterium avium* (Fischer *et al.*, 2004), dipterans in mycobacterial infections in cattle and pigs (Fischer *et al.*, 2001), and cockroaches in hospital NTM infections (Pai *et al.*, 2003). The only experimental study that have somewhat shown active involvement of an insect in the transmission of a mycobacterial disease is in the case of the transmission of *M. ulcerans* (Marsollier *et al.*, 2002)

Marsollier and colleagues showed that water bugs of the family Naucoridae (Hemiptera: Nepomorpha) could take up *M. ulcerans* while feeding, which multiply in the salivary glands and are transmitted to a susceptible host via bites of the bugs (Marsollier *et al.*, 2002; Marsollier, Deniaux, *et al.*, 2007). A wide survey evaluating the relative abundance and composition of invertebrates in BU endemic and non-endemic communities in Ghana, however, provided evidence of the unlikelihood of biting aquatic insects being actively involved in the transmission of BU (Benbow *et al.*, 2008). *Mycobacterium ulcerans* due to its waxy feature has a higher affinity to the exoskeleton of Belostomatidae (Hemiptera: Nepomorpha) and can be maintained in the aquatic food web (Mosi *et al.*, 2008; Wallace *et al.*, 2010).

The involvement of insects, specifically mosquitoes, in the transmission of *M. ulcerans* has also been suggested in Australia where a strong dose-response relationship between the detection of *M. ulcerans* in mosquitoes and the risk of human disease has been detected (Lavender *et al.*, 2011). A recent study in Benin however suggested mosquitoes and other flying insects not be involved in the ecology of the pathogen (Zogo *et al.*, 2015). Again, another study in Australia proposed possums to be acting as reservoirs, and mosquitoes as vectors infecting both humans and possums from contaminated environments through their bites (Fyfe *et al.*, 2010). In West Africa, the hemipteran Families Belostomatidae and Naucoridae have been implicated in several studies but the composition and diversity of these two insect groups in endemic communities have not been adequately described (Doannio *et al.*, 2011; Ebong *et al.*, 2012; Ribeiro *et al.*, 2014). A significant positive correlation between the abundance and the prevalence

of BU has been reported for the two insect families in Cameroon and suggested as evidence that they may be locally important in the transmission and/ or environmental persistence of *M. ulcerans* (Carolan *et al.*, 2014). Taxonomic insight into the distribution of these two families of the Hemiptera in established BU endemic areas is therefore needed. Information on the genetic diversity of these two families in West Africa is lacking even though the WHO recommends confirmation of the roles these hemipterans may be playing in BU infection and maintaining *M. ulcerans* in the aquatic environment (WHO, 2014a).

Infections caused by NTM such as *M. avium* complex (MAC), *M. intracellulare*, *M. chimaera* and *M. bovis* have been well documented in both humans and animals (Biet *et al.*, 2005; Van der Merwe & Michel, 2010). Buruli ulcer is primarily described as a human infection but skin infections positive for *M. ulcerans* have also been observed in a wide range of mammals. The significance of such zoonotic infections to public health issues of BU is however not known. Mammals in which infections have been observed naturally include armadillos, possums (Fyfe *et al.*, 2010), alpacas (O'Brien *et al.*, 2013), grasscutters, cynomolgus monkeys (Walsh *et al.*, 2007), guinea pigs and experimental infections have been proven in mouse models (Addo *et al.*, 2005)

Studies on the ecology of BU will therefore be incomplete if human activities, animals and the environment together with associations that exist among them are not considered. The employment of the "One Health" concept in the study of BU ecology is therefore apposite.

The One Health strategy for the control of zoonotic diseases has gained significant success. It is now even highly recommended in research of suspected

zoonotic infections with environmental links (Fisman & Laupland, 2010). One Health concept recognizes that the health of humans, other animals, and ecosystems are interconnected and therefore involves applying a coordinated, collaborative, multidisciplinary, and cross-sector approach in addressing risks that originate at the interface of humans, other animals, and ecosystems (Monath *et al.*, 2010). The One Health model has been adapted by the WHO for neglected zoonotic diseases (NZD) with recommendations for developing joint medicalveterinary integration of human and animal surveillance and research activities in the control of diseases such as rabies, echinococcosis, brucellosis and trematodosis (WHO, 2007).

1.2 Rationale

An important factor in developing effective prevention and control strategies for NZD is identifying the relevant transmission routes between reservoir and human host (Loh *et al.*, 2015). Pathogens transmitted between the environment, wildlife, livestock and humans represent major challenges to human and domestic animal health. Among such pathogens are members of the genus *Mycobacterium* which is well represented by *M. bovis* (the etiological agent of bovine tuberculosis), *M. avium* ssp. *paratuberculosis* (the etiological agent of Johne's disease) and *M. avium* ssp. a*vium* (the etiological agent for Crohn's disease) (Biet *et al.*, 2005; Mura *et al.*, 2006). In recent times, *M. ulcerans* infection has gained public health importance due to the sporadic emergence of new disease foci (WHO, 2014a).

While various studies conducted on the ecology and epidemiology of *M.*

ulcerans has added significant information on the biology of the infection, the pertinent question of exact environmental reservoirs and the mode of transmission from the environment to and among humans and small mammals remain to be answered (Fyfe *et al.*, 2010; Merritt *et al.*, 2010). Several studies highlight the significant challenges which include the inability to adequately identify and sample infected environments and communities, the difficulty of culturing *M. ulcerans* from environmental samples and insufficient demographic and social data of the affected populations and communities (Benbow *et al.*, 2008; Williamson *et al.*, 2008; Merritt *et al.*, 2010; WHO, 2015). A comprehensive and extensive designed sampling methodology that will be based on determination of livelihood and economic strategies of affected populations exposing humans and small mammals to precise infected environments would eliminate some of these challenges. This study aspired to use an effective systematic approach which involve community maps and the sampling of humans and identified risk environments. Fine genetic markers such as typing of Viable Number Tandem Repeats (VNTR) would then be applied in tracing routes of transmission of *M. ulcerans* from environmental sources to humans.

Insects are involved in the transmission of many infections and diseases, passively or actively, and NTM infections are not an exception (Pai *et al.*, 2003; Fischer *et al.*, 2004). Detection of genetic targets on the plasmid or genome of *M. ulcerans* in several invertebrate taxa collected from surface waters in BU endemic areas (Portaels *et al.*, 1999; Marsollier, Severin, *et al.*, 2004; Mosi *et al.*, 2008; Williamson *et al.*, 2008), isolation of a viable culture from an insect of the Family Gerridae (Portaels *et al.*, 2008) and observed association between infected

mosquitoes and human infections in Australia (Johnson *et al.*, 2007; Lavender *et al.*, 2011) emphasizes the likelihood that insects may play significant roles in the transmission of the pathogen in endemic areas. In West Africa, two aquatic insect families of the Order Hemiptera, Family Naucoridae and Family Belostomatidae, have been implicated in the transmission of *M. ulcerans* due to their cosmopolitan distribution in BU endemic aquatic habitats, the high positivity with *M. ulcerans* genetic material and their propensity of biting humans when disturbed (Portaels *et al.*, 1999; Marsollier *et al.*, 2003; Mosi *et al.*, 2008; Marion *et al.*, 2010; Doannio *et al.*, 2011). There is however a general lack of information on the diversity of these aquatic hemipterans in water bodies in BU endemic areas in West Africa where the disease is most endemic. As any vector incriminating study should be preceded by a comprehensive account of the diversity of the suspected vectors in the study area, there is the need to properly study the diversity of these aquatic bugs in the fresh water bodies of BU endemic areas. Unavailability of taxonomic keys for the identification of aquatic insect groups has challenged most aquatic entomologists in ecological studies especially within the West African region. Few reports (Dejoux *et al.*, 1981; Sankare, 1991; Edia *et al.*, 2007) have focused on invertebrate fauna and most descriptions of such groups end at the Family taxon (Carolan *et al.*, 2014).

1.2 Main Objective

To demonstrate the significance of the application of multidisciplinary tools in describing the ecology of *M. ulcerans* transmission in selected communities in Ghana.

1.2.1 Specific Objectives:

The specific objectives of the study were to;

1. Identify Buruli ulcer risk behaviours and livelihood strategies, and risk water contact areas in four BU endemic communities in Ghana.
2. Determine *M. ulcerans* molecular diversity in risk environments identified in the four BU endemic communities by the use of ground maps and Variable Number Tandem Repeat typing.
3. Analyze relatedness of *M. ulcerans* VNTR types in human *M. ulcerans* infections and in risk environments and suggest routes and activities of human *M. ulcerans* infections.
4. Catalogue the Belostomatidae (Hemiptera: Heteroptera) and Naucoridae (Hemiptera: Heteroptera) in aquatic habitats across selected water bodies in Ghana and Cote d'Ivoire and relate generic and species diversity to *M. ulcerans* preferred water body types.

CHAPTER 2

LITERATURE REVIEW

2.1 Non-tuberculous Mycobacteria

Non-tuberculous mycobacteria (NTM), also called environmental opportunistic mycobacteria, are found both in natural and human influenced environments. NTM can infect and cause disease in humans, animals and birds (Falkinham, 2002). Non-tuberculous mycobacteria are not contaminants but rather normal inhabitants of these environment which include water sources, soils and

dust (Falkinham, 2015). Over 150 individual species of NTM have been described. A range of conditions predispose humans, animals and birds to a variety of NTM infections which include skin, joint, pulmonary and nosocomial (hospital acquired) infections. There is usually no person-to-person spread of NTM infections and treatment is usually by a combination of antibiotics (Falkinham, 2015).

2.1.1 Habitats

Non-tuberculous mycobacteria are natural inhabitants of soils, lakes, rivers and streams. They are however shown to originate in soils from which they may enter surface waters (Falkinham, 2015). Non-tuberculous mycobacteria are also well adapted to human-manipulated environments, such as water distribution systems and plumbing of houses and hospitals (Falkinham, 2011; Falkinham, 2015; Falkinham *et al.*, 2015). Habitat predisposition of various NTM is in part due to their faster growth in acidic conditions as observed with stimulated growth of *M. avium* by humic and fulvic acidic environments (Kirschner *et al.*, 1999). In rivers and streams, NTM usually adhere to objects such as rocks and leaves where they form biofilms. The nature of their membrane drives its attachment to surfaces. Some non-tuberculous mycobacteria have the ability grow in protozoan cells and can survive in cysts formed during unfavourable conditions, a characteristic that has also been suggested for the survival and multiplication of *M. ulcerans* (Cirillo *et al.*, 1994; Mura *et al.*, 2006; Wilson *et al.*, 2011).

2.1.2 Infection Pathways

The surface hydrophobicity due to their lipid-rich outer membrane is responsible for their attachments to surfaces of both natural and humanengineered environments. This same characteristic enables them to be transmitted to humans and animals via many routes: aerosolization and inhalation, swallowing and aspiration, and entry into wounds, either through injury or surgical intervention (Atkins & Gottlieb, 2014; Williamson, Mosi, *et al.*, 2014; Falkinham, 2015). *Mycobacterium avium* complex (MAC) causing pulmonary, joint and bone infections in humans, *M. fortuitum* infecting wounds in humans and *M. paratuberculosis* causing Johne's disease in the small intestines of ruminants are examples (Katoch, 2004; Guglielmetti *et al.*, 2015). Nontuberculous mycobacteria usually cause localized or disseminated disease in humans and animals that are immunocompromised (Katoch, 2004).

2.1.3 *Mycobacterium ulcerans* as a Non-tuberculous Mycobacterium

M. ulcerans, although described as a non-tuberculous mycobacterium, has only recently been included in comprehensive studies of mycobacterial taxonomy, physiology and genetics unlike other species like *M. marinum* and *M. xenopi* (Falkinham, 1996). *Mycobacterium ulcerans* is the causative agent of Buruli ulcer, a mostly tropical infection of the skin and subcutaneous tissues in humans.

There are uncertainties on the sources of *M. ulcerans* infection and reservoirs. The aquatic environment usually ponds, lakes and slow flowing rivers are believed to habour the pathogen (Falkinham, 1996; Merritt *et al.*, 2010). Genetic material of *M. ulcerans* has been detected in these environments and only one isolation of a viable culture from an aquatic insect has been reported (Portaels *et al.*, 2008;

Williamson *et al.*, 2008). The precise mode of transmission from the environment to humans however still remains to be established.

A common characteristic of *M. ulcerans* and its associated disease worldwide is its association with aquatic habitats. The emergence and prevalence of the infection has also been extensively described to be linked to some form of environmental modification usually surface water related. A rise in the number of BU cases have been reported with unprecedented flooding of water bodies (Hayman, 1991; Meyers *et al.*, 1996), wetlands created as a result of dams (Johnson *et al.*, 1999; Merritt *et al.*, 2005), agricultural irrigation systems and paddy farming (Portaels *et al.*, 2001; Wagner, Benbow, Brenden, *et al.*, 2008), sand mining and mining activities (Johnson *et al.*, 1999; Kibadi *et al.*, 2008). Increased sedimentation and eutrophication with the associated low dissolved oxygen concentration created by these environmental changes may be responsible for enhancing the growth of *M. ulcerans* in these environments and hence the frequency of human contact with the pathogen (Guerra *et al.*, 2008). Although these associations have been found to be strong, their precise influence on the survival and multiplication the pathogen is still not known.

2.2 Buruli Ulcer: The Disease

Mycobacterium ulcerans disease, commonly referred to as Buruli ulcer, affects many poor populations in rural areas of over 30 tropical and subtropical countries. The disease is reported to have been first described in the late 19th century in retrieved hospital notes of a physician who worked in Uganda (Clancey, 1964; Uganda Buruli Group, 1971; Meyers, 1995). Buruli ulcer, which is an infection of

the skin and the underlying tissues and infrequently the bone, was however later adequately described in the middle of the twentieth century together with its causative organism, *M. ulcerans* (MacCallum & Tolhurst, 1948).

Buruli ulcer is currently the third most prevalent mycobacterial infection after tuberculosis and leprosy worldwide and was even the second most prevalent mycobacterial infection in Cote d'Ivoire between 1998 and 2000 (Kanga & Kacou, 2001). Although a considerable amount of information is available on the epidemiology of the infection, the mode of transmission of the *M. ulcerans* as well as the precise environmental reservoirs still remain unknown, a situation that has hampered prevention and control efforts (WHO, 2014a).

2.2.1 History

"Buruli ulcer – like" infections have been reported to be described in the hospital notes of a British physician, Sir Dr. Albert Cook, who worked in Uganda in the late 19th century. The disease was however clinically described in 1948 when similar infections were observed in the Bairnsdale area of Australia

(MacCallum & Tolhurst, 1948). The infection was then referred to as the Bairnsdale ulcer. However, earlier in the 1920s to 1930s, comparable infections had been reported in the North Eastern Congo (Janssens *et al.*, 2005). The current common name of the infection, "Buruli ulcer", was coined by a working group when they observed a high emergence of the infection in the Buruli County of

Uganda in the late 1960s to early 1970s (Uganda Buruli Group, 1971; Meyers, 1995).

The emergence of the infection in higher numbers and severity in the countries of West Africa in the 1980s suggested BU to be recognized as a disease of public health importance. The World Health Organization (WHO) subsequently formed the Technical Advisory Group (TAG) on Buruli ulcer to advise on the way forward in fighting the infection. Several countries, under the direction and support of the WHO, formed operative national control programmes to gather prevalence data, and implement control and preventive actions (WHO, 2008). Currently, BU has been reported in 33 countries in Africa, the Americas, and the Western Pacific (WHO, 2014a). The vast majority of cases occur in tropical and subtropical regions but new foci have been reported in Japan (Nakanaga *et al.*, 2011).

2.2.2 Global Distribution

There is still no certainty on the actual distribution and burden of BU as there is frequent detection of new endemic areas. The general global reporting of BU infections however shows a tropical and subtropical distribution. West Africa is the most affected (Figure 1.1). According to the WHO Global Health Observatory for Neglected Tropical Diseases (NTDs), the control programmes of Cote d'Ivoire, Ghana and Benin officially reported 7,993, 3,919 and 2,448 confirmed cases between 2010 and 2015, respectively (WHO, 2015).

Buruli ulcer has also been reported in some Central African Countries, Australia and most recently in Japan (Merritt *et al.*, 2010; Nakanaga *et al.*, 2011; Yotsu *et al.*, 2012). Isolated cases have been reported in non-endemic countries such as Canada and the USA usually from travelers who have been to endemic territories (Makeda *et al.*, 1999; Lavender *et al.*, 2012). Cases reported from Central Africa seem to have declined over the past three decades with a new focus

of infections clearly now in West Africa. The emergence of similar skin infections in Japan although described to be caused by a subspecies of *M. ulcerans* (*M. ulcerans* ssp. *shinshuense*) suggested the emergence of new endemic areas in Asia (Tsukamura *et al.*, 1989). The reporting of many more infections confirms the endemicity of the infection in Japan (Nakanaga *et al.*, 2011; Yotsu *et al.*, 2012).

2.2.3 Distribution in Ghana

Only one national survey for the prevalence of BU in Ghana has been conducted. The overall crude national prevalence rate of active lesions was 20.7 per 100,000, but prevalence as high as 150.8 per 100,000 was reported for BU endemic district. This case search demonstrated widespread disease and gross underreporting compared with the routine reporting system. Passive reporting of cases has not allowed for the accurate data on national prevalence (Amofah *et al.*, 2002; Etuaful *et al.*, 2005). The Ashanti Region is one of the most endemic regions in Ghana and the Amansie District is known to be highly endemic (Adu *et al.*, 2011; Sarfo *et al.*, 2013). Average BU prevalence in the four study communities was reported to be 8.2/1000 individuals in 2013 (HFG, 2014).

2.2.4 Clinical Symptoms

Buruli ulcer is generally an ulcer of the skin with widespread necrosis of subcutaneous fat, confirmed to be caused by *M. ulcerans*. The disease which often begins as a painless swelling referred to as the nodule can also present in early stages as a large painless area of induration, the plaque, or a diffuse painless swelling of the leg, arm or face, the oedema (WHO, 2014a). These, when left untreated or well managed, eventually ulcerate into mostly painless debilitating

ulcers with undermined edges (Figure 1.1). In rare cases, the infection could spread deeper, occasionally affecting the bone (Adu *et al.*, 2011; Pommelet *et al.*, 2014).

The papule is defined as a painless, raised skin lesion, less than 1cm in diameter (often seen in Australia). Nodules extend from the skin into the subcutaneous tissue. It is 1-2 cm in diameter, usually painless but itchy and commonly found in Africa. The plaque is firm, painless and elevated but well demarcated, over 2 cm in diameter. The oedematous form is usually diffuse and extensive with ill-defined margins, firm and painless affecting all of a limb or other part of the body. The oedema may be accompanied by fevers. The ulcerative forms, which are defined into various categories based on diameter of ulcers, usually present with undermined edges and are peripherally undurated. The floor of the ulcers may have cotton-like appearance with a necrotic slough (WHO, 2015)

The severity of *M. ulcerans* infection has been observed to vary geographically which is attributed to the differences in the virulence in different geographically related isolates of the mycobacterium. Isolates from Africa (Ghana, Benin and Cote d'Ivoire) induce higher inflammation, necrosis and bacillary loads compared Australian and Asian strains (Portaels *et al.*, 1996; Ortiz *et al.*, 2009). Secondary bacterial infections may also be responsible for the geographical differences in the manifestations of *M. ulcerans* infection and have been implicated in the delayed healing during drug therapy (Yeboah-Manu *et al.*, 2013). Subsequently, although the disease is usually painless, in severe cases, pain with accompanied fevers has been observed. Fatalities due to BU infections are rare and have been reported in very few cases (Chauty *et al.*, 2007).

2.2.5 Immune Response and Virulence

Mycobacterium ulcerans is free living in its natural aquatic environment forming biofilms with other microorganisms and attached to different kinds of surfaces. *Mycobacterium ulcerans* however infects human and other animal tissue where it causes debilitating skin destruction. The Ugandan group estimated, from observations, four to thirteen weeks as the incubation period (Uganda Buruli Group, 1971). The incubation period from infection or exposure to the onset of clinical symptoms have been more accurately estimated to be four and a half months in Australia (Trubiano *et al.*, 2013). The lack of accurate health care patient data, late reporting of infections as well as problems of recollection of patient movement in endemic areas makes it difficult to estimate the incubation periods in endemic areas in West Africa where the infection is most prevalent.

The pathogenesis of *M. ulcerans* disease is atypical because its destructive activities are caused by a polyketide toxin, mycolactone. *M. ulcerans* was the first mycobacterial species for which there is evidence of the production of destructive macrolide toxin (George *et al.*, 1999). Mycolactone induces apoptosis and necrosis of many cell types and inhibit recruitment of inflammatory cells at the site of infection (George *et al.*, 1999; George *et al.*, 2000). Mycolactone also plays a central role in the extracellular localization of the bacteria and modulation of immunological responses to *M. ulcerans* (Adusumilli *et al.*, 2005). Observations in rodents experimentally infected with mycolactone-producing *M. ulcerans* strains suggested inflammatory cells are killed by necrosis when toxin concentrations are high. Inflammatory cells more distant from the necrotic center and hence exposed to lower toxin concentrations are thought to be killed via apoptosis resulting in

extracellular bacteria being surrounded by an area of coagulation necrosis (Oliveira *et al.*, 2005).

Clinical presentation and pathogenicity of *M. ulcerans* have been observed to be more severe in West Africa than in infected areas of Australia. Mycolactone variants, found to be conserved within specific geographical areas, may be responsible for these differences in virulence. A correlation was confirmed with the use of footpad infection model in BALB/c mice where African strains were found to be highly cytotoxic and produced persistent acute inflammatory responses throughout the infection compared to less virulent Mexican strains that produced chronic inflammation with granuloma-like structures without necrosis and ulceration (Oliveira *et al.*, 2005).

2.2.6 Pathogenic Mechanisms of *M. ulcerans*

M. ulcerans grows extracellularly both in the human body and in organ culture assays and BU tissues show predominantly free bacilli in extensive necrotic areas (Tyrell *et al.*, 1975; Dobos *et al.*, 1999; Johnson *et al.*, 1999). The first noticeable lesion on a human skin is usually a necrotic zone in the subcutaneous fatty tissue with surprisingly little inflammatory reaction in the surrounding tissues. *M. ulcerans* produces a toxin, mycolactone, which has been shown to be responsible for the pathogenic and immunosuppressive activities (Johnson *et al.*, 1999).

Due to the activity of this toxin, the tissue necrosis of BU infection extends further than the clumps of acid-fast rods in typical infections. Mycolactone, with the molecular formula $C_{44}H_{70}O_{9,}$ is a pale yellow compound composed of a 12

membered ring to which two polyketide-derived side chains are attached (Stinear *et al.*, 2004; Silva *et al.*, 2009). Mycolactone A/B exists as a 3**:**2 equilibrating mixture, with the major and minor components being Z-Δ4′,5′- and E-Δ4′,5′-isomers, respectively, in the unsaturated fatty acid side chain (Kishi, 2011). The discovery of a toxin associated with a mycobacterial species was very significant in understanding the pathogenic activities of *M. ulcerans* (George *et al.*, 1999). Mycolactone was also the first toxin isolated from a mycobacterial species (George *et al.*, 2000). Figure 2.1 shows the molecular structure of the two variants of mycolactone, mycolactone A and B. Mycolactone A/B causes a cytopathic effect on mouse fibroblast L929 cells characterized by cytoskeletal rearrangement with rounding up and subsequent detachment from tissue culture plates. Mycolactone A/B causes cell cycle arrest at the G0/G1 phase, leading to cell death by apoptosis (Kishi, 2011).

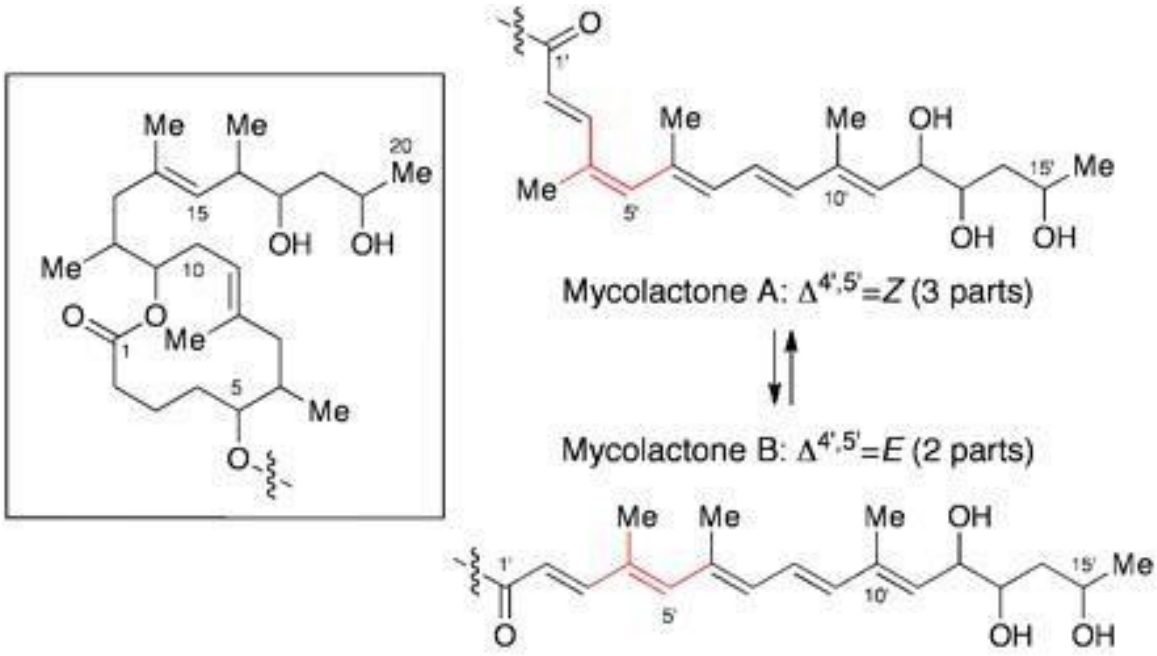


Figure 2.1 Chemical structure of mycolactone A and B (Kishi, 2011).

2.2.7 Diagnosis, Treatment and Management

First line BU diagnosis is the presentation of any of four clinical symptoms of the infection within an endemic area: the nodule, the papule, an oedema or a characteristic ulcer with undermined edges (WHO, 2014a). World Health Organization, however, recommends that the presentation of any of these symptoms should be recorded as suspected BU infection and all suspected cases must then be confirmed by laboratory diagnosis. Less than 50% of all reported suspected cases of BU are confirmed by any of the four confirmatory tests: microscopy (Ziehl-Neelsen Stain Test), histopathology, culture or the detection of genetic targets by PCR (Yeboah-Manu *et al.*, 2011). The PCR assay which targets genomic markers such as the insertion sequence *IS2404* has the highest sensitivity of over 90% but is confined to highly resourced laboratories (Herbinger *et al.*,

2009). Sensitivity of the other tests vary significantly as their success as confirmatory tools is based on the type and stage of clinical lesion, type of sample, transportation and the processing of samples (Bratschi *et al.*, 2014; WHO, 2014a).

Buruli ulcer treatment has progressed significantly over the past two decades evolving from the aggressive surgical removal of affected tissues to a higher focus on combination antibiotic treatment combined with surgery only if needed (Huang & Johnson, 2014). This however greatly depends on presentation and diagnosis of early stages of the infection (Nienhuis *et al.*, 2010). The use of antibiotics that are effective against the pathogen has considerably lowered treatment cost. The WHO currently recommends eight week administration of rifampicin and streptomycin for all stages of the infection after confirmation and surgery to correct any deformities or remove dead tissue (WHO, 2014a). The establishment of corrective rehabilitation and physiotherapy centers has immensely helped in reducing disabilities and restricted joint movements of treated patients leading to improved health related quality of life (Hamzat & Boakye-Afram, 2011).

Buruli ulcer educational activities in affected communities have focused mainly on the need to report the early signs of the infections, usually nodules to health facilities. This enables rapid diagnosis and confirmation so antibiotic treatment can commence early. This initiative has been reported successful in the reduction of cases with extensive ulcers in many affected communities (Converse *et al.*, 2011). In Ghana, non-governmental organizations (NGOs) have aided the National Buruli Ulcer Control Programme (NBUCP) to undertake many educational programmes leading to a reduction in the numbers of extensive cases reporting to health facilities. The cooperation of the volunteer system, a community

person trained to identify cases and administer antibiotics, has also facilitated care and prevented the difficulty of patients having to travel long distances to access care (Ahorlu *et al.*, 2013).

Buruli ulcer treatment and management however remains expensive. In some countries, with the help of governments and aid organizations, medications are free but this is not universal. Even in situations where the medications are available, health personnel are not motivated to clean and dress the spiteful wounds caused by the infection. In complicated and extensive cases, the economic cost to the family is high particularly due to the long hospitalization periods (Amoakoh & Aikins, 2013). The fact that poor populations are the most affected aggravates this situation especially where another family member has to stay in the hospital to give care to the infected individual.

2.2.8 Epidemiology

Buruli ulcer was earlier described to be more prevalent in females than males and usually on limbs and mostly reported in children between the ages of five and fourteen years (Amofah *et al.*, 2002). More recently however, this has been observed not to be the situation (Bratschi *et al.*, 2013). The infection affects all sexes and age groups and is more related to activities of people in infected aquatic environments in endemic areas (Raghunathan *et al.*, 2005). This assertion is however limited due to the lack of adequate knowledge on the mode of transmission of the pathogen, the latent period and people who may be infected but may never show disease.

The distribution of the BU mostly in rural and poor population in many disease foci has been attributed to economic activities in infected environments. Human induced environmental disturbances have also been linked to the onset and increase in infections (Merritt *et al.*, 2010). New infections were reported in Uganda with refugee usage of the Nile, in Australia with the use of dam water for irrigation, in Ghana in mining areas and sand winning areas and in Benin in rice farming areas. These environmental disturbance and BU infection correlations have been explained by these activities creating favourable conditions for the proliferation of the pathogen (Wagner, Benbow, Brenden, *et al.*, 2008; Williamson *et al.*, 2008). Currently, no study has however adequately substantiated these observations.

2.2.9 Socio-Economic Impact of Buruli Ulcer

The debilitating nature of BU infection especially in West Africa has immense economic impacts on affected persons, their families, communities and the health care system (Ahorlu *et al.*, 2013). Health care providers managing and treating BU need specialized training and motivation. The provision of specialized units and personnel in health care facilities, dedicated to BU treatment, burdens the health care system of affected countries. This burden is significantly reduced through advocacy and aid from donors and nongovernmental organizations.

A study on the health facility and economic cost of BU treatment in a treatment facility in Ghana revealed an annual financial cost of $121,189.16 which represented 13% of the total treatment cost of all ailments in the hospital (Asare & Aikins, 2014). The estimated treatment cost and economic cost per capita was

$1,615.86 and $1,914.79 respectively which did not even cover household patient costs. This cost is, however, significantly reduced when cases are identified early and treated only by antibiotic therapy (Ahorlu *et al.*, 2013). The socio-economic burden of BU on affected individuals, their families, communities and countries is very high and efforts to reduce these costs by early reporting will significantly impart costs and control efforts.

2.3 Biology and Genetics of *M. ulcerans*

Even though only one study has successfully obtained a true culture of *M. ulcerans* from an environmental source, the viability of *M. ulcerans* in the aquatic environment is not in doubt (Portaels *et al.*, 2008; Merritt *et al.*, 2010). *M. ulcerans* is described as an aquatic environmental saprophyte living in close association with other microorganisms, forming a matrix, which may help in their survival and multiplication (Marsollier, Stinear, *et al.*, 2004; Marsollier, Brodin, *et al.*, 2007).

Environmental factors that favour *M. ulcerans* survival and multiplication within aquatic environments are poorly understood but the environmental and multi-host nature of *M. ulcerans* suggest that its environmental dynamics are as a result of complex interplay of several abiotic and biotic factors (Garchitorena *et al.*, 2015). *M. ulcerans* is observed to grow better under low oxygen, high temperature and mildly acidic pH, conditions which are usually met in swamps and other stagnant and slow flowing water bodies (Marsollier, Stinear, *et al.*, 2004; Stinear *et al.*, 2007; Garchitorena *et al.*, 2014).

2.3.1 Associations with Protozoans

There are suggestions that *M. ulcerans* may persist in the environment commensally, associated with other organisms that protect the mycobacterium from unfavorable physical parameters of the environment. Evolutionary analysis of the genome of *M. ulcerans* also suggests a gradual adaptation of the pathogen to a specific niche (Yip *et al.*, 2007). Free living amoebas are widespread inhabitants of water, soil and air. These amoebae are usually predators of other microorganisms such as bacteria and may act as reservoirs for some internalized microorganisms (Greub & Raoult, 2004). The possible roles of free living amoebas as reservoirs for amoeba resistant bacteria (ARBs) have been proposed and expansively studied especially in *Legionella* (Adeleke *et al.*, 1996) and in others including various species of *Mycobacterium* and *Francisella tularensis* (Greub & Raoult, 2004). The implications of such amoebae as reservoirs on the ecology, epidemiology, transmission, virulence and public health importance of environmental mycobacteria, however, remains to be better defined.

The involvement of free living amoebae in the biology (multiplication and transmission) of *M. ulcerans* was first proposed by Portaels and colleagues and has been reiterated recently (Portaels *et al.*, 2001; Wilson *et al.*, 2011). Even though relationships between other aquatic ARBs and amoebae have been extensively studied, the role of free living amoebae being reservoirs of *M. ulcerans* in the aquatic environment or being involved in transmission of the pathogen to humans and other mammals has not gained adequate attention. The ability of *Acanthamoeba castellanii* to engulf and retain mycobacteria in their cells has been reported (Steinert *et al.*, 1998). Other studies with Legionella-likeamoebal pathogens

(LLAPs) have confirmed similar relationships while a study has even gone further to show an increase in virulence and invasion in *M. avium*, a mycobacterium closely related to *M. ulcerans*, after association with *Acanthamoeba castellanii* (Cirillo *et al.*, 1994; Cirillo *et al.*, 1997). Engulfed bacteria have also been shown to persist in encysted amoeba and to possess the ability to lyse the amoebae when environmental conditions become favourable (Winiecka-Krusnell & Linder, 2001).

2.3.2 Detection and Characterization of *M. ulcerans*

Although *M. ulcerans* exists as a saprophyte within aquatic environments, its isolation and successful culture from such environments has been challenging. This is attributable to overgrowth by faster growing organisms in attempts to culture from environmental samples (Portaels *et al.*, 2008). The pathogen can however be more easily isolated from samples taken from lesions of infected persons. *Mycobacterium ulcerans* has only once been successfully isolated from an aquatic hemipteran after a series of mouse footpad passages (Portaels *et al.*, 2008).

The detection of *M. ulcerans* in environmental samples has therefore been primarily based on the detection of several genetic targets of the mycobacterium. Almost all hypothesis including insect involvement in transmission, invertebrate reservoir and possibility of animal infections have all been based on various genetic targets (Portaels *et al.*, 1999; Benbow *et al.*, 2008; Mosi *et al.*, 2008; Williamson *et al.*, 2008). These targets include the insertion sequences *IS2404* and *IS2606*, enoyl reductase gene (ER), and keto reductase gene (KR).

2.3.3 Molecular Characterization of *M. ulcerans*

M. ulcerans reference strain Agy99, used in comparative genome analysis, consists of a circular chromosome of 5632 Kb and a plasmid pMUM001 of 174Kb (Qi *et al.*, 2009). Genetic analysis also suggest that *M. ulcerans* to have diverged from the fish pathogen *M. marinun* between 470,000 and 1,200,000 years ago by acquiring the virulence plasmid pMUM001, the two strains still sharing over 80% nucleotide sequence identity. Comparative genomic analysis of the two suggests *M. ulcerans* to be evolving from the generalist-environmental bacterium to a niche adapted specialist in a mammalian host (Stinear *et al.*, 2007). Different genotyping tools such as restriction fragment length polymorphism (RFLP), VNTR among others have been used to resolve genetic diversity among *M. ulcerans* isolates and relationships between types have been observed to be influencing geographical distribution and virulence (George *et al.*, 1999; Stinear *et al.*, 2007).

Multiple genetic targets for detection of *M. ulcerans* have been particularly useful, since PCR is still by far the most effective method used in detecting the organism in the environment (Ross *et al.*, 1997; Narh *et al.*, 2014). Other detection methods such as culture, Ziehl-Neelsen staining and histopathological examination are used mainly for clinical samples (Herbinger *et al.*, 2009; Yeboah-Manu *et al.*, 2011). Identification of multiple molecular targets and the use of several more stringent and finer fingerprinting techniques have led to the characterization of the pathogen into geographical variants and their relatedness to type of mycolactone produced and virulence. Detection and discrimination tools used for *M. ulcerans* range from variable number tandem repeats–typing (VNTR-typing) to single nucleotide polymorphisms

(SNPs) and microarray analysis (Ablordey, Hilty, *et al.*, 2005; Ablordey, Swings, *et al.*, 2005; Roltgen *et al.*, 2010).

Variable number tandem repeats (VNTR) are locations in the genome where short sequences of DNA occur in a repetitive pattern usually adjacent to each other (Ablordey, Swings, *et al.*, 2005). These repeats may vary in number per genome of closely related organisms and therefore have been found useful in differentiating such closely related species. In *M. ulcerans* and other closely related mycolactone producing mycobacteria (MPM), numerous VNTR both within functional and nonfunctional genes have been identified and used to differentiate *M. ulcerans* from other MPM (Ablordey, Swings, *et al.*, 2005; Lavender *et al.*, 2008). Studies targeting locus 6, locus 19, MIRU1 and ST1, all with variable repeats, have successfully been used to resolve an apparent genetic homogeneity within and between geographical isolates (Hilty *et al.*, 2006). Hilty and colleagues revealed three different genotypes with clonal clustering and suggested genetic diversity of *M. ulcerans* in Ghana (Hilty *et al.*, 2006).

VNTR-typing has been useful in BU transmission studies. It has been used to source track human and animal infections of *M. ulcerans* from environmental sources in endemic communities in Ghana and differentiate Australian strains geographically especially in infections among travelers (Lavender *et al.*, 2012; Narh *et al.*, 2015).

Single Nucleotide Polymorphism typing (SNP typing) detects a single base pair mutation at a specific locus, revealing genetic variations between members of a species. The SNP typing tool has also been used to reveal subtle differences in seemingly identical strains (Marth *et al.*, 1999). SNP analysis of *IS2404* to genotype

83 *M. ulcerans* isolates from African countries identified 11 types that differentiated regional strains into three haplotypes (Kaser *et al.*, 2009).

The development of a real-time PCR SNP typing tool for *M. ulcerans* patient isolates, collected from different parts of Ghana, Cote d'Ivoire, Democratic Republic of Congo, Benin, Togo and Angola, identified three clades. Using 65 SNP, six haplotypes around the Densu River in southern Ghana and isolates from Togo formed a clade, isolates from central Ghana clustered with one Ivoirian isolate and other African isolates formed the third clade (Roltgen *et al.*, 2010). Large scale application of SNP typing for epidemiological studies would, however, involve the use of DNA microarrays on chips.

2.4 Transmission of Buruli Ulcer

The exact mode of transmission of Buruli ulcer is still not known. The WHO constituted Technical Advisory Group (TAG) on BU has as one of its priorities research into the mode of transmission of the infection (WHO, 2014a). Many hypotheses have been postulated and tested and yet many still remain to be tested. The mode of transmission of an infection is important in the devising and formulation of any effective control strategies.

2.4.1 Risk Factors of Infection

Although a thorough analysis of known risk factors for *M. ulcerans* infection may help identify likely transmission routes and eliminate unlikely pathways, very few studies have systematically examined these (Jacobsen & Padgett, 2010). The most commonly identified risk factor for *M. ulcerans* infection

is living in close proximity to a water body and frequent contact with the water increasing the risk of infection (Aiga *et al.*, 2004; Sopoh *et al.*, 2010).

Detection of the pathogen in aquatic areas used by communities, although important in the presence of BU, is not directly correlated with infection as genetic material of the pathogen has been detected in water bodies in both BU endemic and BU non-endemic community related water bodies (Ross *et al.*, 1997; Williamson *et al.*, 2008). Water bodies that bear the brunt of human activity that had greatly altered the environment seem to increase the risk of infection (Portaels *et al.*, 2009; Merritt *et al.*, 2010). The focal nature of BU prevalence also implies living in an endemic area is a risk for acquiring the infection. No human to human transmission of the pathogen has been documented although an isolated case of BU infection from a human bite has been reported (Debacker *et al.*, 2003).

2.4.2 Invertebrate Involvement

Invertebrates, especially insects, have been involved in the transmission of many humans and animal infections. They usually act as reservoirs, dispersal and inoculation agents of bacteria, fungi, protozoans and viruses. In some instances, invertebrates may be the actual cause of the disease as in tick and louse infections (Gratz, 1999). The possible active role of insects in the transmission of *M. ulcerans* to humans was first suggested when the insertion *IS2404*, found in the genetic material of the pathogen, was detected in insects collected from water bodies in Benin but not in the stems or roots of aquatic plants (Portaels *et al.*, 1999). They proposed a hypothesis that suggested an inoculation or dispersal role to the *Naucoris* sp (Hemiptera: Naucoridae) and *Diplonychus* sp (Hemiptera:

Belostomatidae) in the transmission of the pathogen. A large survey conducted in Ghana also detected the pathogen in several other aquatic invertebrates (Benbow *et al.*, 2008; Williamson *et al.*, 2008). In Australia detections were made in mosquitoes (Lavender *et al.*, 2011). Various studies have tested these hypotheses but the exact roles, if any, of these aquatic bugs or other invertebrates such as mosquitoes in the transmission of the pathogen remains to be properly defined (Merritt *et al.*, 2010; Doannio *et al.*, 2011). The detection of the pathogen from aquatic sources and the cultivation and characterization of a viable isolate from an aquatic hemipteran, *Gerris* sp, collected from water bodies in BU endemic communities in Benin have strongly suggested human infection directly or indirectly from aquatic environments (Portaels *et al.*, 2008).

Although several taxa of aquatic invertebrates have tested positive for various genetic markers of *M. ulcerans*, the Family Naucoridae and Family Belostomatidae (Order Hemiptera: Suborder Heteroptera) are the most implicated (Marsollier *et al.*, 2002; Benbow *et al.*, 2008; Mosi *et al.*, 2008; Merritt *et al.*, 2010; Doannio *et al.*, 2011). The ecology, biology and distribution of these two families in water bodies of BU affected communities may be the reason for these assertions. Members of the Family Naucoridae and Family Belostomatidae are known to inflict painful bites to humans as a defensive mechanism and are also generally aggressive predators in their natural aquatic environment, feeding on other invertebrates (Merritt & Cummins, 1996). Artificial infections of members of the Family Naucoridae and their ability to bite and infect the tails of mice with *M. ulcerans* have been shown in animal model experiments (Marsollier *et al.*, 2002).

M. ulcerans has also been observed to have a strong affinity to cuticular surfaces due to its sticky nature but failed to colonize the organs of experimentally infected Belostomatidae suggesting a more likely mechanical rather than biological involvement of the Belostomatidae in the biology of *M. ulcerans* (Mosi *et al.*, 2008). Other invertebrates including members of the Families Dytiscidae (Class Insecta: Order Coleoptera), Class Mollusca, Class Copepoda and Family Chironomidae (Class Insecta: Order Diptera) have also tested positive for genetic markers for *M. ulcerans* (Marsollier, Severin, *et al.*, 2004; Benbow *et al.*, 2008; Williamson *et al.*, 2008; Merritt *et al.*, 2010).

2.4.2.1 Naucoridae

Members of the Family Naucoridae, commonly called Naucorids, are aquatic hemipterans belonging to the suborder Heteroptera, infraorder Nepomorpha and the superfamily Naucoroidea. The family Naucoridae is represented worldwide by 413 species in 39 genera and 7 subfamilies (Sites, 2000). Members of the Family Naucoridae are also commonly known as the creeping water bugs. They are generally oval in shape with various degrees of dorsoventral flattening. The naucorids are predaceous, sucking the body fluids of other aquatic invertebrates and have even been reported to inflict painful bites on humans (Sites, 2000). The Naucoridae are primarily aquatic (all life stages) with adults mostly employing compressible gas gill respiration (Matthews & Seymour, 2010).

The family Naucoridae was first implicated in the transmission of BU when genetic material of the pathogen, *M. ulcerans*, was detected by PCR in insects collected from aquatic areas in Buruli ulcer endemic communities in Benin and

Ghana (Portaels *et al.*, 1999). The hypothesis that these insects may be mechanical or passive reservoirs and by their bites be the source of trauma and introduce the pathogen into human skin was thus for proposed and this was followed up by a series of studies to test the hypothesis. Marsollier and colleagues were subsequently successful in causing an infection in the tail of mice after a bite from artificially infected Naucoridae (Marsollier *et al.*, 2002). Insects used in the study were collected from swamps in western part of France, a geographical region which had never reported cases of Buruli ulcer. Moreover, ecological studies in Ghana suggested the involvement of insects as vectors as highly unlikely when no significant differences were observed in diversity and numbers when Naucoridae and other invertebrates collected from endemic and nonendemic communities were compared (Benbow *et al.*, 2008).

There is currently a general lack of knowledge on the diversity and ecology of family Naucoridae in the tropical regions of the world (Sites, 2000;
Sites & Mbogho, 2012). The need to fill this gap especially in BU endemic communities and their associated water bodies is principal especially when their roles in the survival and transmission of *M. ulcerans* is still plausible. Present knowledge of fauna of the Naucoridae in Africa remains very low. The most extensive recorded works on the Naucoridae in Africa was by La Rivers when a catalogue of this family was written for Africa, a catalogue that has only twice been updated since the nineteen seventies (La Rivers, 1971; La Rivers, 1974; La Rivers, 1976). Insect taxonomist working in Africa have appeared to concentrate on insects that have a direct economic importance to humans, agricultural plants and animals much to the neglect of others whose impact are presumed less direct. Faunistic surveys and reviews documenting the taxonomic

composition and distribution of the species of Naucoridae in some African countries have only recently been conducted for Tanzania after their first descriptions many years ago (Sites & Mbogho, 2012; Mbogho & Sites, 2013). Apart from being implicated in the transmission of BU, naucorids had only been reported as effective predators of mosquito larvae and pupae, including species of Anopheles, hence suggesting them as possible biological control agents of malaria (Wladimirow & Smirnov, 1932; Sites & Nichols, 1990).

Characteristics used for Naucoridae identification to species level include type of fold of the front of head, width of head compared to the anterior margin of scutellum, width of head compared to half of the greatest width of pronotum, ratio of width of body to length, embolar on hemelytra and midlateral projections on subgenital plates (Figures 2.2 and 2.3).

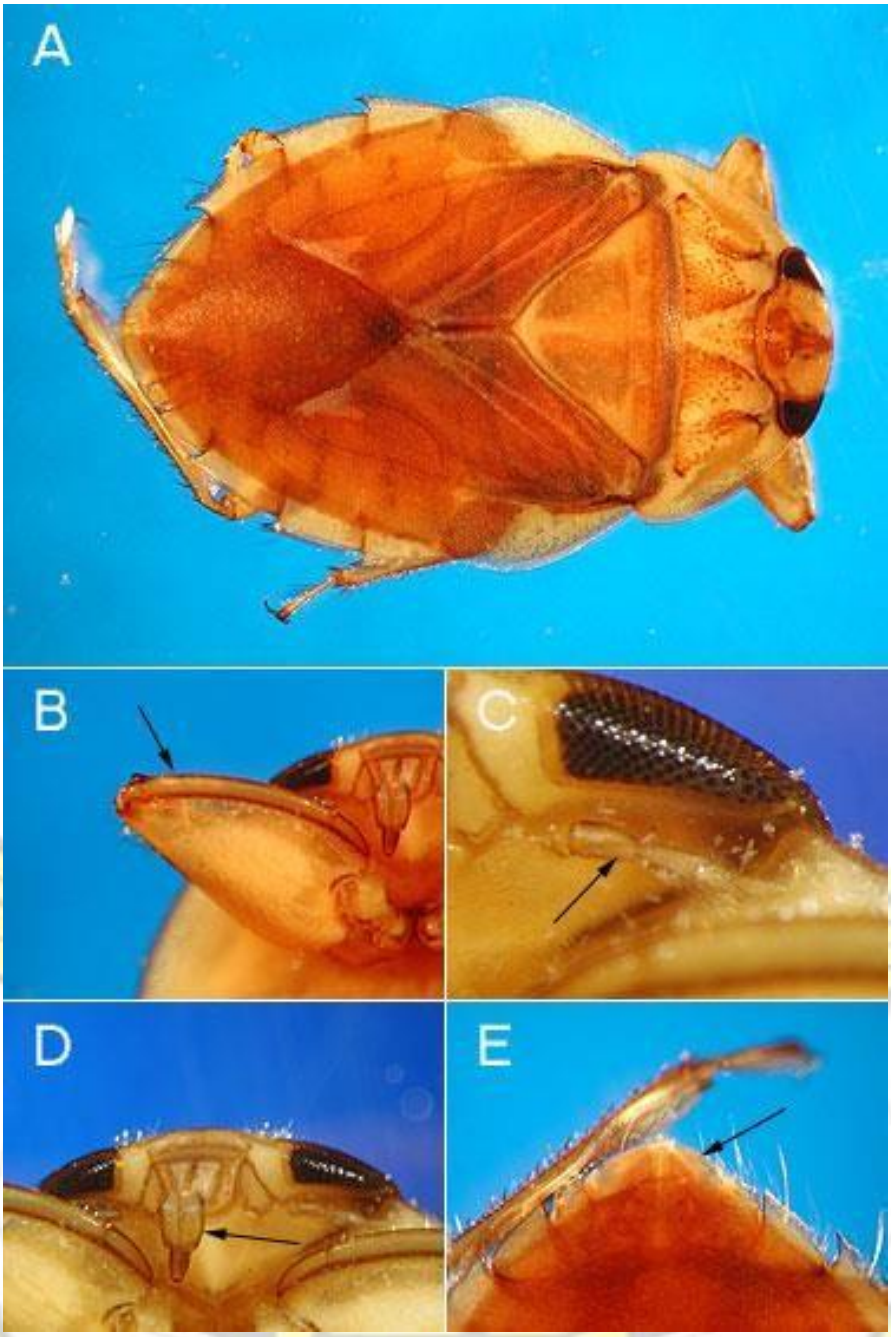


Figure 2.2 Distinguishing characteristics of the Naucoridae

A. Dorsal view of the Naucoridae showing its oval body. **B**. Raptorial forelegs for catching and holding prey **C.** Antennae small and located under the eye **D**. 3-4 segmented cylindrical beak. **E**. Air straps lacking at the apex of the abdomen (Macro-Invertebrate Lab, Valley City State University, 2005)

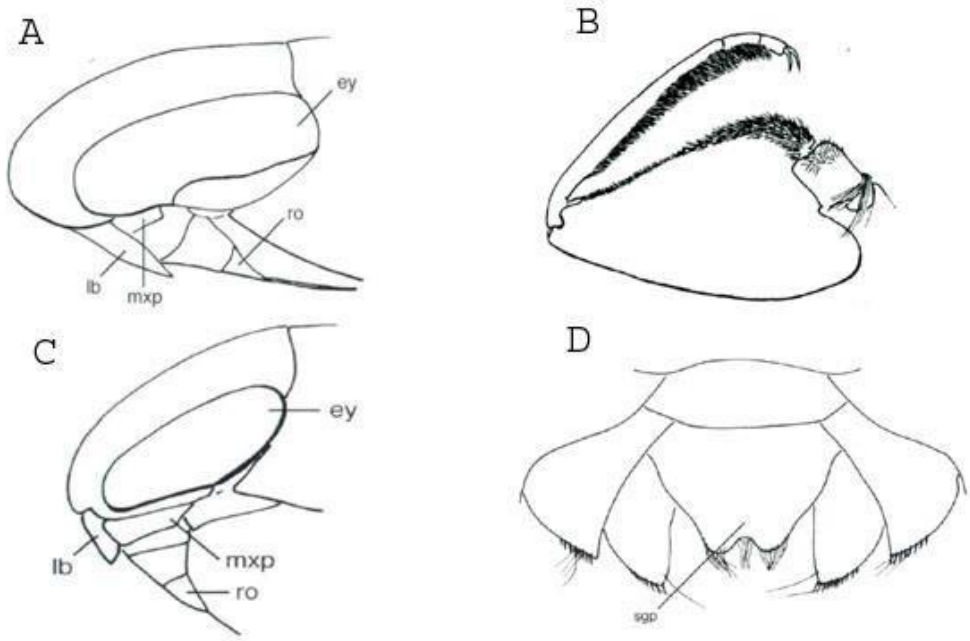


Figure 2.3 Some distinguishing characteristics of genera of the family Naucoridae (Zettel *et al.*, 1999).

A. Lateral view of the head of *Laccocoris* sp. (ey – eye; ro – rostrum; mxp – maxillary plate; lb – labrum). **B.** Ventral view of the foreleg of *Laccocoris* sp. **C.** Lateral view of the head of *Naucoris* sp. (ey – eye; ro – rostrum; mxp – maxillary plate; lb – labrum). **D.** Ventral view of the abdominal segment of *Laccocoris* sp. (sgp – subgenitalplate of female)

2.4.2.2 Belostomatidae

The Belostomatidae are another family of aquatic invertebrate largely implicated in the transmission of Buruli ulcer. These insects, commonly referred to as the giant water bugs, are classified into the order Hemiptera, suborder Heteroptera and infraorder Nepomorpha. Members of the family are cosmopolitan and have been collected in both the temperate and tropical regions of the world

(Merritt & Cummins, 1996). Distinguishing morphological features of this hemipteran family include flattened legs; hind tarsi with two apical claws; fore legs adapted for grasping prey, the femora enlarged and the tibiae curved; ocelli absent; antennae shorter than the head, inserted beneath the eyes (Merritt & Cummins, 1996). They are typically encountered in freshwater streams and ponds. Most species are relatively large (2 cm or more in length) with some of the largest, such as *Lethocerus* spp, exceeding 12 cm in length. All members of the family Belostomatidae are predaceous, entirely aquatic as nymphs and pass much time under water as adults. Belostomatidae feed on a variety of aquatic invertebrates, including insects, and in the case of larger species, small vertebrates, e.g., fish and tadpoles (Usinger, 1963).

The family Belostomatidae has rarely been described to be involved in the transmission or as the cause of any human and animal infections or diseases. This group has been described as biological control agents of mosquito larvae and gastropods (Consoli *et al.*, 1995). Three species of *Belostoma* (Insecta, Heteroptera: Belostomatidae) have been reported as intermediate hosts of digenetic trematodes of the genus *Stomylotrema* in birds (Digiani, 2002).

M. ulcerans genetic material being detected in members of this Family has implied their possible role in the transmission of BU through bites but this assertion when tested in rearing experiments suggested a rather passive or a more accidental role (Mosi *et al.*, 2008; Doannio *et al.*, 2011). Similar to the Naucoridae, the general lack of taxonomic information on the diversity of the Belostomatidae in BU endemic areas of West Africa has hampered vectorial capacity studies of these insect groups.

Classical description of the Belostomatidae of tropical Africa has been scarce not because of the lack of specimen but rather because of a lack of trained specialists (Ribeiro *et al.*, 2014). The rarity of some taxa has also contributed to this problem because some species remain poorly described or are known only from one or two specimens. The family Belostomatidae are represented by nearly 150 species distributed almost all over the world (Hildago, 1935; Iglesias *et al.*, 2008).

Features used for Belostomatidae classification include the development level of the keel, the presence or absence of grooves on the anterior femora, height of the basal segment of the beak and reduction in the size of the hemelytron (Figure 2.4) Others features are the similarity of the tibia and tarsus of the hind leg, transverse swelling interrupting the depression of the interoculus at the posterior end and convexity of the interoculus (Figure 2.5).

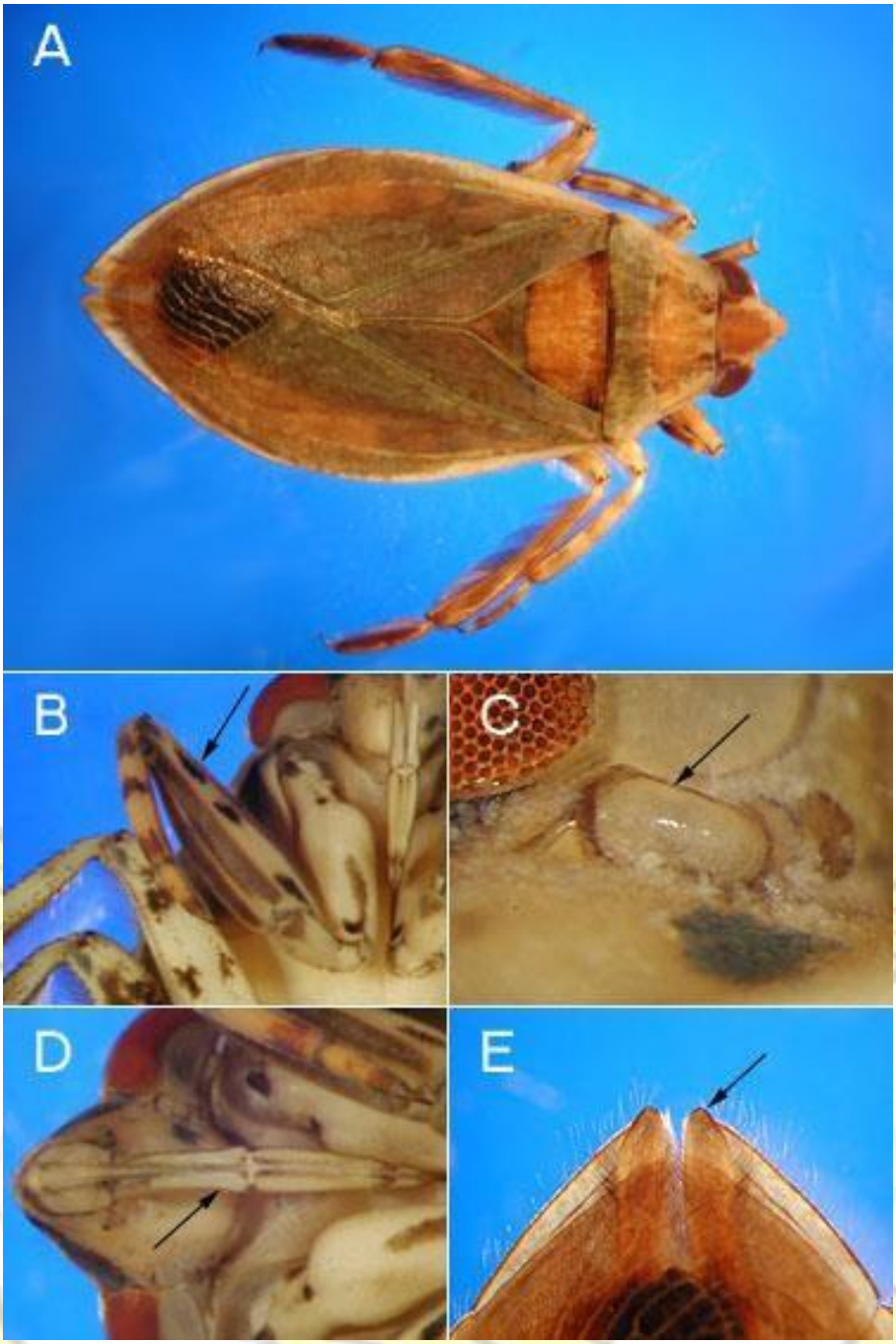


Figure 2.4 Some distinguishing characteristics of the Belostomatidae

A. Body oval flattened. **B**. Raptorial fore legs for grasping prey. **C**. Antennae under the eyes and shorter than head. **D**. Segmented cylindrical beak. **E.** Apex of abdomen with a pair of air straps to help in respiration (Macro-Invertebrate Lab, Valley City State University, 2005)

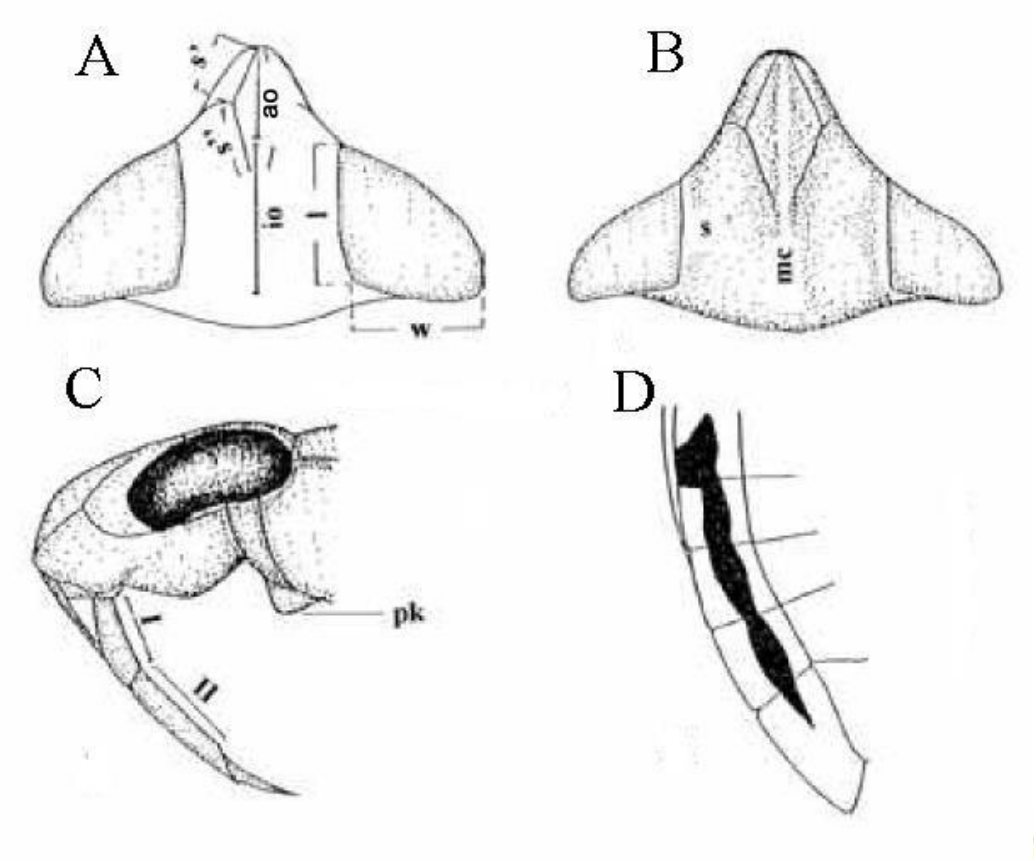


Figure 2.5 Some distinguishing characteristics of *Diplonychus* spp (Estévez & Polhemus, 2001).

A. Head: dorsal view (ao - anteoculus; io – interoculus; l – length of eye; s' – suturae anteclipeus-maxillary plate; s" – anteclipeus-loral plate; w- width of eye).

B. Head: ventral view (mc – median carina; s – sulcus). **C**. Head: lateral view (I – first segment of the beak; II – second segment of the beak; pk – prosternal keel. **D**. Abdominal pilosity.

2.4.3 Involvement of Small Mammals

The role of mammals in the ecology of a number of mycobacterial infections has been extensively exploited. Generally, pathogens that are transmitted between the environment, wildlife, livestock and humans represent a major challenge for control, prevention and management (Biet *et al.*, 2005). As several mycobacterial pathogens appear to exhibit such relationships, *M. ulcerans* inclusive, it is imperative to identify all ecological players in their survival and

transmission for the development of feasible preventive or control measures. Although *M. ulcerans* is described as an environmental saprophyte, the detection of high concentrations of genetic material of the pathogen in the faeces of possums sampled in Australia suggested a possible role of small mammals in its survival and or transmission to humans (Fyfe *et al.*, 2010). A study conducted by Durnez and colleagues in some BU endemic areas of Benin, which sampled 68 animals and 90 faecal samples, however did not detect *M. ulcerans* genetic material. They however suggested further research on African small mammals (Durnez *et al.*, 2010). Buruli ulcer-like skin lesions observed on possums, which tested positive for the insertion sequence *IS2404*, in Australia reiterates the importance small mammals may be playing in the survival, multiplication and transmission of the pathogen (Fyfe *et al.*, 2010).

2.5 The One Health Concept

Control and management of infections are influenced by interrelated events or factors. It is therefore important that studies into such infections acknowledge the complexity of all related factors (Lerner & Berg, 2015). A strong belief in solving multifaceted problems in the health sector with a multifaceted approach has been reported to yield better outcomes than single unrelated research approach to health problems (Monath *et al.*, 2010). The One Health concept recognizes that the health of people is connected to the health of animals and the environment and therefore assesses health in three levels:

Individual level, Population level and Ecosystem level (Lerner & Berg, 2015). The One Health framework has offered the opportunity for the WHO programme on Neglected Tropical Diseases (NTD) to create a common platform for veterinarians

and clinicians to advice on the control of neglected zoonotic diseases (NZD) such as rabies, brucellosis, tuberculosis and echinococcosis (WHO, 2007; Fisman & Laupland, 2010).

2.5.1 One Health in Neglected Zoonotic Diseases

The One Health concept aims to promote the integration of human, animal and environmental health through collaborations among clinicians, veterinarians, environmental public health experts and others. The concept promotes interdisciplinary and transdisciplinary approach to research on neglected zoonotic infections. The WHO in its 2008 report on the meeting of the International Task Force for Disease Eradication adopted this concept leading to an increase and expansion of its use in many areas of disease research (WHO, 2008). By One Health principles, artificial barriers among different professionals are broken while the individual domain is still protected, each discipline being a strong part of a whole, informing each other and thereby strengthening each other (Monath *et al.*, 2010). One health has now become a lead concept in research, capacity building and translational efforts (Zinsstag *et al.*, 2014).

A one health approach however does not mean that all possible aspects of a disease have to be included in every study but rather implies that the practicality of any correlations to species, populations and ecosystems must be considered when designing research whenever relevant (Lerner & Berg, 2015). An example of a study based on the One Health Concept is being undertaken by the Swedish University of Agricultural Sciences, which comprises human well-being, animal welfare, human-animal interactions and company efficiency in abattoirs and animal laboratories: (Lerner & Berg, 2015).

Buruli ulcer is a mycobacterial infection of the skin of humans and some mammals. The causative organism is a mycobacterium that has been detected in aquatic environments and infections believed to be from these environments (Merritt *et al.*, 2010; Williamson, Mosi, *et al.*, 2014). The exact mode of transmission is, however still not known. Studies on the ecology of such a pathogen will be incomplete is aspects of its habits and factors that may be driving infection are explored exclusively. The One Health concept of research, which is multidisciplinary and integrated, is consequently appropriate for such a study.

2.5 Study Communities

All four study communities are in the Amansie Central District of the Ashanti Region of Ghana. The Amansie Central District is located in the southern part of the Ashanti Region, within Latitudes 06.000 N and 06.300 N and Longitudes 1.000 W and 2.000 W (Figure 3.1, insert). The Amansie Central District has a total land area of about 710 square kilometers and is divided into five sub districts. These are Jakobu, Twapiase, Fenehia, Numereso and Fiankoma.

Numereso and Fiankoma are endemic for Buruli ulcer. The four communities selected for this study were Wromanso (**N** 06.03256, **W** 001.89761), Bepotenten (**N** 06.09213, **W** 001.96604), Monia-Gyaman (**N** 06.05113, **W** 001.92242) and Sukuumu (**N** 06.05190, **W** 001.94341) all in the Numereso Sub district. The district is a rural district in the Ashanti Region of Ghana, with an overall annual per capita expenditure of GHC 3,119 which is above the national average (Ghana Statistical Service, 2014).

3.5.1 Bepotenten

Bepotenten is a BU endemic community in the Numereso sub-district with an estimated population size of 247. The inhabitants predominantly belong to the Ashanti tribe with farming as the main occupation. Most of the youth in this community were however observed to be involved also in small scale mining (locally called galamsey) along the banks of the two major rivers (Offin River and the Oda River). The community has three public refuse areas and two public latrines. Two working borewells were seen in the community. Houses were mainly built from clay with roofing of thatch and there is no health facility in this community. Reported BU prevalence for Bepotenten is 0.012% (HFG, 2014).

3.5.2 Sukuumu

Sukuumu was described as the most BU endemic community in the district by the disease control officers in the district. With an estimated population of 1,406 inhabitants, Sukuumu was the biggest of the four selected communities and has a health post manned by a medical assistant. Houses in this community are mainly made of cement blocks and roofed with aluminum sheets, indicative of a higher social status compared to Bepotenten. There was no community latrine in this community as households had their own dug out latrines. Inhabitants in Sukuumu are predominantly dangmes who have migrated into the area from the Greater Accra Region for farming. The Offin River flows close to the community together with a smaller stream, the Twingum stream. The majority of households also reared domestic animals usually for household consumption. Sukuumu has two functioning borewells and a BU prevalence of 0.0078% (HFG, 2014).

3.5.3 Monia-Gyaman

Monia-Gyaman is a twin community about 5km from Sukuumu, with an estimated population size of 454. Youth in this community are highly involved in dredge mining in the Offin River which flows about 3 km from the community. Mixed crop farming is the main occupation of the inhabitants. One functioning borewell and one well dug close to the Akotia Stream were the main water sources to the community. Another stream known as the Ampomaa Stream also flows close to the community and inhabitants usually wade through this stream on the way to their farms. This community also had a school and a public latrine for community use. Buildings were constructed with a mixture of cement and clay with thatch and aluminum roofs. Buruli ulcer prevalence is 0.0066% (HFG, 2014).

3.5.4 Wromanso

Wromanso is a small community with an estimated population of 312. The community is surrounded by a thick forest cover which is about a kilometer away from the community. Mining activities have greatly interrupted this forest cover. Vast bare lands of abandoned surface mining are particularly seen near the Offin River. Similar to the other three communities, inhabitants of Wromanso are also predominantly farmers. A small pond, the Bebunu pond and a borewell are the sources of water to the community. Wromanso also has a common refuse damp and a community pit latrine. Houses are a mix of mud houses with thatch roofs and cement houses with aluminum roofs. Buruli ulcer prevalence is 0.0064% (HFG, 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Sites

The selection of communities and water bodies for all the studies was associated with the specific objective of the aspect of the study. The criteria for site selection therefore differed for the various objectives as described in the following sub sections.

3.1.1 Risk Environments and Socioeconomic Activities

A BU survey conducted in the Amansie District reported an overall district prevalence of 0.32 per 1000 individuals (HFG, 2014). Four BU endemic communities were selected in the Amansie Central District of the Ashanti Region of Ghana after a two week scouting (Figure 3.1). Numbers of inhabitants in the four communities were obtained from the Amansie Central District Health Directorate (ACDHD). This was 2,419 (247, 312, 454 and 1,406 for Bepotenten, Wromanso, Monia-Gyaman and Sukuumu respectively). Buruli ulcer prevalence in the four communities were 12.1, 6.4, 6.6 and 7.8 per 1000 inhabitants for Bepotenten, Wromanso, Monia-Gyaman and Sukuumu respectively (HFG, 2014). These were all higher than the district overall district prevalence, the reason for which these four communities were selected for the study. Identified water bodies in these communities were also sampled for NTM presence for transmission route studies.

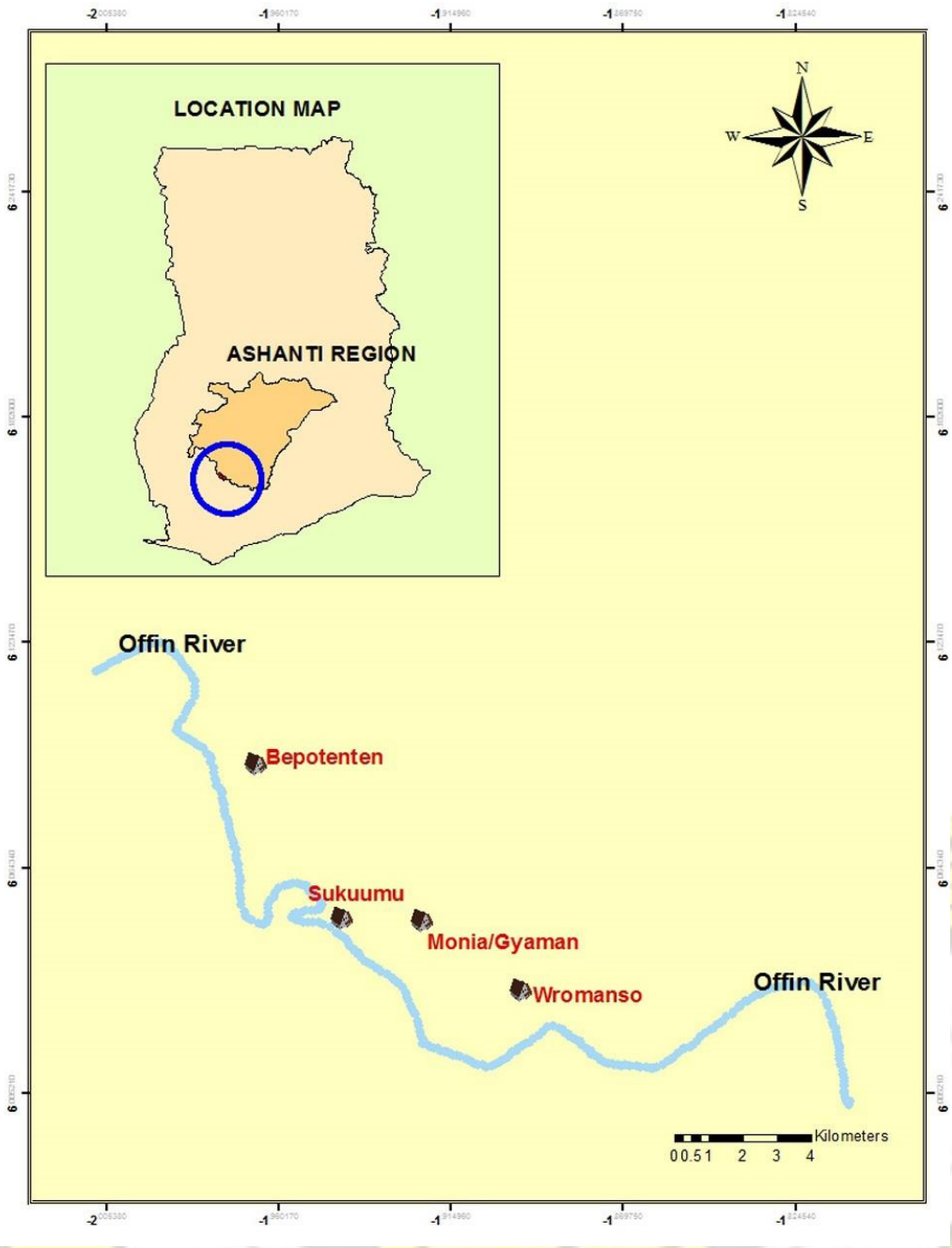


Figure 3.1 Location map of the Amansie Central District showing the locations of the four study communities (ESRI, 2011).

3.1.2 Belostomatidae and Naucoridae Diversity

The Families Belostomatidae and Naucoridae are the most implicated in the transmission of BU in the West African region. Although several other macroinvertebrates were collected, this study focused on these two families.

Macroinvertebrates were collected from water bodies in two West African countries namely Ghana and Cote d'Ivoire. The collection period spanned seven years from 2006 to 2012 and this was part of bigger projects involving the use of the one health concept and other ecological methods in transmission and ecology studies of the infection. The samples were, therefore, collected in many projects over the time period. Samples were collected mainly from water bodies in communities with BU disease history but few were also from BU non endemic communities in Ghana (Volta Region of Ghana and parts of the Greater Accra Region of Ghana). Communities were preselected based on the presence of at least one water body, reported BU disease and aquatic macroinvertebrates. Criterion for separating communities into BU endemic and BU non endemic was based on data of BU case reports from the Ghana National Buruli ulcer Control Programme and District BU data. A single BU case reported from a community was adequate to designate a community as BU endemic. Samples of invertebrates from non-endemic communities especially in the Volta region in which cases of BU were reported during the study were still kept and analysed with the nonendemic community samples for their possible involvement in transmission. Ninety eight water bodies were sampled for invertebrates from the two countries, 54 endemic and 35 BU non endemic communities. A complete list of all communities and water bodies from which samples were taken is given in appendix 6. The locations and BU statuses of communities from which invertebrate samples were identified and obtained are shown in Figure 3.2. Water bodies sampled included large rivers, streams, lakes and ponds.

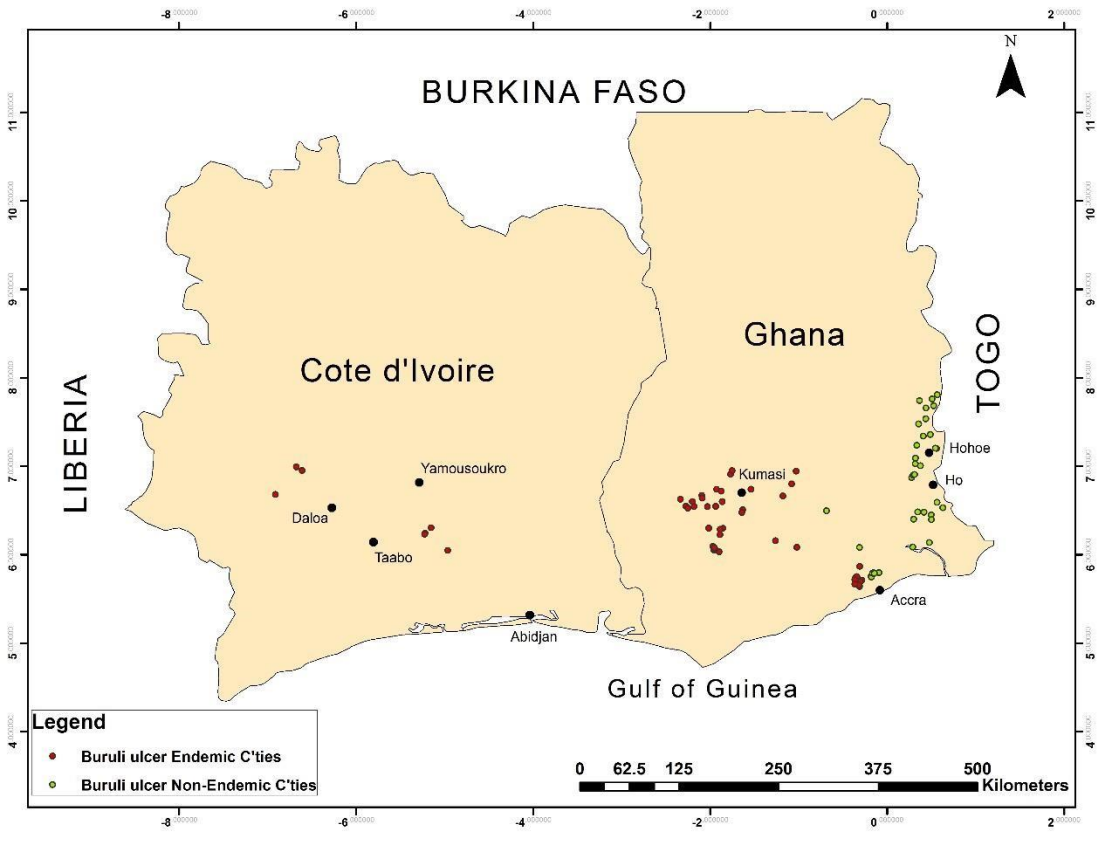


Figure 3.2 Map showing communities and their BU disease statuses from which macroinvertebrates were collected in Ghana and Cote d'Ivoire (ESRI, 2011).

3.2 Ethical Approval

Ethical approval (FWA 00001824; IRB00001276; IORG0000908) for recruitment into the study was sought from the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana. This also covered the administration of questionnaires. Community entry procedures were followed. All participants consented before recruitment into the study (Appendix 1).

3.3 Sample Size and Study Design

The sample size calculator for questionnaire surveys was used in determining sample sizes for administration of questionnaires (Sopoh *et al.*, 2010). For the estimated population size of 2,419, expected frequency of 85%, a design effect of 1.0 for a simple random sample design and assuming the 4 communities as clusters, an estimated sample size for each cluster was calculated as 46 questionnaire respondents per cluster (community). The total minimum estimated sample size was therefore 184. The study design was cross sectional. The design, which was based on the one health concept, was an integration of mapping selected communities for water contact points, using structured questionnaires for risk information and linking community disease to identified environments using molecular methods. Sample sizes were not calculated for the insect diversity studies.

3.4 Questionnaire Administration for Risk Determination

Two hundred and twenty four (224) questionnaires were administered in the four communities. Three teams were formed to administer questionnaires in each community. Each team was made up of one research assistant and one community health nurse or a disease control officer. The purposeful sampling technique was used in the administration of questionnaire in the four communities. Questionnaires were translated to the local dialects (Twi and

Dangme) with the help of community opinion leaders, tested and retranslated into English after use. They were administered to one person usually an adult within randomly selected households, after the community was partitioned for the different teams. Participant's demographic data (name, age, gender, status in

community), duration of stay in the community, Buruli ulcer disease status, water usage and contact, livelihood and economic strategies and contact with game and household animals were collected (Detailed questionnaire: Appendix 3).

Presumptive BU lesions were confirmed by *IS2404* PCR in the laboratories of the Noguchi Memorial Institute for Medical Research. Characteristic scars were confirmed by treatment history.

3.5 Community Mapping and Description

The four communities (Bepotenten, Sukuumu, Monia-Gyaman and Wromanso) selected for risk environment were mapped marking landmarks, aquatic areas, paths and some households. A Garmin 62 GPS (Garmin®, Kansas, USA) was used for taking all coordinates. Information from the questionnaires administered earlier was used to identify water contact areas that could not be easily identified in community maps.

3.6 Active Case Search and Human Sample Collection

The volunteer system set up by the office of the Amansie West District Directorate of Health for BU patient identification and treatment was used in active case search and subsequent collection of patient samples in the four communities. After community leaders and opinion leaders were met and permissions obtained, the community was sensitized by the beating of the "gonggong" to announce the presence of health officials from the district health office

on an agreed date. A BU screening team was set up in the community for screening of all skin lesions. Samples were taken for all suspected BU cases

(WHO, 2014b) which were later confirmed by PCR. Fine needle aspirates (FNA) were taken for nodules and swabs for lesions by a trained laboratory technician (Figure 3.3). This was done after informed consent or assent for minors had been obtained from the patient (Appendix 1 and Appendix 2). Fine needle aspirates were kept in sterile 2 mL screw-cap (STARSTEDT, Numbrecht, Germany) tubes and swabs in 15 mL sampling tube. In few instances, the team had to follow up on cases in their households upon reliable information. Patient samples taken were stored on ice or refrigerated at 4^{o}C until the PCR confirmation processes were performed.

Figure 3.3 Patient sampling (swab collection) at Monia-Gyaman (Author, 2013)

3.7 Environmental Sample Collection

Environmental sampling followed procedures described by Williamson *et al*. (2008) with minor modifications. Briefly, at each water body site, four types of samples were collected: water filtrates (3 samples), biofilms (5 samples), detritus

(3 samples) and soil (3 samples). Fourteen samples were therefore collected at each water body site (Figure 3.4). All sampling equipment used were thoroughly washed with 70% bleach and decontaminated with 90% v/v ethanol and DNA AWAY (Molecular BioProducts, San Diego, California, USA) between sampling sites and sample types.

For soil, a sterile scalpel was used to collect about 50 g of soil from the water-land interface (bank) of the water body and two from the riparian zone 5m apart into a 15 mL falcon tube (BD Biosciences, San Jose, USA). Samples were then preserved in 5 mL 100% ethanol (Pharmacos, Haryana, India). Detrital samples consisted of dead and rotten leaves, rotten stem barks and twigs and dead grass blades found within the water body. These were collected onto a white tray and pieces taken into a 15 mL falcon tube using a knife and a pair of forceps. Detrital samples were also preserved in 5 mL of 100% ethanol.

Biofilms are usually attached to surfaces and therefore needed to be dislodged. Stems, twigs and leaves of dominant aquatic vegetation that are permanently in contact with water were cut into Ziploc bags (30X38 cm). Approximately, 100 mL of sterile double distilled water was added, sealed and biofilm dislodged by hand robbing the plant in the bag vigorously for about 1 minute. Fifty milliliters (50 mL) of the water was then poured out into a 50 mL falcon tube and preserved on ice. For water filter samples, about 2 litres of open surface water was scooped from various areas of the water body to provide a mixture. Exactly 50 mL of this mixture was pressure filtered through a 0.45 µm nitrocellulose filter (Whatman Inc, Buckinghamshire, UK) in a 50 mL filter

syringe. The nitrocellulose filter was then removed and wrapped completely in aluminum foil and kept on ice.

Figure 3.4 Field activities and sampling sites (Author, 2013).
A. Meeting opinion leaders at Bepotenten before questionnaire administration. **B**. Akotia stream at Monia-Gyaman. **C**. Offin River showing immense mining activities and the destruction of natural environment. **D**. Small scale dredgemining activities on the Offin River.

3.8 Macroinvertebrate Sample Collection

At each water body, a brief description was performed that included the type, size, flow, and vegetation cover. Sections of the water body to be sampled for invertebrates were also determined based on representation and accessibility of littoral areas of the water body. Using a 500 µm mesh size dip net, designed to

capture all life stages of aquatic invertebrates, ten sweeps of the littoral areas of the water body was performed (Figure 3.5). Collected content in the dip net was then carefully emptied into a white tray. Invertebrates were then carefully picked using a pair of forceps into 100% v/v ethanol in labeled vials. Picking of invertebrates was done for a maximum duration of 20 min with care not to pick similar species of invertebrates in cases where the abundance of some species in the collection is very high. The collection of Hemiptera was prioritized. The process was repeated two more times thereby obtaining a total invertebrate diversity in 30 dip net sweeps per water body. In situations where picking of invertebrates in the field was hampered (usually due to time constraints and adverse weather conditions), the entire content of the dip net sweep was preserved in a jar containing 100% v/v ethanol and picking done in the laboratory.

Figure 3.5 Collection of invertebrate samples using the D-frame net (Author, 2012).

3.9 Confirmation of Human Buruli Ulcer Samples

Human samples were confirmed based on the recommended laboratory method for the confirmation of suspected Buruli ulcer samples (WHO, 2014b).

3.9.1 Pre-DNA Extraction

Using the FNA, 2 mL of 1X Phosphate Buffer Saline (PBS) was added to 300 µL of sample in a 2 mL screw-cap tube and vortexed briefly. With the swabs, 2 mL of 1X PBS was pipetted into the sample collection tube containing the swab. The tube was then vortexed for 5 min to dislodge bacterial cells. The solution was later transferred to a 2 mL screw-cap tube. For both types of sample tubes, 1 mL was pipetted into a new 2 mL screw-cap tube, vortexed briefly and spun at 15,400

RCF (Suprema 21, TOMY, Tokyo, Japan) for 5 mins. About 800 µl of supernatant was then pipetted off and preserved at 4ºC for later use. The remaining 200 µl (plus pellet) was used in the DNA extraction procedure.

3.9.2 DNA Extraction

The DNA extraction for patient samples was performed with the Qiagen DNeasy blood and tissue kit (QIAGEN, Venlo, Netherlands) following the manufacturer's protocol. For FNA samples, 850 µL of 1X PBS was added from which 500 µL was aliquoted into a new 2 mL screw cap tube and centrifuged at 15,400 RCF for 15 min to pellet bacterial cells. About 300 µL of supernatant was pipetted off and 180 µL of buffer ATL and 20 µL of proteinase K were added to the tube containing the pellets. The resulting mixture was then vortexed for 10 sec and incubated at 56°C for 3 hours after which 200 µL of buffer AL was added, vortexed briefly and incubated at 70°C for 30 mins. About 250 µL of 100% ethanol was then added, vortexed and transferred to a spin column. The column was then centrifuged at 3,500 RCF for 1 min and flow-through discarded. The column was then washed by adding 500 µL of buffer AW1, centrifuged at 3,500
RCF for 1 min and flow-through discarded. Washing was repeated with buffer AW2. The column was then dried by spinning at 13,300 RCF for 3 min, put into freshly labeled 1.5 mL tubes, 150 µL of buffer AE added and centrifuged at 3,500 RCF to elute DNA. DNA was stored at -40°C until further use.

3.9.3 Polymerase Chain Reaction

Insertion sequence 2404 (*IS2404*) was targeted for confirmation of *M. ulcerans* infection (BU) in human BU samples as recommended by WHO (WHO,

2014b). For each PCR run, both negative and positive controls were included. Amplification for *IS2404* loci was performed in a nested PCR. The nest 1 was performed in a 25 µL reaction containing 1X PCR buffer (Promega, Wisconsin, USA), 1 mM $MgCl_2$ (Thermo Scientific, Waltham, Massachusetts, USA), 300 µM each of deoxyribonucleotide (Thermo Scientific, Waltham, Massachusetts, USA), 700 nM of each primer, Pg1 and Pg2 (Table 3.1), 1U GoTaq Polymerase (Promega, Madison, Wisconsin, USA) and 5 µL of genomic DNA. In the nest 2, 1µl of PCR product from nest 1 was used as template in a 25 µL reaction. All other reagents, volumes and concentrations remained same except for the primer set which were 500 nM each of, Pg3 and Pg4 (Table 3.1). Cycling conditions were as follows: Preheating at 95°C for 2 min, followed by 40 cycles each of; denaturation at 94°C for 30 sec, annealing at 64°C for 1 min and extension at 72°C for 1.5 min. Final extension was at 72°C for 10 min and reaction held at 4°C.

3.9.4 Gel Electrophoresis and UV Visualization

Agarose gel electrophoresis was used for resolution of *M. ulcerans* PCR amplicons. Seven microlitres of PCR products were run on a 2% agarose (SigmaAldrich, St. Louis, Missouri, USA) gel, stained with ethidium bromide (SigmaAldrich, St. Louis, Missouri, USA), using 1X Tris Acetate EDTA (0.8 mM Tris, 0.8 mM glacial acetic acid, 10 mM EDTA) as running buffer, at 100V (Power PAC 3000, BIO RAD, Marnes-la-coquette, France) for 50 min. The gel was visualized under a UV transilluminator (ClearView, Cleaver Scientific Ltd, Warwickshire, United Kingdom). For each run, 100 bp ladder was used as size marker. Gel

pictures were taken using a Gel Logic Imaging System (Kodak, Tokyo, Japan) and band sizes scored by the aid of the marker.

3.10 Processing of Environmental Samples

3.10.1 Pre-Extraction Processing

The four types of environmental samples collected (filters, biofilms, detritus and soil) as described earlier were processed differently before DNA extraction.

3.10.1.1 Filters

Filters preserved in aluminum foil were removed and cut into four pieces using a sterile pair of scissors. The scissors was decontaminated between samples by wiping off with ethanol in tissue followed by DNA AWAY (Molecular BioProducts, Waltham, Massachusetts USA). The four quarters were then put into sterile screw cap tubes for DNA extraction.

3.10.1.2 Biofilms

The 50 ml falcon tube containing the biofilm-distilled water mix was concentrated by spinning at 11,300 RCF for 5 min in a high speed refrigerated centrifuge at 4°C (Suprema 21, TOMY, Tokyo, Japan). About 20 mL of the water was slowly decanted and the remaining spun again at 13,300 RCF for 5 min. Approximately 15 mL of the water was again decanted off and the rest spun at 15,400 RCF for 5 min after which 5 mL was pipetted off leaving a more concentrated biofilm-distilled water mixture of about 10 mL. For each DNA extraction, 1 mL of this solution was pipetted into a 2 mL screw-cap tube, centrifuged at 15,400 RCF in a mini centrifuge (Centrifuge 5415D, Eppendorf,

Hamburg, Germany) for 5 min, 700 µL of the supernatant pipetted off and the remaining 300 µL used for further studies.

3.10.1.3 Detritus

Detrital samples were processed in ethanol (from the same tube the sample was preserved in from the field). For each sample, about 1g of detritus was cut with a pair scissors into a sterile 2 mL screw-cap tube, 400 µL of the 100% v/v ethanol added. The tube was then centrifuged at 15,400 RCF for 5 min after which 300 µL of the ethanol was pipetted off. The DNA was extracted from the remaining 100 µL and the detrital sample.

3.10.1.4 Soil

The 15 mL Falcon tube containing soil sample preserved in ethanol was vortexed vigorously to dislodge any attached material into the ethanol. About 500 µL was immediately pipetted into a 2 mL screw-cap tube, spun at 15,400 RCF for 5 min. The supernatant was then pipetted off and DNA extracted from the remaining mixture.

3.10.2 DNA Extraction

Extraction of DNA from all environmental samples basically followed protocols described by Williamson *et al*. (2008). To each sample in a 2 mL screw cap tube, 450 µL of Lysis buffer (0.1 M Tris (pH 8.0), 0.05 M EDTA, 0.5 M NaCl, 1.3% SDS and 50 µg/mL RNase A) and 1 g of glass beads (Sigma-Aldrich, St. Louis, Missouri, USA) were added. The tubes were then bead-beaten by vortexing (Mini vortexer, VWR Scientific Products, Radnor, Pennsylvania, USA) for 10 min. The sample was then incubated at 65°C in a water bath and spun at 1,500 RCF for

2 min. Exactly 400 µL of supernatant was pipetted into a new 1.5 mL tube (Posi-click tube, Denville, New Jersey, USA), 150 µL of 5 M potassium chloride added and incubated at -40°C overnight. The sample was later centrifuged at 1,500 RCF for 30 min and 400 µL of supernatant transferred to a new 1.5 mL tube containing 600 µL of binding buffer (1 M Guanidine hydrochloride and 63% V/V ethanol solution). After vortexing briefly for 5 sec, 700 µL of the resulting mixture was transferred into a spin column (MOBIO, Ontario, Canada) which was spun at 1,500 RCF for 2 min and the flow-through discarded. The spin column was washed by adding 500 µL of washing buffer (100 mM Tris (pH 8.0), 1 mM EDTA, 500 mM NaCl and 67% V/V ethanol solution) and spun at 1,500 RCF for 2 min and the flow-through discarded. The wash procedure was repeated once but with 100% ethanol. The column was then spun at 1,400 RCF for 5 min to dry. About 200 µL of elution buffer [10 mM Tris (pH 8.0)] was added to the column and incubated at room temperature for 5 minutes.

The column was then spun at 1,500 RCF for 2 min to elute DNA. The DNA was stored at -40°C until further use.

3.10.3 Detection of Mycobacteria

To identify environmental mycobacteria, samples were first screened using mycobacterial 16S rRNA primers which is indicative of the presence of mycobacterial genetic material. Samples positive for 16S rRNA were further screened for the insertion sequence *IS2404* which is suggestive of the presence of mycolactone-producing-mycobacteria (MPM). The *IS2404* positive samples were then further screened by viable number tandem repeat (VNTR) profiling for

categorizing MPMs. Details of primers used for all PCR amplifications and their associated references are listed in Table 3.1.

3.10.3.1 16S rRNA Detection

16S rRNA PCR was performed in a 25 µL reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 400 µM each of deoxyribonucleotide, 160 nM each of forward and reverse primers, 1 U GoTaq polymerase and 5 µL of genomic DNA. About 300 ng/µL BSA was added for environmental samples to minimize or eliminate inhibition. Pre-cycling was done at 95°C for 5 min, followed by 34 cycles each of; denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 45 sec. Final extension was set at 72°C for 10 min and reaction held at 4°C. Identification of characteristic band sizes for 16S rRNA were done on Agarose gel as described in section 3.9.4.

Table 3.1 List of primer sequences used for PCR amplifications

Primer	Forward and reverse sequences	Expected Band Sizes	References
IS2404 **(Nest 1)**	**Pg1**: 5'-AGGGCAGCGCGGTGATACGG-3' **Pg2**: 5'-CAGTGGATTGGTGCCGATCGAG-3'	400	Ablordey *et al.* (2012)
IS2404 **(Nest 2)**	**Pg3**: 5'-GGCGCAGATCAACTTCGCGGT-3' **Pg4**: 5'-CTGCGTGGTGCTTTACGCGC-3	200	Ablordey *et al.* (2012)
16S rRNA	**PA**: 5'-AGAGTTTGATCCTGGCTCAG-3' **MSHA**: 5'-AAAAAGCGACAAACCTACGAG-3'	600	Hughes *et al.* (1993)
Locus 6 (VNTR)	**F**-5'GACCGTCATGTCGTTCGATCCTAGT 3' **R**-5' GACATCGAAGAGGTGTGCCGTCT 3'	Variable	Williamson *et al.* (2008)

Locus 19 (VNTR)	**F**-5' CCGACGGATGAATCTGTAGGT 3' **R**-5' TGGCGACGATCGAGTCTC 3'	Variable	Williamson *et al.* (2008)
ST1 (VNTR)	**F**-5' CTGAGGGGATTTCACGACCAG 3' **R**-5' CGCCACCCGCGGACACAGTCG 3'	Variable	Williamson *et al.* (2008)
MIRU1 (VNTR)	**F**-5' GCTGGTTCATGCGTGGAAG 3' **R**-5' GCCCTCGGGAATGTGGTT 3'	Variable	Williamson *et al.* (2008)

3.10.1.2 *IS2404* Detection

Detection of insertion sequence *IS2404* in environmental was done as described for human samples in section 3.9.3.

3.10.1.3 VNTR profiling

Detection of the four VNTR loci was performed as described by Williamson *et al.* (2012), Hilty *et al.* (2006) with slight modifications. Four loci (Table 3.2): MIRU 1, Locus 6, ST1, and Locus 19, were amplified in separate reactions with similar master mix concentrations. Each was performed in a 25 µL reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 400 µM each of

deoxyribonucleotide, 160 nM each of the specific forward and reverse primers, 1 U GoTaq polymerase and 5 µL of genomic DNA. Cycling conditions for locus 6, locus 19 and MIRU 1 were 95°C for 2 min followed by 40 cycles each of, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min and finally 72°C for 10 min. The reaction was then held at 4°C. For ST1, conditions were 95°C for 2 min, 40 cycles each of; 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min, 72°C for 10 min and the reaction kept at 4°C.

3.10.1.4 VNTR Analysis and Strain Designation

Repeat numbers for all VNTR loci were calculated based on published data (Ablordey, Hilty, *et al.*, 2005; Stragier *et al.*, 2005; Hilty *et al.*, 2006; Hilty *et al.*, 2007; Lavender *et al.*, 2008; Williamson *et al.*, 2008). Expected PCR product sizes and corresponding repeats for each VNTR locus are shown in Table 3.2.

Table 3.2 PCR product (band) sizes and corresponding VNTR Repeats

VNTR Loci	Band size (Repeat number)				Repeat length (bp)
MIRU1	404 (1)	457 (2)	510 (3)	563 (4)	53
Locus 6	454 (1)	509 (2)	565 (3)	621 (4)	56
ST1	369 (1)	423 (2)	477 (3)	531 (4)	54
Locus 19	288 (1)	344 (2)	400 (3)	456 (4)	56

3.11 Macroinvertebrate Samples

Macroinvertebrate samples collected from water bodies were cleaned and prepared for taxonomic identification. The process comprised of removal of all aquatic invertebrates from excess substrates that might have been picked in dipnet sweeps (cleaning and picking) and assigning specimen to taxonomic lots prior to taxonomic identification. Figure 3.5 illustrates the step by step processing in situations where crude samples collected and brought to the laboratory in jars.

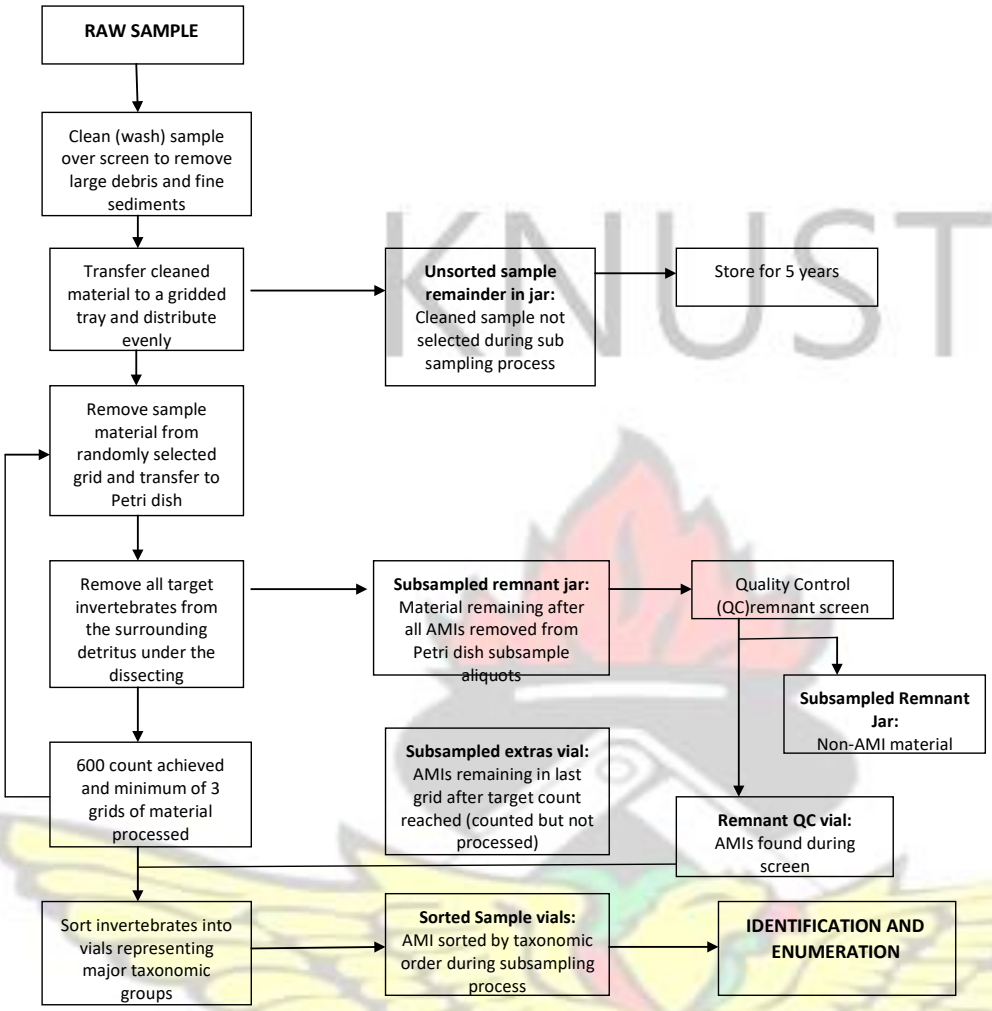


Figure 3.5 Stepwise processing of aquatic macroinvertebrate (AMI) samples

3.11.1 Sample Cleaning

During this process, fine sediment and large debris were removed from the sample yielding "cleaned" AMI (aquatic macroinvertebrates) specimens in the gridded subsampling tray. Briefly, the entire content of the jar was poured into the 500-µm sieve over an ethanol catch container. Water was gently spayed over and through the sample in the sieve using a squirt bottle, gently shaking the sieve until the water runs clear and all fine sediment had been removed. Large objects (e.g., rocks, sticks, leaves and other large detritus) were removed with a pair of forceps rinsing each over the sieve after inspection for clinging AMI and then discarding

them. The rinsed sample was then transferred to a gridded white sample tray using water to rinse any remaining sample in the sieve into the sample tray. Samples already picked in the field and preserved in vials were cleaned by pouring out the entire content of the vial onto a petri dish and picking out invertebrates into a new vial filled with ethanol. Twigs, leaf pieces and other items that might have been picked in the field were discarded after all invertebrates had been picked.

3.11.2 Sample Processing

The sample processing involved subsampling steps, picking and sorting. Here, each sample was evenly spread onto a gridded sub-sampling tray making sure sample thickness in the tray was not greater than 12 mm and with a target of 100 organisms per grid (50 mm X 60 mm). Three grids were then selected randomly (using random numbers). Each grid subsample was then divided into eight using a clean razor. One-eighth grid (1.88 cm x 2.5 cm) was the smallest aliquot size that could be manipulated efficiently. Each one-eighth subsample was then transferred with a pair of forceps to a petri dish under a dissecting microscope at low power (10X). The 70% v/v ethanol added to completely cover the sample and the petri dish gently agitated to evenly distribute the subsample within the dish. The petri dish was then scanned and AMI picked into vials ensuring the target of 100 AMI for the whole eight subsamples (12-15 organisms per aliquot). The remaining sample from the gridded tray was then transferred back into the unsorted sample remainder jar, using ethanol to rinse the sample from the tray into the jar. Aquatic macroinvertebrates picked (both on the field and in the laboratory) were then sorted into the following taxa in separately labeled vials: Ephemeroptera, Plecoptera, Odonata, Trichoptera, Hemiptera,

Coleoptera, Diptera (other than Chironomidae), Chironomidae, Oligochaeta, Turbellaria, Hirudinea, Ostracoda, Bivalvia, Gastropoda, Isopoda, Amphipoda, Decapoda, and Others (Merritt & Cummins, 1996).

3.11.3 Identification and Enumeration of Belostomatidae and Naucoridae Samples

Vials containing sorted members of the order Hemiptera were separated from the others for each sample site. For each site, all contents of the vials were poured into petri dishes and all members of the Family Belostomatidae and Family Naucoridae picked (based on characteristics described below) and counted into separate vials. The Belostomatidae and Naucoridae were identified to generic and species taxa based on available taxonomic keys. All taxonomic characteristic examinations were done under the Motic K-500L stereo microscope.

3.11.3.1 Taxonomy of the Belostomatidae

Descriptive keys used to classify the Belostomatidae were based on an amalgamation and complementary alignment of available taxonomic keys (Menke, 1960; Lauck & Menke, 1961; Needham & Needham, 1962; Menke, 1979; Merritt & Cummins, 1996). Full taxonomic key used to classify the Belostomatidae in this study is given in Appendix 4. All Belostomatidae were classified to generic level as no keys were available to clearly differentiate below this level for the collections.

3.11.3.1 Taxonomy of the Naucoridae

Keys used for the taxonomy of the collected Naucoridae were a combination of keys from a number of sources (Needham & Needham, 1962; Dejoux *et al.*, 1981; Merritt & Cummins, 1996; Sites & Mbogho, 2012; Mbogho & Sites, 2013).

3.12 Statistical Analysis

All community maps were drawn using ArcMap 10.2 (Environmental Systems Research Institute, Redlands, California, USA) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, California, USA). Associations between age groups and gender, and community responses were tested by the Chi square contingency for test of independence in Microsoft Excel 2010 MegaStat (Microsoft Corporation, Redmond, Washington, USA). Association between age groups and gender, and BU infection was tested by simple logistic regression in Epi Info 7.2 (CDC, Clifton Road, Atlanta, USA). Multivariate logistic regression (Epi Info 7.2, CDC, Clifton Road, Atlanta, USA) was used to explore associations between water use and livelihood strategies, and BU. The VNTR types were compared by simple similarity profiles. Belostomatidae and Naucoridae dependence on water types was tested by the Chi-square contingency test for independence (MegaStat, Microsoft Corporation, Redmond, Washington, USA).

CHAPTER 4

RESULTS

4.1 Sampling Community Maps and Demographics

4.1.1 Community Maps and Characteristics

Geographical positioning system (GPS) coordinates taken for houses and other landmarks together with a draft drawing of each community were used to draw an aerial map of each community. This enabled for the identification of all water areas (permanent or temporary) that may be available in the community together with those that were identified in questionnaire responses.

4.1.1.1 Bepotenten

Bepotenten which was the smallest of the four study communities is made up of about 50 houses (Figure 4.1). The only road is the one that led into the community from Yawkrakrom and terminates in the middle of the community. A path then leads to the borewell and another down a hill, eastwards towards the two contact points on the Oda River. The Oda River which flowed into the Offin River (about 3.5 Km from the community) was the only surface water body close to the community and a source of water that supported the single borewell with a pump located at the northern part of the community. Artisanal mining was also being undertaken at the upper and lower reaches of the river.

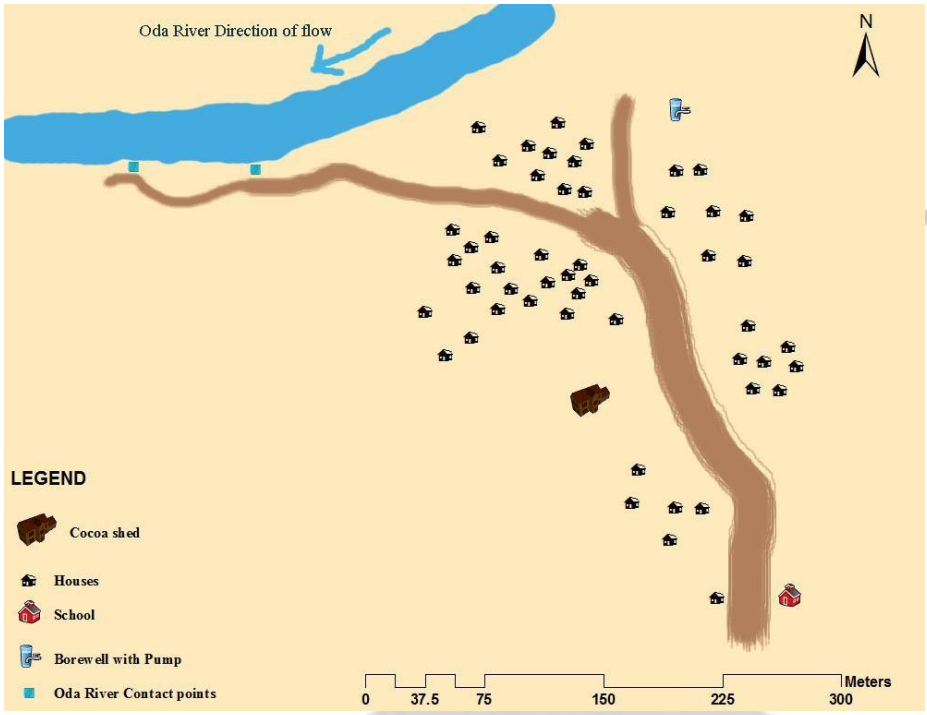


Figure 4.1 Sketch of the Bepotenten community (Author, 2015).

4.1.1.2 Sukuumu

Sukuumu was the largest of the four study communities and distinctly the most economically active (Figure 4.2). A health centre that is to serve communities in its catchment area was being constructed. The community, which is made up of approximately 150 houses, has two schools (primary side and junior high) on each side of the main feeder road. A single borewell fitted with a pump was available for use by all community inhabitants. The mapping of the community however revealed one main pond (Twingun 2) about 50 m from the last house in the community. A well cut out path and clearings around this pond suggested vibrant fetching of water from this pond (10 m in diameter).

Tracing the source of water to this pond revealed that it was artificially dug and regularly desilted by youth in the community. The source of water to this pond is the Twingun Stream (Twingun 1) which was traced to the other side of the main

road. Another contact point to this stream was found and marked. Inhabitants also fetch water from this stream usually on their way to farms.

The Sukuumu community is also surrounded by massive small scale artisanal mining activities. Most of the youth in the community participate in this activity mostly close to the Offin River which is about 700 m away from the community. Community inhabitants cross the Offin to other nearby larger communities mainly for economic activities. Intensive swimming and bathing activities also occur in the Offin River. Another water contact point for the Sukuumu community was the Akotia stream which lies about 1,500 m away from the community. This was the main source of water for a hamlet (few houses forming a small community usually belonging to one family), that had formed close to this water body.

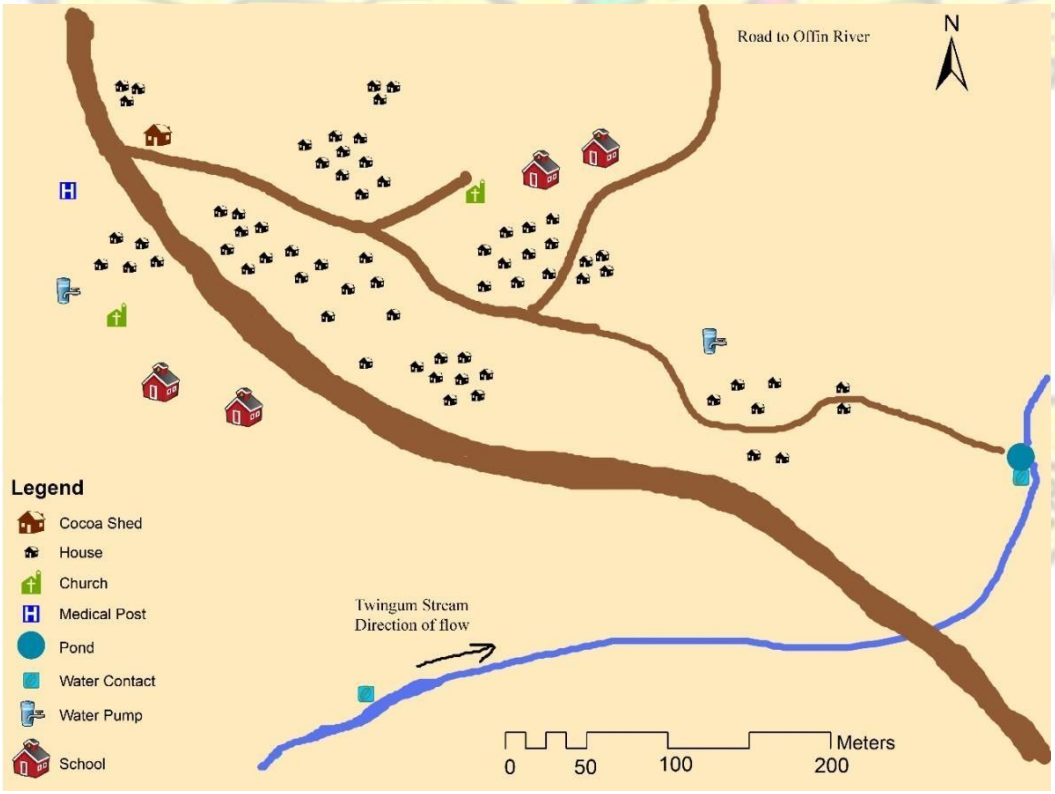


Figure 4.2 Sketch of the Sukuumu community (Author, 2015).

4.1.1.3 Monia-Gyaman

The Monia-Gyaman community also lies close to the Offin River (1000 m away).

It is made up of about 70 houses, a school and two borewells (Figure 4.3). One borewell is situated in the middle of the community close to a pond of water. This pond, formed from pit during the drilling of the borewell, had collected water but was not used by the community for any purpose. A second borewell had been dug closer to the end of the community (near the contact point of the Akotia Stream) but without a fetch pump. This borewell was not being used. Two streams flowed close to the community; both on paths that led to and from the community. These were the Ampomaa and the Akotia streams. Community inhabitants regularly waded through these streams on their way to their farms. At the time of community mapping and sampling, the Akotia stream in MoniaGyaman had been severely disturbed by the movement of an earthmover (for mining purposes) through the river bed. Activities at the Offin River included bathing, swimming and artisanal mining which could lead to potential outbreak of waterborne diseases.

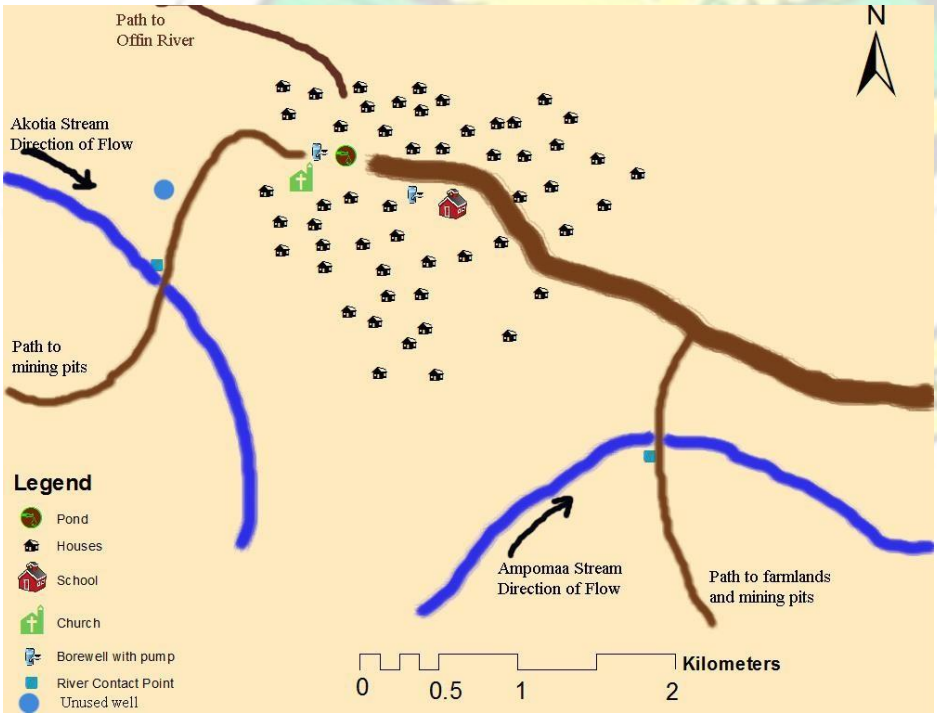


Figure 4.3 Sketch of the Monia-Gyaman Community (Author, 2015).

4.1.1.4 Wromanso

The Wromanso community also has a school and two borewells (Figure 4.4). These two borewells are being used in the community of about 80 houses. This community is the last on the feeder road and therefore mining activity here was very high and destructive. Massive degraded land (from mining activities by Chinese miners) was observed from the community to the Offin River which was about 2 km away from the community. Community inhabitants mostly crossed the Offin River but would hardly swim or bath in it with the fear of falling into pits dug in the river bed during dredge mining activities. A pond, the Bebunu pond which is close to the community, is another major source of water the community depends on apart from the two borewells.

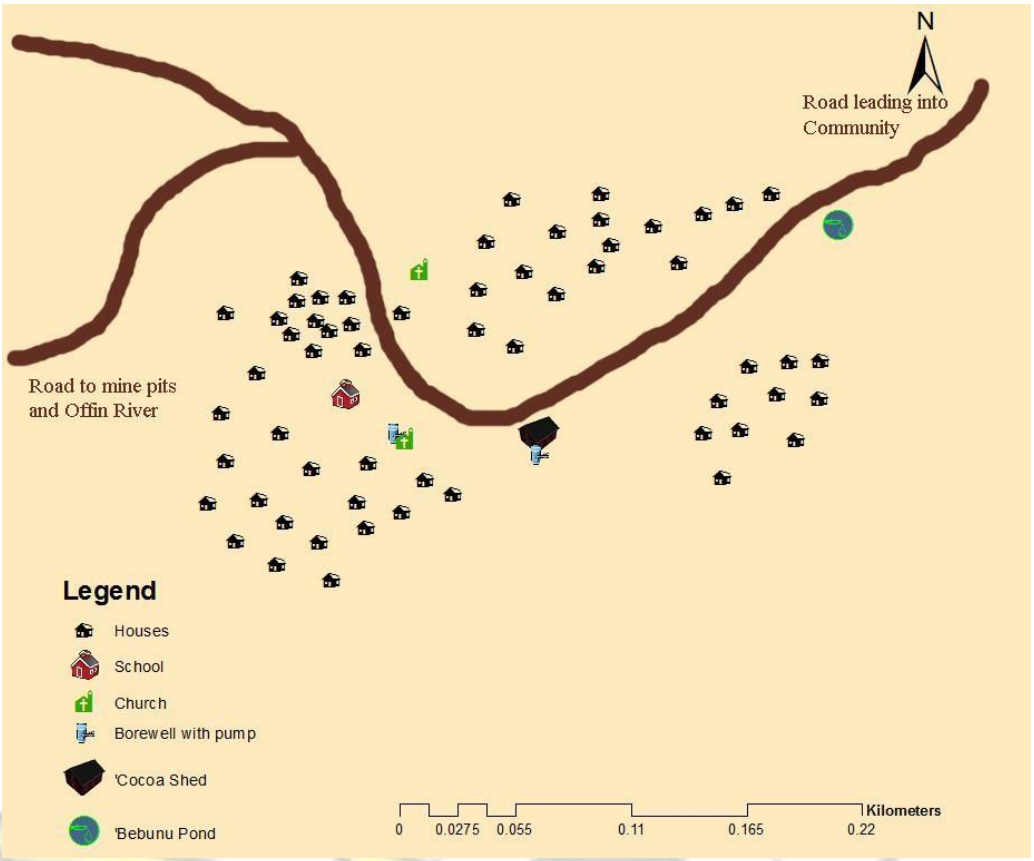


Figure 4.4 Sketch of the Wromanso community (Author, 2015).

4.1.2 Respondents' Characteristics

A total of 224 respondents comprising of 139 (62.05%) females and 85 (37.95%) males were interviewed in the four study communities (Table 4.1). The higher number of females was characteristic for all the four study communities and did not differ significantly among the four communities (p=0.7262). Age distribution (grouped by a 15 year range) did not differ significantly among the communities. Only 6 (2.86%) active BU cases were identified among questionnaire respondents while another 18 (8.04%) had completely treated and healed lesions (Table 4.1). Samples taken from the six suspected cases were confirmed by *IS2404* PCR while healed lesions were identified based on treatment history and the

characteristic scaring of BU lesions. Questionnaire respondents grouped into the "BU case" and "BU non-case" groups were therefore 24 (10.71%) and 200 (89.29%) respectively. No significant associations were observed for age groups and gender in relation to the occurrence of BU infection (Table 4.2). Due to the low numbers of respondents above 75 years (1/224), responses from this age group were excluded from data analysis to satisfy conditions for the use of tests. This was vital to any other analysis as this information suggests age and gender not to be influencing other observed relationships.

4.1.3 Educational Background and Buruli Ulcer knowledge

Approximately a quarter (25.23%) of the respondents had not had any formal education with the other 75% having varying levels of education. They were Middle School Leaving Certificate/Junior Secondary School (7.66%), Primary (38.74%) and Secondary (25.23%). Only seven (3.12%) of the respondents had attained tertiary education. In general, almost 88.9% (199) of the respondents acknowledged BU to be endemic in their community, and 94.62% knew about the disease but only 58 (29%) admitted actually having seen clinical manifestations of the infection. Of this, 16 (27.59%) had seen infections on the arm and 41 (70.69%) on the legs. Educational background did not influence knowledge of BU (Figure 4.5) or the associated symptoms when subjected to a Chi square test ($p=0.6731$). The most common symptom described was the open festering wounds on patients (77.84%). Others also described fevers, headaches and itching (9.28%, 5.67% and 5.15%, respectively) as symptoms of BU infection.

Table 4.1 Characteristics and Buruli ulcer infection status of respondents from the four communities

Variable	Total (%)	Bepotenten	Wromanso	Monia-Gyaman	Sukuumu	p-value
Estimated population	2419	247	312	454	1406	
Questionnaires administered (%)	224 (100)	51 (22.77)	53 (22.66)	58 (25.89)	62 (27.68)	
Age groups (n=223)						**0.726**
0-14	5 (2.24)	2	2	1	0	
15-29	83 (37.22)	20	18	24	21	
30-44	73 (32.74)	14	16	19	24	
45-59	41 (18.39)	8	9	10	14	
60-74	16 (7.17)	5	6	2	3	
75-89	3 (1.35)	1	1	1	0	
90-100	2 (0.90)	1	1	0	0	
Gender (n=223)						**0.326**
Male	85 (37.95)	14 (27.5)	20 (37.74)	25 (43.10)	26 (41.9)	
Female	139 (62.05)	37 (72.5)	33 (62.26)	33 (56.90)	36 (58.1)	
BU infection status (n=224)						
Non case	200 (89.29)	50 (98.00)	50 (94.45)	46 (79.30)	54 (87.10)	
Active cases	6 (2.68)	0 (0.00)	2 (3.77)	0 (0.00)	4 (6.45)	
Treated cases	18 (8.04)	1 (2.00)	1 (1.89)	12 (20.70)	4 (6.45)	

Associations tested by the Chi square contingency test for independence.

Table 4.2 Buruli ulcer infection in various age groups and gender

Variables	BU status		p-value
	Case	Non Case	
Age groups in years (N=223)			**0.153**
1-14	2	3	

15-29	13	70	
30-44	5	68	
45-59	2	39	
60-74	1	15	
75-89	1	2	
90-100	0	2	
Gender (N=224)			**0.831**
Male	13	72	
Female	11	128	

Associations tested by the chi square contingency table test for independence and logistic regression for age groups and gender respectively.

Close to 80% (172) of the respondents knew of the consequences of the infection, most identifying deformities and possible amputation as the outcome of untreated infections. A vast majority (93.69%) believed that the hospital is the best place to seek treatment for BU infections.

The enquiry on perceptions on how the disease is transmitted and which groups are most infected showed a strong perception for activities around water bodies being the main risk factor of infection with all ages and occupational groups being at equal risk. Sixty nine percent (69%) considered activities around water bodies to predispose one to the infection. Thirty one percent (31%) and 11% believe poor hygiene and insect bites, respectively, were the causes of infection. About 6% however attributed the infection to curses and spiritual attacks. Only three of the BU case respondents, however, associated their infections to a water body contact activity.

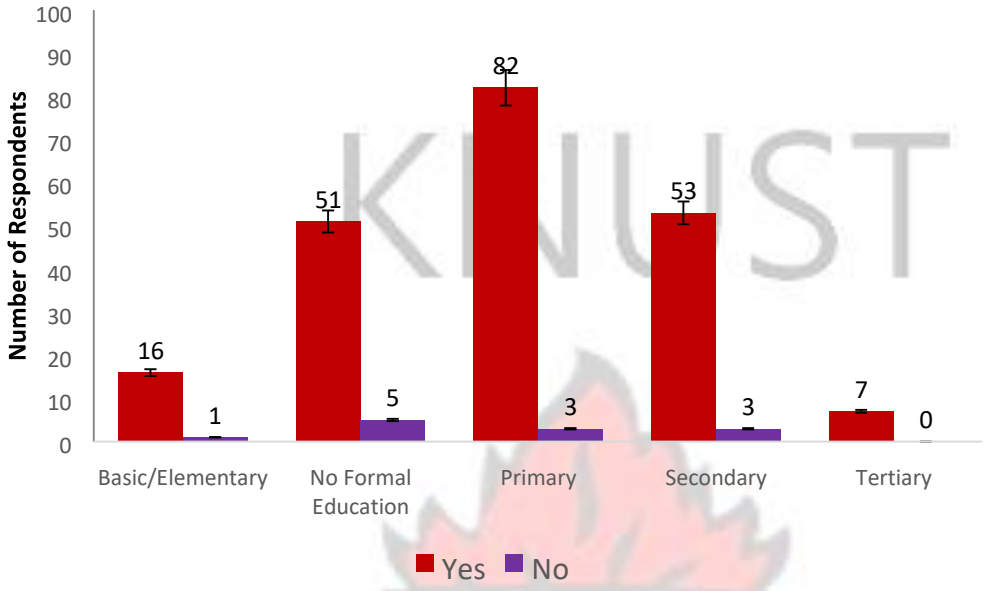


Figure 4.5 Buruli ulcer disease knowledge and educational level attained (p=0.6731).

4.1.4 Water Use, Livelihood Strategies and Buruli Ulcer Disease

Covered borewells fitted with fetching pumps were found in all four communities and were the most used source of water (89.40%). Even though responses to the continual functioning of all borewells indicated that all borewells had always function and had hardly broken down or been unable to reach the water table, 112 (50%) of respondents also used other sources of water mainly for bathing and swimming purposes (Table 4.3). Only 13 (5.8%) of the respondents do not use the borewells in the communities for any purpose and solely depend on surface water bodies. Although no relationship was observed for general use of surface water and disease infection, swimming and bathing in surface waters was associated with a significantly greater risk of BU infection with an odds ratio of 3.28 (95% CI=1.03-10.47, p=0.045). Wading through temporary ponds formed on

paths to farms and other areas of the community, usually in rainy seasons, did not significantly increase the risk of BU infection (OR=1.39, 95% CI=0.56–3.42, p=0.47).

The main livelihood strategy of the study communities was agriculture (96%) with majority engaged in mix cropping of cocoa, corn and cassava. A small number of inhabitants however engaged in animal husbandry and some other form of commercial activity. None of these livelihood strategies was however associated with an increased risk of acquiring BU infection (Table 4.3). Only seven (2.54%) respondents engaged in hunting game animals as an economic activity. Nevertheless, about a half of respondents still trapped various game animals in their farms usually for domestic consumption (47.27%). The crop type as well as the type of field cultivated also did not significantly increase the risk of BU infection (Table 4.3). The odds of acquiring BU was however higher in respondents cultivating tomatoes (OR=3.28, 95% CI=0.88-12.21) although not significant (p=0.08). In fact, nineteen of the twenty four BU infected respondents are engaged in tomato cultivation.

4.1.5 Community's Surface Water Contact Points

Six surface water bodies and ten associated water contact points were identified to be patronized for various purposes by people in the study communities (Table 4.4).

Table 4.3 Surface water usage, main livelihood strategies, environmental contact and Buruli ulcer disease burden in study participants

	Frequency (%)
Main water source usage (N = 224)	

Borewells	194 (89.40%)				
Surface water sources (ponds, streams, rivers)	112 (50.00%)				
	BU case	**BU non case**	**Odds ratio**	**95% CI**	**p-value**
A. Use of surface water sources	N = 14	N = 98			
Laundry/Dishwashing	10 (71.40)	32 (32.65)	1.072	0.386 - 2.975	0.95
Drinking/Cooking	10 (71.40)	49 (50.00)	1.194	0.409 - 3.486	0.76
Bathing/Swimming	14 (100.00)	86 (87.75)	3.284	1.025 - 10.471	0.04**
B. Livelihood Strategies	N = 24	N = 200			
Agriculture	22 (91.67)	193 (96.50)	0.439	0.085 - 2.275	0.33
Animal Husbandry	0 (0.00)	6 (3.00)	0.000	0.000 - 0.000	0.97
Commerce	6 (25.00)	34 (17.00)	1.505	0.552 - 4.107	0.43
C. Main Cultivated Crops	N = 22	N = 193			
Cocoa	21 (95.45)	162 (83.94)	1.56	0.433 - 5.685	0.49
Plantain	11 (50.00)	113 (58.55)	0.732	0.305 - 1.758	0.49
Cassava	14 (63.64)	129 (66.84)	0.788	0.319 - 1.948	0.61
Yams	13 (59.09)	84 (43.52)	1.299	0.518 - 3.256	0.58
Tomatoes	19 (86.36)	117 (60.62)	3.279	0.881 - 12.210	0.08
Corn	20 (90.91)	157 (81.35)	0.577	0.131 - 2.534	0.47
D. Fields Types for Crop Cultivation	N = 22	N = 193			
Dry fields	21 (95.45)	163 (84.45)	1.511	0.419 - 5.451	0.53
Irrigated fields	11 (50.00)	96 (49.74)	0.750	0.313 - 1.797	0.52
Inundated fields	1 (4.54)	25 (12.95)	0.287	0.036 - 2.303	0.24

No association was observed for surface water use and BU infection (p=0.39). All associations explored using multivariate logistic regression. ****** denotes significance

The Offin River was common to all study communities and is usually bathed in, swam or crossed in canoes to farms and to other communities (Figure 3.1). Sixty percent and 62% of respondents from Monia-Gyaman and Wromanso respectively admitted daily contact with the Offin River. Other surface water bodies

encountered were the Oda River in Bepotenten and the Akotia Stream, in Sukuumu and Monia-Gyaman. Laundry, dishwashing, drinking and cooking were the main purposes for which community inhabitants use these water bodies.

Swimming and bathing happened only in the large rives (Offin and Oda).

Table 4.4 Surface water used by study communities and approximate distance from communities

Community	Waterbody	Type	Approx. Distance from community (meters)	Usage		
				Laundry/ Dishwashing	Drinking/ Cooking	Bathing/ Swimming
Bepotenten	Oda	River	100	✓	✓	✓
Sukuumu	Offin	River	800	✓	✓	✓
	Akotia	Stream	1,500	✓	✓	**X**
	Twingum 1	Stream	50	✓	✓	**X**
	Twingum 2	Stream	150	✓	✓	**X**
Monia Gyaman	Akotia	Stream	400	✓	✓	**X**
	Ampomaa	Stream	700	✓	**X**	**X**
	Offin	River	1,100	✓	✓	✓
Wromanso	Offin	River	2000	✓	✓	✓
	Bebunu	Pond	20	**X**	✓	**X**

✓ Indicates positive response, **X** indicates negative response. Ten water bodies were used by the four study communities.

4.1.6 Animal Contact and Disease

Although very few respondents were involved in animal husbandry or hunting as a source of livelihood, nearly 70% still had contact with animals through household rearing and 45% sometimes hunted for game on their way to and from their farms or on their farms. Animals reared in households included chicken, goats, dogs and cats. Figure 4.6 shows the percentage respondents in the BU case and BU

non-case groups rearing animals in their household. No associations were observed for the types of animals in the household and the occurrence of the infection when the association was tested by the Chi square test. A significant association was, however, observed with respondents involved in hunting (both for economic or domestic purposes) and the occurrence of the infection (Figure 4.7). Although 40% of respondents in the BU case group hunt for grasscutters (*Thryonomys* spp) compared to less than 5% in the BU non-case group, logistic regression analysis showed hunting of grasscutter to rather be associated with a lesser risk of BU infection although not significantly (OR=0.38, 95% CI=0.09–1.58, p=0.18). An association was also observed for the hunting of squirrels (Family Sciuridae), 33.3% and 13.5% in the BU case and non-case group respectively. The hunting of squirrels was significantly associated with an increased odds of BU infection (OR=5.85, 95% CI=1.53–22.40, p=0.01) albeit a low number of respondents being engaged in this activity (15%). Bush rats were the most caught as game with the others being duikers, bush chicken and pangolins.

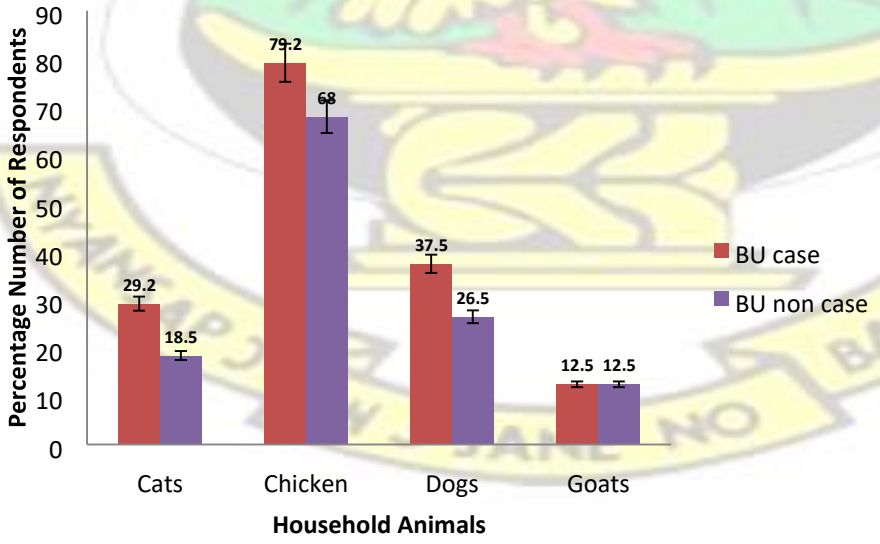


Figure 4.6: Proportion of community inhabitants with or without BU rearing animals in households (p=0.885)

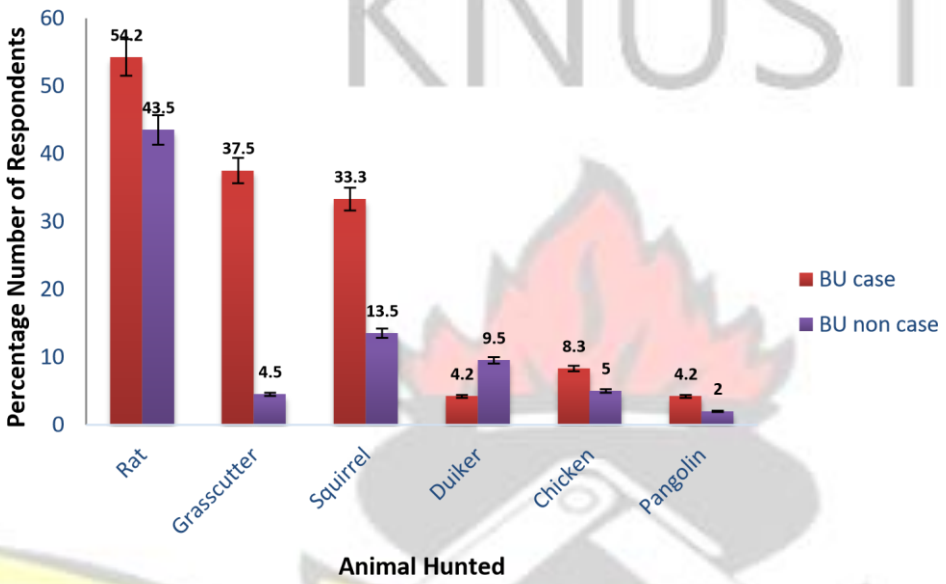


Figure 4.7 Proportion of community inhabitants with or without BU engaged in hunting (p=0.0042).

4.2 Non-tuberculous Mycobacteria Distribution in Study Communities

4.2.1 Human Samples

Fifteen (15) clinical samples were obtained from suspected BU lesions and nodules after community awareness and active case search were conducted and consent of participants obtained. This was done together with health personnel from the district health directorate. All suspected cases were referred to the Amansie Central Health Directorate for treatment soon after confirmation by PCR as required. Two swab samples and 13 fine needle aspirates were taken for

confirmation and molecular characterization. Two suspected BU samples each were collected from Bepotenten and Wromanso, seven samples from Sukuumu and four samples from Monia-Gyaman. Of these, 14/15 were confirmed to be BU disease by *IS2404* PCR (Table 4.5). An FNA sample taken from a patient at Bepotenten tested negative by PCR.

Using four VNTR targets: MIRU1, Locus 6, ST1 and Locus 19 (in that order), clinical samples were effectively characterized into four types based on the copy numbers of each VNTR target (Table 4.5). These are: '**W**' for profile 1 1 2 1, '**X**' for profile 1 1 2 2, '**Y**' for profile 1 2 2 1 and '**Z**' for profile 1 2 2 2. The single positive sample obtained from Bepotenten was of the 'Y' type. The 'Y' type was also the most common and was detected to be causing BU disease in all four communities. Sukuumu and Monia-Gyaman presented the most diverse *M. ulcerans* types. Types 'X', 'Y' and 'Z' were responsible for BU infection in Sukuumu and Types 'W', 'X' and 'Y' were detected in Monia-Gyaman BU patient samples (Table 4.5). Two patient samples from Sukuumu could not be completely assigned a type as the locus 19 target failed to amplify.

4.2.2 Environmental Samples

One hundred and forty (140) environmental samples were obtained and analyzed, 14 from each water body (3 filters, 5 biofilms, 3 detritus and 3 soil samples). The 16S rRNA positivity in environmental samples was 27.1% (38/140) of which 25/38 (65%) were positive for *IS2404*. The VNTR analysis was therefore conducted on 18% (25/140) of all environmental samples collected. As expected, VNTR profiles detected in environmental samples were more diverse compared to human BU samples. Other VNTR profiles detected in environmental sample such

as profiles 1 1 2 2, 1 2 1 2 and 3 1 1 2 in Twingun 2 were not assigned types as they were not detected in human disease samples (Table 4.6).

Three assigned types ('W', 'X' and 'Y') were detected in the water bodies sampled in Sukuumu. Type 'Y' was the most common *M. ulcerans* type and was detected in one or more water bodies in all four communities. A VNTR profile type similar to that designated as 'B' (profile: 3 1 1 2) by Williamson and colleagues in 2008 was detected in the Twingum 2 pond in Sukuumu. Samples obtained from the Offin River presented the lowest success of VNTR target amplifications, being partially amplified at Sukuumu and not at Monia-Gyaman and Wromanso.

Table 4.5 Clinical and molecular confirmation of patient samples and VNTR-Type characterization of *M. ulcerans* positive samples (FNA-Fine needle aspirate)

Community	**Diagnosis**					**NTM/MU Characterization**				
					16S	**VNTR Profile**				**TYPE**
	Patient ID	**Clinical**	**Type of Sample**	**Molecular confirmation (*IS2404*)**		**MIRU1**	**L6**	**ST1**	**L19**	
Bepotenten	B1	Nodule	FNA	Positive	Positive	1	2	2	1	**Y**
	B2	Nodule	FNA	Negative	Negative	-	-	-	-	
Sukuumu	S1	Nodule	FNA	Positive	Positive	1	1	2	2	**X**
	S2	Nodule	FNA	Positive	Positive	1	2	2	1	**Y**
	S3	Nodule	FNA	Positive	Positive	1	1	2	-	**NA**
	S4	Nodule	FNA	Positive	Positive	1	2	2	2	**Z**
	S5	Nodule	FNA	Positive	Positive	1	1	2	2	**X**
	S6	Nodule	FNA	Positive	Positive	1	1	2	-	**NA**
	S7	Nodule	FNA	Positive	Positive	1	2	2	2	**Z**
Monia Gyaman	M1	Lesion	Swab	Positive	Positive	1	1	2	2	**X**
	M2	Lesion	Swab	Positive	Positive	1	1	2	1	**W**
	M3	Nodule	FNA	Positive	Positive	1	2	2	1	**Y**
	M4	Nodule	FNA	Positive	Positive	1	1	2	2	**X**
Wromanso	W1	Nodule	FNA	Positive	Positive	1	2	2	2	**Z**
	W2	Nodule	FNA	Positive	Positive	1	2	2	1	**Y**

NA: VNTR profile was not be assigned as Locus 19 failed to amplify.

Table 4.6 DNA profiling of environmental samples

Community	Waterbody	Sample Positivity (%)		NTM/MU Characterization				TYPE
		16S	*IS2404*	VNTR Profiles				
				MIRU1	L6	ST1	L19	
Bepotenten	Oda	4/14 (28.5)	4/4 (100)	1	2	2	1	**Y**
				1	2	2	2	**Z**
				-	2	3	-	**NT**
Sukuumu	Offin	8/14 (57)	5/8 (62.5)	1	2	2	-	**-**
				1	2	7	-	**NT**
	Twingun 1	3/14 (21.5)	1/3 (33.3)	1	-	1	-	**NT**
	Twingun 2	5/14 (35.7)	4/5 (80)	1	1	2	2	**X**
				1	1	1	2	**UNTM**
				1	2	2	1	**Y**
				1	2	1	2	**UNTM**
				3	1	1	2	**UNTM** or **B***
	Akotia	7/14 (50)	2/7 (28.6)	1	1	2	1	**W**
				-	1	4	1	**NT**
Monia Gyaman	Offin	0/14 (0)	0/0 (0)	-	-	-	-	**-**
	Akotia	5/14 (35.7)	4/5 (80)	1	2	2	1	**Y**
				1	2	X	X	**NT**
	Ampomaa	3/14 (21.5)	2/3 (66.7)	1	2	7	X	**NT**
				1	2	2	1	**Y**
Wromanso	Offin	2/14 (14.3)	2/2 (100)	-	-	-	-	**-**

Bebunu	1/14 (7.2)	1/1 (100)	1	2	2	1	**Y**
			1	2	2	2	**Z**

ND denotes type not determined due to incomplete profile amplifications or no amplification. NT denotes Not Typed, UNTM denotes Unidentified NTM, B* denotes type detected by Williamson *et al.* (2008).

4.2.3 Matching Environmental and Human Variable Number Tandem Repeat profiles

The NTM-VNTR profiles detected in environmental samples were of a higher diversity compared to that from human clinical samples, with other profiles that were not assigned types being detected in environmental samples. Results, however, focused on *M. ulcerans* profiles that had been detected as causing human BU infections in the study communities. Figure 4.8 shows the resolution and comparison of human disease and environmental profiles on a gel image. Three profiles, 'X', 'Y', 'Z', were detected in patient samples collected from Sukuumu. Sukuumu showed a close to perfect match of human clinical types and types in the environment with only type W being detected in the environment but not in human disease. A comparable situation was observed for Wromanso where only types 'X' and 'Y' were detected in both human and environmental samples. Type 'Y' was detected in clinical samples from all study communities as well as all environmental samples collected from all the communities (Table 4.7).

Matching clinical types to environmental types for each of the four communities, there seem to be localized infection from community associated water bodies. In Bepotenten, Sukuumu and Wromanso, all types detected in clinical samples matched environmental samples collected from community associated water bodies. This was however not the case for Monia-Gyaman where types 'X' and 'Y' were detected in patient samples but only 'Y' type was detected in associated water bodies (Ampomaa and Akotia streams). In all, 80% of profiles detected in BU disease were similar to profiles detected in environmental samples.

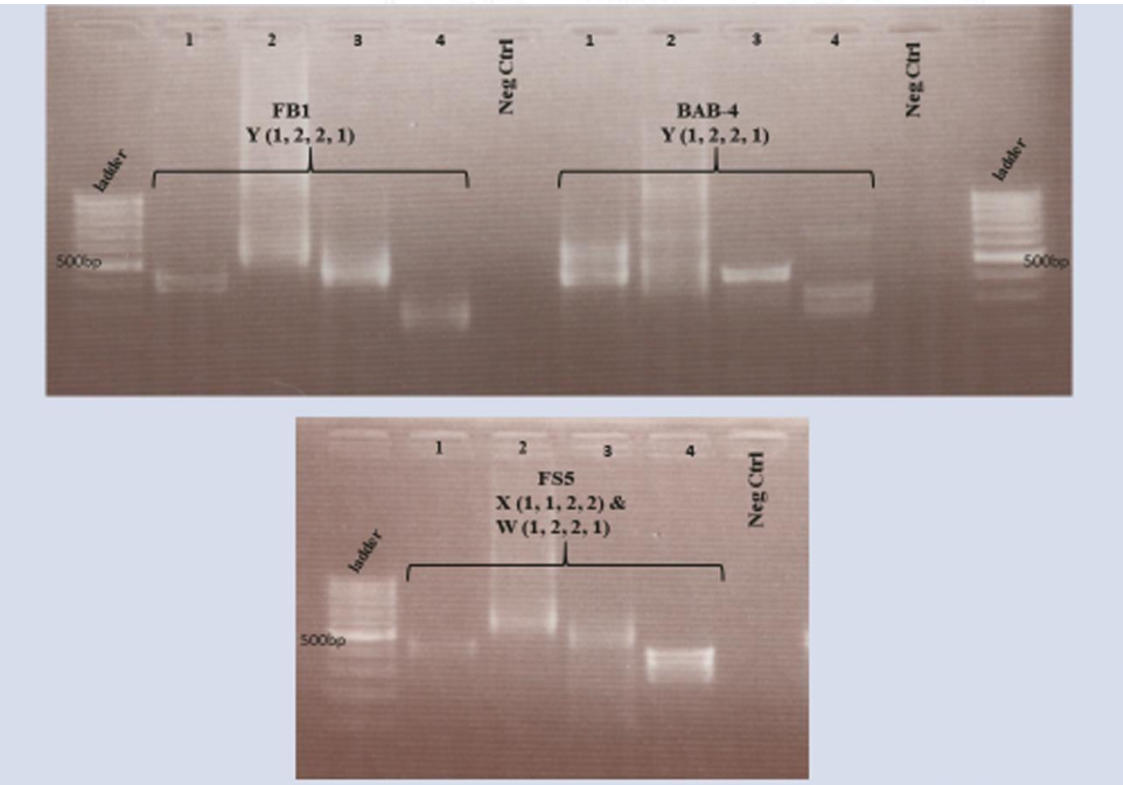


Figure 4.8 Gel picture showing VNTR profiles of environmental and human *M. ulcerans* types.

FB1 is a human sample from Bepotenten. BAB-4 is a biofilm sample from Oda River. FS5 is a human sample from Sukuumu.

Table 4.7 *M. ulcerans* VNTR types in human BU cases and environmental samples.

Community	MU VNTR profile Type	Human Disease	Environmental Samples
Bepotenten			
	W		
	X		
	Y	✓	✓

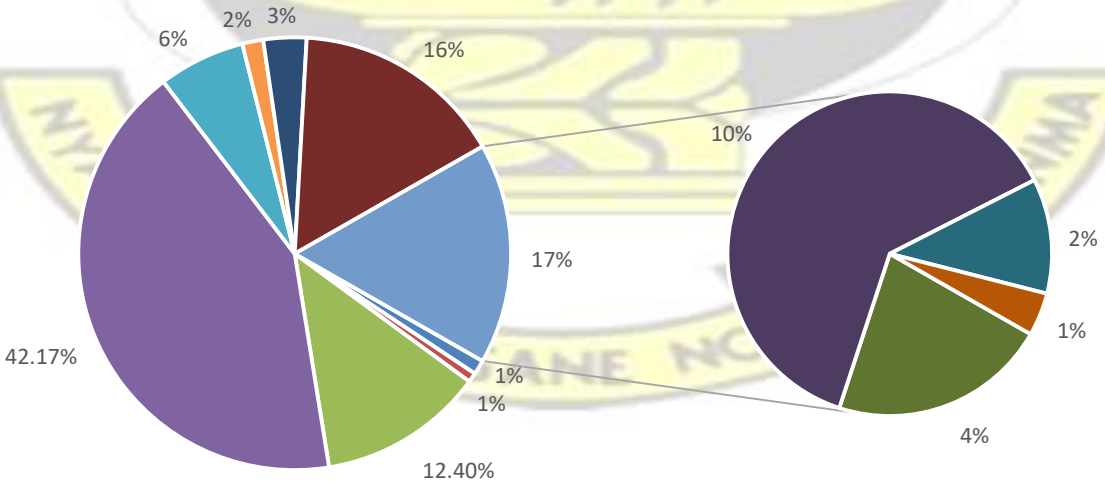
	Z		✓
Sukuumu			
	W		✓
	X	✓	✓
	Y	✓	✓
	Z	✓	
	B		✓
Monia Gyaman			
	W		
	X	✓	
	Y	✓	✓
	Z		
Wromanso			
	W		
	X		
	Y	✓	✓
	Z	✓	✓

4.3 Distribution of Belostomatidae and Naucoridae

A total number of 2,113 members of the Order Hemiptera belonging to eleven identified families were collected from 89 water bodies of different types in Ghana (82) and Cote d'Ivoire (7). Thirty three (33) other Hemiptera for which Family taxon could not be assigned due to the unavailability of keys were also collected (unassigned). Almost 11% (232/2113) of all hemipterans collected were from the seven water bodies in Cote d'Ivoire. The complete list of all Belostomatidae and Naucoridae collected and identified as well as other Hemiptera collected is tabulated in Appendices 6, 7 and 8.

The Belostomatidae were the most collected Hemiptera with 891 individuals forming 42.17% of all Hemiptera collected (Figure 4.9). Members of the Family Pleidae, Notonectidae and Naucoridae were also collected in high numbers forming 15.85% (335), 10.31% (218) and 12.40% (165), respectively. Together, the Naucoridae and the Belostomatidae formed over 54% of all Hemiptera collected (1153/2113). Seven percent and 16% of Belostomatidae and Naucoridae were respectively collected from Cote d'voire. Other Hemiptera collected were distributed in the Families Hydrometridae, Nepidae, Gerridae, Ranatridae, Veliidae, Corixidae and Macroveliidae.

Further identification was done only for the Belostomatidae and the Naucoridae. A total of 446 individuals (125 Naucoridae and 321 Naucoridae) of the two families that were further needed to be classified to the genus and species level which were mainly from water bodies associated with BU endemic communities.



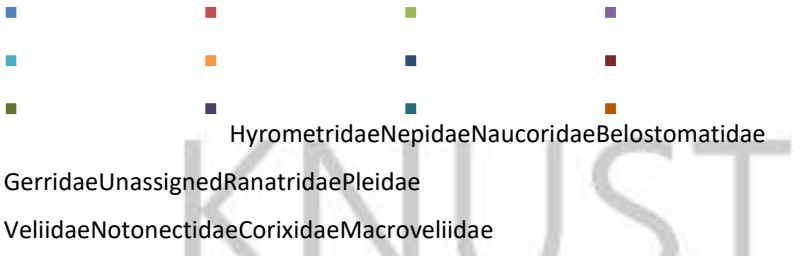


Figure 4.9 Distribution of families of collected Hemiptera.

4.3.1 Family Belostomatidae

Three hundred and twenty-one (321) individual members of the family Belostomatidae were classified to the Genera level based on available keys. This represented a random selection of about 36% of all Belostomatidae collected. Insects that lost body appendages were also excluded from identification. Further classification to species level was hampered by the lack of descriptive keys for collected individuals especially juvenile forms of members of this family. All Belostomatidae were classified into four Genera: *Abedus, Diplonychus, Lethocerus* and *Benacus*. Table 4.8 shows the abundance of the four genera in two water body flow types, lotic (flowing: rivers, streams, and springs) and lentic (stagnant: ponds and lakes).

Abedus sp was the most abundant and most geographically widely distributed of the Belostomatidae genera collected being identified in all but six lotic and five lentic water bodies respectively (Appendix 6). Seventy nine and 73 individuals of the *Abedus* genera representing 47.3% of all Belostomatidae were collected from lentic and lotic water bodies respectively. Only four individuals of the genera *Benacus* were identified. Three of these were collected from a lotic pond in Atwima Mponuah

District of the Ashanti Region of Ghana and one from the Akotia stream in Monia Gyaman in the Amansie Central District also in the Ashanti Region of Ghana.

Numbers of the Belostomatidae collected from lotic and lentic water sources were similar and there seemed to be no preference of the Belostomatidae for any of the two water flow types. Similarly, genera preference in terms of abundance and diversity followed a similar trend. A test of independence of genera to type of water flow revealed a non-significant relationship ($p=0.2068$). Thus, the distribution of Belostomatidae was not dependent on type of flow (Table 4.8).

4.3.2 Family Naucoridae

Generally, lower numbers of members of the family Naucoridae were collected and the availability of keys enabled individuals to be identified to the species level. Eight species of this family, belonging to five genera were identified in 125 individuals collected from 24 water bodies (15 lotic and 9 lentic). The distribution of the different species of the Naucoridae in lotic and lentic water bodies is shown in Table 4.8.

Almost 90% (112/125) of all Naucoridae identified were collected from lotic water sources indicative of a preference for flowing water bodies. Test for dependence of species of the Naucoridae on water flow showed a very strong relationship ($p<0.05$). The most abundant species identified was *Ctenipocoris africanus* which was described by Leach in 1815. Seventy-four (59.2%) individuals of this species were collected from nine of the 15 lotic water bodies and two of the nine lentic water bodies. Other species identified included *Aneorocoris insolitus* (Montandon, 1897) and *Naucoris obscuratus*

(Montandon, 1913) which formed 22.4% and 8% of the identified collections respectively. Single individuals of two species, *Laccocoris spurcus congoensis* (Poisson, 1949) and *Neomacrocoris vuga* (Sites, 2015) were collected from two different water bodies. *Laccocoris spurcus congoensis* from Bodada in the Jasikan District of Ghana and *Neomacrocoris vuga* from Adaklu Amedzivie in the Ho district of Ghana.

Table 4.8 Individual insects of the families Belostomatidae and Naucoridae identified in lotic and lentic water bodies

Family		Type of flow		
	Species	**Lotic**	**Lentic**	**Total**
Belostomatidae (N = 321)				
	Abedus sp	73	79	152
	Diplonychus sp	57	58	115
	Lethocerus sp	27	23	50
	Benacus sp	4	0	4
TOTAL		**161**	**160**	**321**
Naucoridae (N = 125)				
	Ctenipocoris africanus (Leach, 1815)	71	3	74
	Aneorocoris insolitus (Montandon, 1897)	27	1	28
	Laccocoris limicola (Stal, 1855)	2	1	3
	Laccocoris spurcus congoensis (Poisson, 1949)	1	0	1
	Neomacrocoris bondelaufa (Sites, 2015)	0	2	2
	Neomacrocoris vuga (Sites, 2015)	0	1	1
	Naucoris obscuratus (Montandon, 1913)	6	4	10
	Neomacrocoris usambaricus (Montandon, 1913)	5	1	6
TOTAL		**112**	**13**	**125**

Distribution of Belostomatidae was not dependent on type of water flow (p=0.2068) Distribution of Naucoridae showed association with type of flow (p<0.05) N = Total number of insects identified in each group.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

A major challenge to developing effective prevention and control strategies for many diseases is identifying the relevant transmission routes to humans (Loh *et al.*, 2015). This challenge is particularly high for non-tuberculous mycobacteria (NTM) or environmental mycobacteria known to cause infections in humans and animals. The transmission of pathogenic NTM is, however, most likely due to the common habitats shared by humans and animals.

M. ulcerans transmissions from environmental sources to humans has been hypothesized to be through a number of routes. Direct inoculation, invertebrate vector involvement, amoebal involvement and aerosolized transmission are few hypothesized and tested routes of transmission (Marsollier *et al.*, 2003; Benbow *et al.*, 2008; Williamson *et al.*, 2008; Merritt *et al.*, 2010; Lavender *et al.*, 2011; Williamson, Phillips, *et al.*, 2014; Zogo *et al.*, 2015). Most ecological studies of the pathogen and probable transmission pathways were designed to collect and process large sample numbers over wide areas. This design may have not yielded the desired results for an infection that is mostly focal in nature even within known endemic districts (Johnson

et al., 2005). Williamson *et al.* (2008), in the largest ecological study of BU transmission, described *M. ulcerans* to be more widely distributed compared to the associated human infection suggesting socioeconomic activities of communities to be playing important roles in infection.

This study was designed based on a One Health concept which strives to develop methods to assess human and animal health and their respective social and environmental determinants offering a more holistic attempt at deciphering transmission of an environmental mycobacterium to humans (Lam *et al.*, 2015). The selection of four study communities, mapping of these communities, assessment of social and economic activities that are related to surface water contact, comparison of *M. ulcerans* strains from systematically determined risk environments and human BU infections using highly sensitive molecular tools such as VNTR suggested the most probable transmission routes in these BU endemic communities.

Insects involvement in the transmission of mycobacterial infections is rare but detection of genetic material and the isolation of a pure culture of *M. ulcerans* from aquatic invertebrate sources however made important the possibility of invertebrates playing roles in the transmission of BU (Portaels *et al.*, 1999; Benbow *et al.*, 2008; Portaels *et al.*, 2008; Williamson *et al.*, 2008; Doannio *et al.*, 2011). Even though members of the hemipteran Families Belostomatidae and Naucoridae collected from BU endemic water bodies have tested positive for *M. ulcerans*, there remains a lack of information on the types of these two families in endemic communities. This study therefore generated a diversity catalogue of these two main aquatic insect groups collected from 87 water bodies (lentic and lotic) in Ghana and

Cote d'Ivoire using available taxonomic keys.

5.1.1 Buruli Ulcer Risk Behaviors

The presence of *M. ulcerans* in environmental sources especially aquatic environments has been demonstrated in endemic areas in West Africa (Portaels *et al.*, 1999; Portaels *et al.*, 2008; Williamson *et al.*, 2008). Aquatic area selection for BU transmission studies was based on information from community informants and not by any systematic mechanisms (Benbow *et al.*, 2008; Williamson *et al.*, 2008). The success of using a questionnaire survey is evidenced in the successful identification and selection of water bodies and all high rate community water contact points for subsequent systematic sampling for *M. ulcerans* and other NTMs. With the use of this method, six water bodies with 10 water contact points were identified rather than one water contact point per community. Positivity rates of *M. ulcerans* and other NTM in environmental samples were higher (17.9%) as compared to other studies that sampled and tested similar sample types in endemic communities; 9% in a Ghana study (Williamson *et al.*, 2008) and 4% in a recent study in Cote d'Ivoire (Zogo *et al.*, 2015)

The prevalence rate of Buruli ulcer in endemic communities is usually known to be low with a typical prevalence of 20.7 per 100,000 inhabitants (WHO, 2014a). The overall estimated prevalence for our study communities was 57.9 per 100,000 inhabitants, higher than what was reported in earlier national prevalence studies in Ghana in 2002. This high prevalence of BU reported in this study could be as a result of the intense and focused active case search in smaller communities. Underreporting of BU is acknowledged as a problem in the global management of this neglected tropical disease as countrywide and global burden are still not known (WHO, 2014b).

Although active case search for early detection, as was undertaken in this study is recommended by this study at the entire endemic district level, the unavailability of funds poses a great challenged this approach.

Knowledge of BU in the study communities was generally high (90%). This is attributable to educational efforts of Non-Governmental Organizations (NGOs) about a year before the commencement of this study in the district (Personal communication with District Director of Health and Community Based Disease Control Officers). Educational background and age did not influence BU awareness as educational programmes in these communities were held in the open, community wide and in the local dialects of the communities. Educational programmes coupled with active cases detection, organized periodically, in endemic communities was shown to be most effective mechanism for the detection of early cases for treatment as evidenced in this study. This should, however, be complemented by the training and resourcing of community based volunteers for routine early case identification (WHO, 2014a).

The emergence of BU has been linked to extensive human disturbances of the environment through activities that usually creates ponds and new water ways such as mining and sand winning (Johnson *et al.*, 1999; Merritt *et al.*, 2005). The study communities lie within the small to medium scale mining zones of the Ashanti Region of Ghana. Although massive surface mining was observed within a few meters of all four communities, artisanal mining was not a livelihood strategy of community inhabitants. These mining activities were mostly being undertaken in the

Offin river bed exposing all communities to its associated pollution and risks as the river flows along (Figure 3.1). Several communities along this river have been reported to be endemic to BU but no study has specifically linked the river to BU infection (Gyasi *et al.*, 2011).

Bathing and swimming in surface water bodies were associated with a greater risk of BU infection in the study respondents ($p<0.05$). Swimming in rivers have been reported to be a risk factor in earlier studies in Ghana but bathing, cooking and drinking river water were not (Aiga *et al.*, 2004). Prolonged and "abrassive" contact with the aquatic environment, such as swimming and wading, thus seem to be associated with BU infection rather than in activities where water is only fetched and thereby contact is brief. Other studies have also reported wading in water to be associated with an increased risk of infection (Raghunathan *et al.*, 2005; Pouillot *et al.*, 2007). These studies and the findings from this study are suggestive of a traumadirect environmental contact activity to be driving BU infection in humans. *M. ulcerans* infection from environmental aquatic sources may infect humans through the skin via trauma usually caused by a rigorous activity such as wading and swimming in infected aquatic environments as hypothesized and tested in some studies (Merritt *et al.*, 2010; Williamson, Mosi, *et al.*, 2014).

Farming was the main livelihood strategy of the study communities but this was not associated with an increased risk of BU infection ($p>0.05$). Similarly, crop type and field type cultivated did not influence acquisition of the infection. Crops cultivated in marshy and waterlogged fields such as rice have been shown to be associated with an increased risk of BU infection (James *et al.*, 2003; Brou *et al.*,

2008). The cultivation of tomatoes, usually a crop that needs regular and high amounts of irrigation and therefore a higher water contact frequency, was however associated with a higher risk of BU infection, although not statistically significant ($p>0.05$). This corroborates the assertion that activities that may cause abrasions on the skin (trauma) and the duration of contact with an infected water source is an important risk factor for infection.

M. ulcerans has been reported to cause BU-like lesions in possums in Australia raising questions of a possible zoonotic association with human infections (Fyfe *et al.*, 2010). Laboratory animal models for BU have also been developed using grasscutter as initial model (Addo *et al.*, 2005). *M. ulcerans* infections have, however, only recently been observed and confirmed in small mammals such as *Mastomys* sp in Cote d'Ivoire and Ghana (Dassi *et al.*, 2015; Narh *et al.*, 2015). Most households (70%) in the study communities reared chicken (69.1%), dogs (27.7%) and goats (12.5%), however, similar to earlier studies in Ghana, livestock rearing in households was not found to be associated with an increase in household human BU infections (Raghunathan *et al.*, 2005). The hunting of game animals which was not a main livelihood of the study communities was however associated with an increased risk of BU infection ($p<005$). Interestingly, rat hunting which was common practice did not increase the risk of infection. The trapping and capture of squirrels, admitted by only 35 (16%) respondents, was associated with a higher risk of BU disease in humans but caution was exercised for this observation as the number of positive responses to squirrel capture was extremely low and therefore needed further exploration. Grasscutter capture appeared to rather protect against BU infection. The probable

significance of small mammals in the transmission, dispersal or survival of mycobacterial pathogens has also been demonstrated in earlier studies (Durnez *et al.*, 2008; Durnez *et al.*, 2010; Fyfe *et al.*, 2010; Dassi *et al.*, 2015; Narh *et al.*, 2015).

5.1.2 Community Maps and Buruli Ulcer Risk Identification

Community mapping of probable areas of any known infectious disease can be effectively used for descriptive purposes and may identify patterns of variations and also propose or predict possible causes of the infection in humans (Joseph *et al.*, 2015). Such maps have also been used to map wildlife epidemics of chronic wasting disease and Bayesian geostatistical prediction of schistosomiasis leading to an enhancement in the implementation of control programmes (Clements *et al.*, 2006; Clements *et al.*, 2008). Risk area mapping therefore helps determine, isolate and display the role of a location as a risk factor and is therefore invaluable in transmission studies of environmental mycobacterial infections.

In this study, basic community mapping was performed for the four BU endemic communities and maps generated to show specific areas of relative risk. In earlier environmental studies, the selection of water contact areas to be sampled as risk areas for *M. ulcerans* and other NTM infections were based on basic information from community members. This was not comprehensive enough and fairly uncommon or unused water bodies were missed (Benbow *et al.*, 2008; Williamson *et al.*, 2008).

In Monia-Gyaman community, information from questionnaire and

community discussions indicated the use of the two borewells in the community and scarcely the Ampomaa stream for all purposes. The community map revealed further water contact points on another stream, the Akotia Stream, which was not mentioned by questionnaire respondents. This was found to be due to the destruction of the stream at the fetching point by an earth moving machine about two months before community entry and questionnaire administration. Community inhabitants, however, wade through this stream daily on their way to farms, to mining pits and other nearby communities. Environmental samples collected from this stream were positive for VNTR type designated as the 'Y' type of *M. ulcerans.* The 'Y' type was the most common VNTR type detected in human disease samples in all four study communities. In Monia-Gyaman, the detection of the ;Y' type in the environment would have been missed for this community if mapping was not performed as it was detected in only the Akotia stream and not the other two water contact sites (Offin River and the Ampomaa Stream).

A similar observation was made for Sukuumu community where only the pond Twingum 2 was identified as the source of surface water to the study community. Mapping of the community exposed a linked small stream that flowed from across the main road and through a cocoa farm to this pond. A water contact point identified on this stream sampled. Only one of the 14 samples taken tested positive for a mycolactone producing mycobacteria (*IS2404* positive). This MPM was not the type found to be associated with BU disease in the community. The associated pond, Twingum 2, showed the highest diversity in VNTR types of *M. ulcerans* infections in the

community and other MPMs (Table 4.6). The difference in ecology of the two aquatic areas may be responsible for the observed differences in MPMs associated with these two linked environments. Although MPMs and other NTM have been isolated from natural waters, their distribution and abundance has differed based on type of water body (flow) as well as the biochemical composition of the water (Falkinham, 2002).

Higher abundance and diversity of NTM had been reported in acid-rich brown swamps and water draining from forest soil compared to lower numbers and diversity in fast flowing and cleaner drinking water sources (Kirschner *et al.*, 1992; Livanainen *et al.*, 1999). Several studies in tropical water bodies have also associated the presence of *M. ulcerans* and its associated disease with slow flowing or stagnant and disturbed water bodies (Johnson *et al.*, 2005; Merritt *et al.*, 2005; Wagner, Benbow, Burns, *et al.*, 2008; Merritt *et al.*, 2010; Williamson *et al.*, 2012). The observed disparity of NTM detected in the Twingum 1 stream and the Twingum 2 pond was therefore expected (Table 4.6). The pond dug up by the community was observed to be biologically richer with invertebrates and vegetation than the associated stream. The pond is therefore a main source of *M. ulcerans* infection to inhabitants of the study community as VNTR types X and Y were detected in samples from this water body and also in human BU infections in the community.

The generation of community maps (Figures 4.1, 4.2, 4.3 and 4.4) as a prerequisite to the identification of risk environments for NTM infections was shown in this study to be very important. This method facilitated the identification of more

risk environments which was linked to community BU infection than would have been sampled without the use of such maps.

5.1.3 Environmental and Human Distribution of *M. ulcerans*

In transmission studies, mechanisms and its associated risks for successful infection should be considered only if it is proven that the disease causative organism and the host or hosts exist. This would allow for the tracing of infection through mechanisms such as vectors, aerosols and direct contact. The exact infection mechanism for BU remains a challenge albeit recent successful studies. Some of these mechanisms are active aquatic insect involvement (Marsollier, Deniaux, *et al.*, 2007; Mosi *et al.*, 2008), mosquito involvement (Lavender *et al.*, 2011) and direct inoculation and localized trauma (Williamson, Mosi, *et al.*, 2014).

Viable number tandem repeat typing has been used to discriminate variant human and environmental isolates from different geographical regions and therefore presents as a good tool for tracking human infections from environmental sources (Ablordey, Hilty, *et al.*, 2005; Ablordey, Swings, *et al.*, 2005). Studies in Australia determined VNTR typing as a valuable tool for determining the geographical origin of patient infection (Lavender *et al.*, 2012).

The four loci (MIRU 1, Locus 6, ST1 and Locus 19) detected in this study discriminated *M. ulcerans* in humans and environmental samples into four VNTR types namely 'W', 'X', 'Y' and 'Z' in the four study communities. One other profile type, designated as 'B', detected in similar studies, was also detected in this study from a pond in Sukuumu (Williamson *et al.*, 2008). In the study by Williamson *et al.* (2008) study, three VNTR loci (MIRU1, Locus 6 and ST1) were amplified for designation of

this 'B' type. In this study four, with the addition of Locus 19, were used. The two types could therefore be similar but not the same.

The localized nature of BU infection was also observed in this study. Apart from Monia-Gyaman where the 'X' type was detected in a human sample and not in the environments sampled, all other human infections matched with environmental profiles from community associated water contact points. This observation strengthens suggestions that *M. ulcerans* is an aquatic saprophyte infecting humans through some form of direct contact (Merritt *et al.*, 2010). This, however, does not rule out the passive or active involvement of other agents such as aquatic insects and other microorganisms such as amoeba (Marsollier *et al.*, 2002; Marsollier, Brodin, *et al.*, 2007; Wilson *et al.*, 2011; Carolan *et al.*, 2014). Although studies in Australia (Johnson *et al.*, 2007; Lavender *et al.*, 2011) have shown evidence of flying insects such as mosquitoes involved in the transmission and dispersal of *M. ulcerans*, the localized infection and the match of profiles indicates that the involvement of flying insects in active transmission highly unlikely in the four study communities (Zogo *et al.*, 2015).

The most common VNTR type detected in both human BU samples and environmental samples was type 'Y'. This type was the most widely circulating and disease causing type in the study communities (Table 4.6). The pond at Sukuumu (Twingum 2) had the most diverse variants of the pathogen with all four types being detected in this pond. Ponds may be providing the most conducive environmental factors for the survival of *M. ulcerans* and other NTMs. A similar richness of variants of *M. ulcerans* was reported by Williamson *et al.* (2008) in a pond in Bonsaaso.

Studies to determine *M. ulcerans* strains causing BU infections in Ghana which used only two targets, MIRU1 and ST1, established three genotypes, designated BD/BAA, BD/B and C/BAA, to be found in Ghana (Hilty *et al.*, 2006). Several studies had used other loci to reveal strains of the pathogen and other MPMs and their distribution (Ablordey, Hilty, *et al.*, 2005; Ablordey, Swings, *et al.*, 2005; Stragier *et al.*, 2005). The four loci used in this study had been successfully used for environmental samples and were also successful in discriminating between environmental types as well as between types of human disease samples (Williamson *et al.*, 2008; Williamson *et al.*, 2012). The strains found to be causing disease in the study communities and in their associated aquatic areas showed similarity to types collected and profiled by earlier studies in the same geographical area (Ablordey, Swings, *et al.*, 2005; Hilty *et al.*, 2006).

5.1.4 Belostomatidae and Naucoridae Distribution

A general critical lack of information on the diversity and biology of insects, especially those that are not of known economic or medical importance in the tropical regions of the world is reported (Polhemus & Polhemus, 2008b). At the end of the 20th century and the beginning of the 21st century, the probable active or passive involvement of aquatic invertebrates especially the Hemiptera of the families Naucoridae and Belostomatidae had been hypothesized, tested and implicated in the transmission of *M. ulcerans* (Portaels *et al.*, 1999; Marsollier *et al.*, 2002; Marsollier, Deniaux, *et al.*, 2007). Recent studies in two BU endemic regions in Cameroun which modeled environmental drivers of Naucoridae and Belostomatidae abundance and BU prevalence found a possible correlation (Carolan *et al.*, 2014). This was interpreted as

evidence that these insect families may be locally playing important roles in the environmental transmission of *M. ulcerans*. The lack of information on insect group diversity in water bodies and aquatic areas has hindered focused vector incriminating research.

The Naucoridae was the first aquatic invertebrate family to be implicated in the transmission of Buruli ulcer and also the most exploited candidate vector in BU infections in humans. This is because of the aggressive behaviour of the Naucoridae and the proven ability to harbour *M. ulcerans* in their salivary glands and effectively causing infections in mammals through their bites (Marsollier *et al.*, 2002). Members of this family are however not very cosmopolitan in distribution showing strong preference to lotic water bodies. In the Philippines, collections of the Naucoridae were mostly from lotic water ecosystems or slow flowing waters with relations with streams (Zettel *et al.*, 1999). Analysing the collections of this study, the preference of the Naucoridae for lotic water bodies was also observed with nearly 90% being collected from lotic water sources. The Naucoridae are well adapted to the unstable environments of lotic waters, having a flattened body to reduce resistance against water flow. Other body and functional modifications such as the prolongation of anteclypeus, back-shifting of labrum, insertion of rostrum into a groove, partial loss of flight ability and plastron respiration due to the difficulties of swimming to the water surface in swift streams assists their habitat preference (Merritt & Cummins, 1996; Zettel *et al.*, 1999).

Two subfamilies of the Naucoridae were collected, Laccocorinae and Naucorinae, similar to a recent collection in Tanzania (Mbogho & Sites, 2013). The collection comprised of eight species belonging to five genera; *Ctenipocoris,*

Aneorocoris, Laccocoris, Neomacrocoris and Naucoris. Ctenipocoris africanus Poisson (1948) was the most collected species and *Naucoris obscuratus* Montandon (1913) among the least collected (Poisson, 1949; Polhemus & Polhemus, 2008a). Differences in habitat preferences of the Naucoridae collection as also observed. *Ctenipocoris africanus* was observed to have the highest preference for lotic water bodies. *Naucoris obscuratus* and *Laccocoris spurcus congoensis* described by Poisson (1949) showed the least preference for lotic water sources being a little higher in the former (Table 4.8). A collection in the Philippines also recorded a similar gradient with *Naucoris* spp having a greater preference for lotic water bodies compared to *Laccocoris* spp (Zettel *et al.*, 1999). Although *Ctenipocoris africanus* is more common in this tropical collection of Naucoridae, in species specific Naucoridae environmental vector implication studies for BU transmission, a focus on the *Naucoris* spp and *Laccocoris* spp is recommended.

The Belostomatidae, with similar habits as the Naucoridae with respect to feeding and their ability to inflict painful bites when threatened, are more cosmopolitan in their distribution and abundance as compared to the Naucoridae. The Belostomatidae are not known to be vectors of any diseases and were only recently suspected of playing a role in the circulation of *M. ulcerans* and possibly in the transmission of BU (Owusu *et al.*, 2015). Mosi *et al.* (2008) found this relationship to be more likely mechanical in nature when mycobacteria were detected on exoskeletal surfaces but not in internal organs of experimentally infected insects An ecological niche modeling of habitat suitability of the Belostomatidae and Buruli ulcer presence also showed strong correlation in a region in Cameroun (Carolan *et al.*, 2014).

The distribution of the Belostomatidae was, however, not influenced by the type of water body at least at the genera taxon. Four genera of the family Belostomatidae were collected: *Diplonychus* Laporte (1833) and *Abedus* Stal (1935) belonging to the subfamily Belostomatinae, and *Lethocerus* Mayr (1853) and *Benacus* Say (1853) belonging to the sub family Lethocerinae (Lauck & Menke, 1961). Only four individuals were classified into the genus *Benacus.* The genera *Benacus and Abedus* had been described by earlier taxonomists as being exclusive to the western hemisphere (Usinger, 1963). and their presence in this collection is interesting This genus *Benacus* has been described as a subgenus of the genus *Lethocerus* by other authors (Pennak, 1953; Bobb, 1974). This may account for the lack of this genus in collections and descriptions from tropical regions of the world (Table 4.8).

A high number of the Belostomatidae collections in this study belonged to the genus *Abedus* (Table 4.8). This genus was described to be confined to the new world extending from California to Panama (Hildago, 1935). The structural characteristics of this genus which included an elevated strong mid-central keel and reduced hemelytra suggest an adaptation to a wide range of aquatic habitats. Mayr (1871) mentions the collection of a single species, *Abedus signoreti*, in Signoret's collection labeled from Guatemala, a country with similar climatic condition as West Africa. The presence of this genus in this collection was therefore not unexpected.

Diplonychus sp has been described in several collections in tropical and sub-tropical

Africa and in the collections of environmental sampling for BU vector implication studies (Doannio *et al.,* 2011). The *Diplonychus* was second in abundance in this collection.

A report of a bite of member of the Family Belostomatidae being the cause of infection in a recent case report from Cameroun has revived the argument in the importance of some members of this Family in the transmission of BU (Marion *et al.,* 2014).

5.1.5 Integrated Approach

This study adopted the 'One Health approach' to decipher the mode of infection from risk environments to community inhabitants of a NTM, *M. ulcerans.* The adopted approach for this study enabled a step by step process of following infection from systematically identified risk environments, through behavioral activities in these environments and the linking of these environments to human BU infections. The study integrated various disciplines to help achieve the objective of the study. The multidisciplinary design of the study employed surveys and questionnaire collection of data, mapping of communities, selection and sampling of water contact environments, molecular detection and typing of pathogen strains detected and matching these to active human BU infections in the associated communities. This is part of a larger study conducted in Ghana and Cote d'Ivoire aimed at using the One Health approach to study the ecology and transmissibility of non-tuberculous mycobacteria from aquatic environments to humans and small mammals. This is the first of such a focused study.

5.2 Conclusion

Integrated use and success of several scientific methods in transmission research was proven in this study. This was informed by the One Health concept approach. The importance of identifying risk environments for BU transmission studies was effectively demonstrated especially in communities that usually obtain water from various surface sources. Activities that were rigorous in these water bodies such as bathing and swimming were found to increase the risk of BU infection enforcing the trauma-contact infection hypothesis.

Community mapping was used in the identification of risk areas of infection in this study. Matching of *M. ulcerans* VNTR profiles from such environmental sources and BU infections in the communities showed an almost perfect match.

Taxonomic keys for the identification of the Families Belostomatidae and Naucoridae were developed from the integration of available keys and descriptions. *Naucoris* spp and *Laccocoris* spp showed a better adaptation to BU risk environments. Four genera collected of the Belostomatidae did not show preference to BU risk aquatic environments.

5.3 Recommendations

- An expansion of this study to cover more communities in comparable similar geographic area using the methods developed and other acceptable methods.
- Further exploration of small mammals especially, mice, rats, squirrels and grasscutters, both in the wild and in homes to establish the roles they may be playing in the ecology of *M. ulcerans* and BU infection.

- Intensify studies on the exploration and description of aquatic macroinvertebrates, especially, the Belostomatidae and the Naucoridae of West Africa.
- A detailed description of the Belostomatidae genera *Abedus*, which was first described in this region of the world in this study.

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APPENDICES

Appendix 1: Informed Consent Form for Patient Sampling

Title: Zoonotic risks of non-tuberculous mycobacteria (NTM) between humans and small mammals (potential transmission of BU) in Côte d'Ivoire and Ghana.

Principal Investigator: Lydia Mosi

Address:
Noguchi Memorial Institute for Medical Research
University of Ghana
P.O.Box LG 581
Legon-Accra

Introduction:

This consent form informs you about the background, aims and the way this study will be conducted. In addition it explains how it will benefit you, the potential risks and how it may affect you. Finally, it informs you of why you have been selected and your rights regarding participating in this study.

General Information about Research

Germs that look like those that cause tuberculosis can affect you especially when you have other diseases. These germs live in the environment and can be in fishes, mammals and humans. One of such group of germs is those that cause Buruli ulcer (BU). This study puts forward that the transmission of these germs depend on the fact that humans come into contact with them in their environment.

The study is divided into three parts and will hopefully give information on the distribution of these germs. This will be done by searching for diseases in humans and small animals like grass cutters with the aim of determining how many have such diseases. The second part will look at how the germs affect animals and humans living close to each other and finally, laboratory identification of these germs in the described

study areas. It is believed that because people are not reporting such infections and there are wrong diagnoses, this is very important for both the people and animals.

In humans, these germs cause disease and represent a major global health problem particularly in developing countries like Ghana and Cote d'Ivoire. The numbers of deaths due to the disease in animals still make up one of the highest losses in revenue in agriculture and local wildlife conservation. In this study, a lot of emphasis will be placed on identifying where *Mycobacterium ulcerans* (the germ that causes Buruli ulcer) is in the environment and in humans within the selected study sites. This is because of the high importance of Buruli ulcer as an emerging neglected tropical disease in Côte d'Ivoire and Ghana. It is also unknown whether this germ can be transferred from animals to people. Information got from this study will be essential in the formulation of policies that will help prevent and eradicate Buruli ulcer.

Samples will be taken from patients with suspicious lesions and ulcers by doctors and other trained individuals. Samples to be collected will be liquid from the lesion or a little skin from the patient using WHO guidelines. Samples obtained will be stored appropriately and transported to CSRS and NMIMR for further processing. All samples will undergo case diagnosis and confirmation.

Risks and Discomfort:

There are no major risks associated with your participation in this study apart from the slight discomfort you may get from parts of the wound being cleaned for sample collection. Samples will be taken only once.

Possible Benefits:

Buruli ulcer treatment is free of charge in hospitals. There will be no direct benefit for subjects' participation in this study. Patients diagnosed with other infections will be counseled accordingly on treatment options. However, the main benefit of your participation is indirect as you will help us identify the prevalence (how many) of environmental mycobacterial diseases in Côte d'Ivoire and Ghana. At the end of the study, you would have been of great help in preventing the horrible effects of the disease in your community.

Confidentiality:

Your records will be kept in a secure location at CSRS and NMIMR. All information collected during the study will be stored in a file which will not have your name on it, but a study number assigned to it. Only the research team will have access to the names associated with the study numbers and for special reasons such as treatment. It is likely that data obtained from tests done on you may be published in medical journals; however, your name will not be used.

Compensation:

Patients identified with Buruli ulcer as well as other lesions will be counseled on treatment choices and the appropriate treatments shall be meted out free of charge.
No money will be paid to participants or any gifts given.

Voluntary participation and Right to leave the Research:

You have the right not to take part in the study if you do not want to, and this will not affect you or your ward in any way. Refusal to participate in or withdraw from this study will not have any penalties or loss of benefits that you may be entitled to. Your position in the community will also not be affected in any way, even if you decide not to participate in the study.

Contact for Additional Information:

Dr. Lydia Mosi
(Principal Investigator)
NMIMR, P.O.Box LG 581, Legon
Tel: +233 54 089 0352
Email: lmosi@noguchi.mimcom.org; lmosi@hotmail.com

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any

questions about your rights as a research participant you can contact the IRB office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org or HBaidoo@noguchi.mimcom.org. You may also contact the chairman, Rev. Dr. Ayete-Nyampong through mobile number 0208152360 when necessary.

You may also contact the following for any further information:

The Chairman
Institutional Review Board/Ethics Committee
Centre Suisse de Recherches Scientifiques
01 BP 1303 Abidjan 01, Côte d'Ivoire

Prof. Bassirou Bonfoh
Centre Suisse de Recherches Scientifiques
01 BP 1303 Abidjan 01, Côte d'Ivoire
Office: +225 23 47 27 90
Email: Bassirou.bonfoh@csrs.ci

Volunteer Agreement

I have read this consent form. I have received satisfactory answers to my questions. I understand that my participation is voluntary. I know about the purpose, methods, risks and possible benefits of the research study to judge that I want to participate. I consent voluntarily to allow my ward to participate in this study. I understand that I have the right to withdraw from the study at any time, and I know that I can call on any member of the study team if I have any questions or concerns.

Name of participant_____

Parent's Name _____ Signature of participant/parent

_____

Witness Name _____ Signature of witness _____

Primary Investigator Signature _____

Date _____ Place _____

(Study ID number assigned to this participation) _____

If volunteers cannot read the form themselves, a witness must sign here**:**

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

Date_____ Signature of Witness_____

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

Date_____

Signature of Person Who Obtained Consent _____

Appendix 2: Child Assent Form

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
INSTITUTIONAL REVIEW BOARD (NMIMR-IRB)

CHILD ASSENT FORM

My name is Dr Lydia Mosi and I am from the Bacteriology Department at Noguchi Memorial Institute for Medical Research. I am conducting a research study entitled Zoonotic risks of non-tuberculous mycobacteria (NTM) between humans and small mammals (potential transmission of Buruli ulcer [BU]) in Côte d'Ivoire and Ghana. I am asking you to take part in this research study because I am trying to learn more about the transmission of BU and other diseases like it that can affect humans and small animals. This will take no time at all and samples will be taken from you only once.

If you agree to be in this study, you will be asked to allow a sample to be taken from your lesion or nodule during treatment at the hospital. This will be done by the doctor or nurse.

Your participation in this study will result in information that will be important in making sure these diseases do not affect many more people in your community.

There are no risks associated apart from a little discomfort you may feel when your wound is being cleaned and the sample is being taken.

You can stop participating at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate.
Your information will be kept confidential. No one will be able to know how you responded to the questions and your information will be anonymous.

You may ask me any questions about this study. You can call me at any time or talk to me the next time you see me. My phone number is 0540890352.

Please talk about this study with your parents before you decide whether or not to participate. I will also ask permission from your parents before you are enrolled into the study. Even if your parents say "yes" you can still decide not to participate.

By signing below, it means that you understand and know the issues concerning this research study. If you do not want to participate in this study, please do not sign this assent form. You and your parents will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled Zoonotic risks of non-tuberculous mycobacteria (NTM) between humans and small mammals (potential transmission of BU) in Côte d'Ivoire and Ghana has been read and or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

Child's Name:……

Researcher's Name:……

Child's Mark/Thumbprint……..

Researcher's Signature:……

Date: ……

Appendix 3 Questionnaire

DATE:……./……../……..

AFRIQUEONE BURULI ULCER PROJECT

PART ONE: SOCIOLOGY ON BURULI ULCER

SECTION A

***IDENTIFICATION OF THE INFORMANT* (head of household or his representative)**

1. Name and surname……..
2. Age…….……
3. Status in the community……
4. BU case or non-case……
5. Profession of the informant……..
6. Marital status……
7. Ethnic group (Optional)……
8. Level of education Primary ☐ Secondary ☐ University ☐
9. Sex : M ☐ F ☐
10. Duration of stay in the community……..……
11. Some prevalent diseases in the community……..……
12. Have you ever suffered from Buruli ulcer? Yes ☐ No ☐

13. If yes, how do you think you contracted it?
……
……...
.....

SECTION B

BACKGROUND INFORMATION ON THE VILLAGE

B1. Is the village increasing or decreasing in population? *If population size is changing, note the reasons below.*

☐ Increasing ☐ Decreasing ☐ No change

_____
_____

B2. Does the population in this village fluctuate significantly during the year? ☐ Yes ☐ No If yes, during what seasons does it increase and decrease, and for what reasons?

_____
_____ B3. Do all people in this village use the same water sources? ☐ Yes ☐ No *If not, explain:*

_____

B4. Do all people in this village have equal access to all water sources? ☐ Yes ☐ No *If not, explain:*_____
_____

SECTION C

HISTORY OF LESION/ ULCER PRESENTATION

C1.What is the name of BU in your local language? _____

C2a. When did the patient or caretaker first notice the lesion/ ulcer? *Establish approximate date through seasonal calendar*. (__/__/____)

_____
_____

C2b. Describe the behavioural activities of the patient when the lesion/ulcer was first noticed *(Be as specific as possible.)*

_____
_____ C3. Where was the lesion/ ulcer located?

☐ Arm ☐ Leg ☐ Torso ☐ Head ☐ Genitals ☐ Other_____

C4a. How did the lesion present during its development? *Check all that apply.*

☐ Nodule ☐ Plaque ☐ Oedema ☐ Ulcer ☐ Osteomyelitis

C4b. How did the lesion present during its development? *Provide a brief narrative of the order of development stages. Note whether the ulcer first appeared from trauma.*

_____
_____

C5a. Are you undergoing any treatment? ☐ Yes ☐ No

C5b. If Yes, Where? _____

SECTION D

IDENTIFICATION AND ANALYSIS OF SOCIAL FACTORS RELATED TO TRANSMISSION AND TREASTEMENT OF BURULI ULCER

Knowledge of Buruli ulcer

D1. Do you know a disease called Buruli ulcer? Yes ☐ No ☐

D2. If yes, could you give the name of this disease in your local language?
……
…….

D3. Does this disease exist in your village? Yes ☐ No☐

D4. If yes do you know people suffering from it? Yes ☐ No ☐

D5. What are some symptoms of this disease that you know?

- Fever ☐
- Headache ☐

- Cough☐
- Itching ☐
- Wound (open wounds) ☐
- Other (precise) ☐……

D6. Which part of the body is particularly affected?

- Arm ☐
- Body ☐
- Hands ☐
- Leg ☐
- Foot ☐
- Eye☐
- Genitals ☐
- Other (precise) ☐…….

D7. Do you know the consequences of this disease? Yes ☐a No ☐b

D8. If yes, cite at least three (3) of them

- …… -
  ……
- …….
- ……..
- ……..

D9. Do you know if it is treatable?

Yes ☐a No ☐b

D10. If yes, How?

……
……
……..

PART TWO : TRANSMISSION OF BURULI ULCER SECTION E

<u>Transmission of Buruli ulcer</u>

E1. For you, what are the causes of the disease?

- Malediction ☐

- Hereditary ☐
- Insect biting ☐
- Poor hygiene ☐
- Working in water ☐
- Other (Precise) ☐……..

E2. What is the main cause (for those who propose many answers)?

……
……
……

E3. Who are the most affected people in this area?

- Women ☐
- Men ☐
- Young boys ☐
- Young girls ☐
- Fishermen ☐
- Hunters ☐
- Farmers ☐
- Others (precise) ☐

E4. Do you think this disease is linked to water?

Yes ☐ No ☐

E5. If yes, which type of water?

.....
.....
.....

E6. At which season are people mostly affected?

- Rainy season ☐
- Dry season ☐
- Any season ☐

E7. What are your sources of water supply in the area (village)?

- Pipe-borne water ☐
- Stream/river/lake… ☐
- Well ☐
- Other (precise) ☐……

E8.What is the main source of water ?.....

E9. What are different activities people from the village have at the river?

- Bathing/swimming ☐
- Drinking water ☐
- Fishing ☐
- Laundry and dish washing ☐
- Other (precise)☐……..

E10. Who is generally responsible for those activities?

- Women ☐
- Men ☐
- Children ☐

E11. At which season do they carry those activities?

- Dry season ☐
- Rainy season ☐
- Any season ☐

E12. At what time of the day do they go to the river?

- Morning ☐
- Afternoon ☐
- Evenning ☐

SECTION F

Intervention Perceptions

F1. Where and How are people affected by Buruli ulcer treated here?

- Hospital/ health centre ☐
- Traditional healer ☐
- Marabout ☐
- Self medication (traditional) ☐ - Self medication (drugs) ☐
- Other (precise) ☐…….

F2. What is the most preferred mode of treatment system in your area?

…….

F3. How do you find the treatment cost of this disease at the hospital?

- Affordable ☐
- Not affordable ☐
- Other (precise) ☐……

F4. Are there sensitization programmes on this disease in this area? Yes ☐ No ☐

F5. If yes, have you ever attended one of those programmes?

. Yes ☐ No ☐

What did you learn ?.....

F6. If No, why?

……
……
……..

F7. Who were the targeted groups through this programme?

……
……
……

SECTION G

Preventive Practices

G1. What are your preventive measures to avoid Buruli ulcer?

- I avoid going to the stream/river ☐
- I take drugs ☐
- I use medicinal herbs ☐
- Other (precise) ☐……

G2. How do you personally fight BU ?

……
……
……

G3. Do you sensitize your relatives on the consequences of Buruli ulcer?

- Yes ☐ No ☐

G4. If yes, what do you recommend them precisely?

- To have a good hygiene ☐
- To avoid bathing/swimming in stream/river/lake ☐
- To wear appropriate clothes while working in water ☐
- Other (precise) ☐

SECTION H

Background information on living areas

H1. Where have you being residing in the last three (3) months?

_____

H2. If patient, where have you being residing in the last three (3) months before lesion appeared?

_____

H3. What time of the year does the informant lives in this house? *Interviewer:*

_____

H4. If the patient spends significant (one month and above) periods of the year in another residence, note the duration above and identify the location(s) below:

_____
_____
_____

H5. When was the house built? *Use seasonal calendar* (___/___/_____)

H6. What is the house made of? *Check all that apply. If Mud is not checked, continue to question D6.*

☐ Cement ☐ Mud ☐ Wood or bamboo ☐ Other: _____ H8.

What type of soil was used in the construction of the house?

☐ Sandy ☐ Clay ☐ Loamy ☐ Other: _____

H9. What are the structures in the compound where the house is found? *Check all that apply and indicate the number.*

☐ Residences ____ How many rooms/doors? __/__

☐ Storage rooms ____

☐ Cooking areas ____

☐ Grain silos ____

☐ Animal enclosures ____

☐ Kitchen gardens adjacent ____

☐ Other: _____

H10. What are the most common methods of waste and sewage disposal? List below

_____

_____

H11. What kind of latrine is used in the house?

_____

H12. Describe the bath house.

_____

H13. What animals are kept in the compound where the house is found? *Check all that apply.*

☐ Goats ☐ Dogs

☐ Sheep ☐ Cats

☐ Chickens ☐ Pigs

☐ Guinea fowl ☐ Cows

☐ Donkeys ☐ Other _____

H14. Does the household keep any other animals in areas outside of the compound? ☐ Yes ☐ No, if *yes, identify them.*

☐ Goats ☐ Dogs
☐ Sheep ☐ Cats ☐
Chickens ☐ Pigs
☐ Guinea fowl ☐ Cows
☐ Donkeys ☐ Other _____

H15. Has household composition changed in the past five years? ☐ Yes ☐ No *If yes, check all that apply and explain below.*

☐ Some members live in field houses during part of each year

☐ Members of the current household lived in other villages ☐

Members of the current household lived elsewhere in this village

☐ Other:
_____
__

SECTION I

Water sources

I1. Where does the household get their water for **drinking and cooking** during the dry season and the rainy season? *Check all that apply and indicate on map.*

Dry season	*Rainy season*	
☐	☐	Pump
☐	☐	Covered well
☐	☐	Open well
☐	☐	Cistern
☐	☐	River (name of river) _____
☐	☐	Pond, swamp or low-lying area, _____
☐	☐	Other _____

I2. Where does the household get their water for **showering and washing clothes** during the dry season and the rainy season? *Check all that apply and indicate on map.*

Dry season	*Rainy season*	
☐	☐	Pump
☐	☐	Covered well
☐	☐	Open well
☐	☐	Cistern
☐	☐	River (name of river) _____
☐	☐	Pond, swamp or low-lying area, _____
☐	☐	Other _____

I3. Was there a time in the last year that water sources that you normally use became unavailable (broken pump, dry or polluted well, etc.) ☐ Yes ☐ No

Identify below, and describe the alternative water sources the household used.

_____
_____
_____

I4. Does the patient ever get water for the household, or accompany the person who does?

☐ Yes ☐ No *If yes, check all that apply and indicate on map.*

Dry season	*Rainy season*	
☐	☐	Pump
☐	☐	Covered well
☐	☐	Open well
☐	☐	Cistern
☐	☐	River (name of river) _____
☐	☐	Pond, swamp or low-lying area, _____
☐	☐	Other _____

I5. Do you have contact with any other water sources? *If a child, include areas where they play. If adult, include areas where they work.*

_____
_____

I6. *Interviewer:* draw on map the paths used by the household to access the water sources indicated in Section E.

SECTION J

Livelihood

J1. List all of the livelihood strategies undertaken by members of the household.

☐ Agriculture

☐ Animal husbandry

☐ Fishing

☐ Hunting

☐ Commerce

☐ Artisan *Identify:* _____

☐ Other: _____

J2. What are the crops grown by the household? *Check all that apply.*

☐ Corn ☐ Tomatoes

☐ Rice ☐ Okra

☐ Palm nut ☐ Yams

☐ Greens ☐ Manioc

☐ Peanuts ☐ Beans ☐ Onions

☐ Other _____

J3a. What kinds of fields are used for cultivation? *Check all that apply.*

☐ Inundated fields

☐ Irrigated fields

☐ Dry fields

☐ Fields tilled each year

☐ Other, _____

J3b. Did the informant spend time around fields, which one and how long?

☐ Yes ☐ No

_____

J3c. *Interviewer:* draw the paths on the map used by the household to access fields, hunting grounds, forests, or bush.

J4. Are there any livelihood strategies or other activities undertaken by the household that expose family members to the environment (forest/bush)?

☐ Yes ☐ No *If yes, describe and explain below using F1 as guide.*

_____
_____
_____

J5a. Do members of this household or friends engage in hunting, or catch wild animals while in their fields? ☐ Yes ☐ No.

J5b. Does the informant engage in these activities, or accompany those who do? ☐ Yes ☐ No

J5c. Does hunting occur ☐ seasonally, or ☐ throughout the year?_____ J5d.

What are the animals most frequently caught?

☐ Grasscutter ☐ Rat

☐ Squirrel ☐ Rabbit

☐ Bush chicken ☐ Snake

☐ Pangolin ☐ Deer

☐ Porcupine ☐ Monkey

☐ Other _____ ☐ Other _____

☐ Other _____ ☐ Other _____

SECTION K

Other family members

K1. Are there any other members of the household that have had BU infections? ☐ Yes ☐ No, *If No, continue to G3. If yes, identify their relationship to the informant.*

☐ Mother

☐ Father

☐ Child_____

☐ Sibling (same mother) _____

☐ Sibling (same father) _____

☐ Other genetic relationship _____

☐ Non-genetic relationship _____

K2a. Do the afflicted members engage in similar work activities as the patient? ☐ Yes ☐ No

K2b. Do the afflicted members work in the same location as the patient? ☐ Yes ☐ No

K3. Does the patient have any close relatives (child, parent, or sibling) who do not live in the compound, but who have had BU infections? ☐ Yes ☐ No, *If No, continue to Section H. If yes, check all that apply and indicate number if multiple cases exist.*

☐ Mother

☐ Father

☐ Child_____

☐ Sibling (same mother) _____

☐ Sibling (same father) _____

SECTION L

Mapping (supplementary section)

L1. If a map of the village is available, locate the house on the map and any other places where the patient resides during the year. Mark the areas in the village where the patient spends significant periods of time.

L2. Locate all of the village water sources for drinking, cooking, washing, playing etc. on the map. Note which of these sources are used by the household, and for what purpose.

L3. Mark the locations where the household gets soil for house construction.

Appendix 4: Taxonomic Keys for Belostomatidae Identification

Based on keys by (Needham & Needham, 1962; Merritt & Cummins, 1996)

1) Antennae shorter than head……...2

- Antennae as long as or longer than head, exposed…….....……3

2) Hind tarsi with indistinct setiform claws …….……..……4

- Hind tarsi with distinct claws…….……..5

3) Head as long as entire thorax; both elongated. Both about 10mm long…..

……. ……..HYDROMETRIDAE

4) Head overlapping thorax dorsally. Front tarsi 1-segmented……...…CORIXIDAE

5) Membrane of Hemelytra reticulately veined ……..……..….6

- Membrane of Hemelytra without veins…….…….…….NAUCORIDAE

6) Apical appendages of the abdomen long and slender, tarsi 1-segmented..NEPIDAE

Apical appendages of the abdomen short and flat, retractile……...……..BELOSTOMATIDAE (7)(7a)

ADULT BELOSTOMATIDAE

7) Mesothorax with strong midventral keel, membrane of hemelytra reduced…… ……..…..*Abedus* Stal

- Mesothorax without midventral keel, membrane of hemelytra not reduced……....8 **8)**

- Basal segment of beak shorter than the second; furrow of wing membrane shallowly

S-shaped. Length more than $1^1/_2$ inches……9 Basal segment of beak longer than the second; furrow of wing membrane nearly

straight with length of about 1inch or less……..10

9) Anterior femora grooved for the reception of the tibia…….……*Lethocerus* Mayr

- Anterior femora not grooved for the reception of the tibia ……....*Benacus* (Say)

10) Lateral band of short pilosity on ventral part of lateral tergite four not reaching margin. Subgenital plate pointed in females and truncated in males….*Diplonychus* (Laporte)

- Pubescence, short hairs covering part of the entire surface of abdominal esternites. Male genetalia, phallobase…….……..…….*Belostoma* (Latrielle)

JUVENILE BELOSTOMATIDAE

7a) Foreleg with two equally developed claws…….*Lethocerus* Mayr

- Foreleg with one long claw…….……8a

8a) Interoculus with narrow, linear depression closely paralleling inner orbit, convexity of interoculus uniform, not interrupted by transverse swelling at posterior

end of depression… ……..……*Diplonychus* (Laporte)

- Interoculus with a broad linear depression distantly paralleling inner orbit, convexity of interrupted by transverse swelling at end of depression..……..*Abedus* Stal

Appendix 5: Taxonomic Keys for Naucoridae Identification

Based on Keys by (Dejoux *et al.*, 1981; Merritt & Cummins, 1996; Sites & Mbogho, 2012; Mbogho & Sites, 2013)

1) Front of head folded posteroventrally. Foreleg pretarsus with two claws. Males with well-developed tomentose patch ventrally on pro- and mesotibia (on females weakly developed)..... Laccocorinae (2)

– Front of head not folded posteroventrally. Foreleg pretarsus with a single claw. Without tomentose patch on pro- and mesotibia..... Naucorinae (6)

2) Length 5.8–7.4 mm. Hemelytra with embolar and claval sutures absent

……..*Aneurocoris insolitus* Montandon

– Length ≥7.5 mm. Hemelytra with embolar and claval sutures distinct

…….....3

3) Pronotum with greatest width approximately twice its length at midline. Female with two protarsal segments..... *Ctenipocoris africanus* Poisson

– Pronotum with greatest width approximately 3× its length at midline. Female with one protarsal segment .....*Laccocoris* (4)

4) Labrum broad, half as long as wide at base; roundedly pointed; reaching at most to middle of penultimate segment of labium. Body large, length 10–13 mm…….....*L. spurcus congoensis* Poisson

– Labrum triangular or acuminate, slightly shorter than wide at base; reaching distal segment of labium. Body shorter, length <10 mm 5

5) Mesosternal tubercle low, broad, and rounded in profile. Pronotum with posterolateral corners broadly rounded or truncated, not pointed or produced caudad. Claval commissure subequal in length to scutellum at midline. Hemelytral membrane broad……..... *L. limigenus* Stål

– Mesosternal tubercle produced, bluntly acute in profile. Pronotum with posterolateral corners slightly produced caudad or at least bluntly pointed Claval commissure approximately twice length of scutellum at midline. Hemelytral membrane narrow.....*L. limicola* (Stål)

6) Head including eyes distinctly wider than anterior margin of scutellum. Body flattened, pyriform. Male with tergites 7 & 8 unmodified. Male parameres elongate and crossing; aedeagus unmodified distally.....

……*Naucoris obscuratus* Montandon

– Head as wide as or narrower than anterior margin of scutellum. Body dorsoventrally robust, ovate. Male with abdominal terga 7 and/or 8 modified with asymmetrical medial lobes. Male parameres present or absent; aedeagus modified distally with complex vesica ……..……7

7) Greatest head width across the eyes is less than half the greatest pronotum width. Male parameres absent……..... *Neomacrocoris* (8)

– Greatest head width across the eyes is more than half the greatest pronotum width.

Male parameres well developed ……..…..... *Macrocoris* (15)
8) Body of adult large, usually ovate; length ≥10.0 mm; width/length ratio usually ≥0.675.....9

– Body of adult smaller and more elongate; length <10.0 mm; width/length ratio <0.675.....12

9) Female
…...…….....10

–Male …….....11

10) Subgenital plate with lateral margins smoothly sinuate to distal spatulate process…..... *N. usambaricus* Montandon

– Subgenital plate with strong mid-lateral projections and distal spatulate process……..... *N. vuga* Sites

11) Phallosoma with acute apical hook projecting to left …….....*N. vuga* Sites

– Phallosoma with left side of apex flattened …….....*N. karimii* Poisson

12) Thick setae scattered sparsely on scutellum and hemelytra, including on clavus, embolium, corium, and membrane. Pronotum slightly flattened

……..... *N. handlirschi* (Montandon)

– Thick setae restricted to embolium and adjacent areas of corium, although thin hairlike setae can be present throughout. Pronotum dorsoventrally robust ……..…13

13) Lateral margin of embolium slightly less arcuate in anterior ⅓ to ½. Phallosoma with rounded gibbosity on right …….....*N. bondelaufa*

Sites – Lateral margin of embolium evenly arcuate throughout. Phallosoma with elongate

flange on right …….....14

14) Pronotum with black spots on lateral ⅓ larger than spots on central ⅔

..... *N. parviceps ocellatus* Poisson

– Pronotum with black spots on lateral ⅓ subequal to spots on central ⅔

.....*N. parviceps parviceps* (Montandon)

15) Pronotum mostly yellow (green in live specimens) with sparse large black spots some specimens with dark punctation in addition to larger dark spots. Connexiva continuously greenish yellow..... *M. flavicollis* Signoret

– Pronotum with profuse small black spots. Connexiva with posterolateral corners dark brown, yellow anteriorly..... *M. laticollis* Montandon

Appendix 6: Hemiptera Collected from all Study Water Bodies

Tables show District or country, water body associated community name, flow type of water body and 'lat lon' location and number of individuals in families identified.

SITE No.	DISTRICT	COMMUNITY	TYPE OF FLOW	Latitude	Longitude	Hyrometridae	Nepidae	Naucoridae	Belostomatidae	Gerridae	Unidentified*	Ranatridae	Pleidae	Veliidae	Notonectidae	Corixidae	Macroveliidae	TOTAL
1	Amansie Central	Monia Gyaman	Lotic	6.05916	-1.94236	1	1	4	4	2	2							**14**
2	Amansie Central	Monia Gyaman	Lotic	6.05392	-1.94514	1			16									**17**
3	Amansie Central	Wromanso	Lentic	6.03256	-1.89761		4		11			3	1		7			**26**
4	Akim North District	Kumasi-Accra Road	Lotic	6.49320	-0.68461			2	4									**6**
5	Amansie Central	Afabrakoso	Lotic	6.22453	-1.88639				8		1							**9**
6	Atwima Mponuah	Bofaso	Lotic	6.59772	-2.19869	1		14	23	4		4			9			**55**
7	Amansie Central	Abuakwaa	Lotic	6.29635	-1.85421	1		4	9									**14**
8	Ho	Adaklu Amedivie	Lentic	6.44893	0.49859			7	21	3		3			1			**35**
9	Ga East	Adigon	Lentic	5.79435	-0.08992	1			6	1	1	2			2	1		**14**
10	Amansie East	Senfi	Lentic	6.50686	-1.63127			2	7	2			10	1				**22**
11	Ho	Adaklu Dzakpo	Lotic	6.47864	0.41836	1	1		6		1	2	2					**13**
12	Atwima Mponuah	Jerusalem	Lotic	6.62637	-2.33293		1	1	6				1					**9**
13	Atwima Mponuah	Akentensu	Lotic	6.66827	-2.09273	1		3	8	1								**13**
14	Kwabre	Achiase	Lentic	6.73669	-1.53734			11	5						7			**23**
15	Jasikan	Nkonya Bumbula	Lotic	7.23443	0.33763			20	23									**43**
16	Akuapim South	Alafia	Lentic	5.86653	-0.30907			8	17	2			1		1	1		**30**
17	Hohoe	Likpe Agbozume	Lotic	7.19891	0.56009			13	41	1	1		165		5			**226**
18	Krachi East	Okaniase	Lotic	7.73876	0.36691				3	2			4	3				**12**
19	Kadjebi	Atta Kofi	Lotic	7.75737	0.50963				1	1				1				**3**

20	Suhum Kraboa Coaltar	Obotumpan	Lotic	6.08105	-0.31027			10	16	1				4				**31**
21	Ga West	Olebu	Lentic	5.63795	-0.30970			4	12			1			2			**19**
22	Ga West	Kotoku	Lentic	5.74113	-0.35399	1	1	7	2	1			5		20			**37**
23	Kadjebi	Asato	Lotic	7.53529	0.43798			1	9	2	2		11	4				**29**
24	Ho	Abutia Kloe	Lentic	6.48063	0.34753		1											**1**
25	Amansie West	Mpatuom	Lentic	6.54440	-1.93550								1		1	2		**4**

Appendix 6 Table Cont'

SITE No.	DISTRICT	COMMUNITY	TYPE OF FLOW	Latitude	Longitude	Hyrometridae	Nepidae	Naucoridae	Belostomatidae	Gerridae	Unidentified*	Ranatridae	Pleidae	Veliidae	Notonectidae	Corixidae	Macrovelidae	TOTAL
26	Ga West	Akotoshie	Lentic	5.75034	-0.34194				1				2		1			**4**
27	Atwima Nwabiagya	Ataase	Lotic	6.90613	-1.76859			1	1	4	1		2		1			**10**
28	Atwima Mponuah	Aniamoa	Lotic	6.54083	-2.02950									1				**1**
29	Bekwai	Nsuta	Lotic	6.15530	-1.25950			1	7									**8**
30	Atwima Nwabiagya	Adagya	Lotic	6.94899	-1.75102						3	1						**4**
31	Atwima Mponuah	Barniekrom	Lotic	6.54168	-2.17884			1	4					2	1			**8**
32	Jasikan	Tapa Bompaso	Lotic	7.47529	0.35664			2	5		2			3	1			**13**
33	Atwima Mponuah	Kobeng	Lentic	6.59742	-1.86081				5									**5**
34	Ho	Adaklu Dorkpo	Lentic	6.39350	0.50163			1	5				1			1	1	**9**
35	Atwima Mponuah	Aboabogya	Lotic	6.54619	-2.27267			5	4		1					1		**11**
36	North Tongu	Dofor Fortipkoe	Lotic	6.08409	0.29076			1	1				1		1			**4**
37	Ga East	Adoteiman	Lentic	5.79539	-0.15261	1		11	61				1	2	5	1		**82**
38	Ga East	Teiman	Lentic	5.75120	-0.18143		2		32					1				**35**
39	Atwima Mponuah	Pamurusu	Lotic	6.52425	-2.24979			6	21					1			14	**42**

40	Asante Akim North	Patrensa	Lotic	6.66068	-1.17576	2		1	15				2		13			**33**
41	Kadjebi	Titiaka	Lotic	7.68075	0.52780			1	2	3			28	1		21		**56**
42	Kpandu	Kpandu Agudzi	Lentic	7.02687	0.31931				5		1			2	7			**15**
43	Atwima Nwabiagya	Abompe	Lotic	6.73738	-1.92522									5				**5**
44	North Tongu	Tadeafenui	Lentic	6.13521	0.47895				4	1			1	1				**7**
45	Ho	Agodeke	Lotic	6.39933	0.30215			1	1	1				1				**4**
46	Ho	Wayanu	Lotic	6.58938	0.56437				1	1								**2**
47	Hohoe	Lolobi Hiyasem	Lentic	7.20189	0.54758	4		3	19	1	9	1	53	5	6			**101**
48	Jasikan	Bodada	Lotic	7.35552	0.48898			1	7	2				1	4			**15**
49	Ga West	Afiaman	Lentic	5.70809	-0.28563			2							3			**5**
50	Jasikan	Takrabe	Lotic	7.33861	0.41121				7	2			8	1				**18**

Appendix 6 Table Cont'

SITE No.	DISTRICT	COMMUNITY	TYPE OF FLOW	Latitude	Longitude	Hyrometridae	Nepidae	Naucoridae	Belostomatidae	Gerridae	Unidentified*	Ranatridae	Pleidae	Veliidae	Notonectidae	Corixidae	Macroveliidae	TOTAL
51	Ga East	Oyarifa	Lentic	5.74378	-0.17533			2	7	6		1		1	3			**20**
52	Kadjebi	Pampamwie	Lotic	7.80563	0.56673			2	7				3		8			**20**
53	Ga East	Danfa	Lentic	5.78748	-0.16475				5		1	1						**7**
54	Ga West	Fise	Lentic	5.69016	-0.30180							2		1	1			**4**
55	Jasikan	Nkonya Asakyiri	Lotic	7.08944	0.32118		2		19			2		1	1			**25**
56	Upper Denkyira	Subin	Lotic	6.08175	-1.01953			3	6					1				**10**
57	Ga West	Nsakena	Lotic	5.65478	-0.32026	1			15			2	14		9	3		**44**
58	Atwima Nwabiagya	Kyenkyentaa	Lotic	6.71566	-1.87409										3			**3**
59	Amansie Central	Kobro	Lotic	6.28616	-1.88503			4	5	5	1		7		10			**32**
60	Kadjebi	Wawaso	Lotic	7.65510	0.44071				2	2				1				**5**
61	Amansie East	Ofoasi Kokobeng	Lentic	6.47629	-1.64132			20	129			3	1		3			**156**
62	Kpandu	Anfoega Glenkor	Lentic	6.86980	0.27900									1				**1**
63	Ga West	Kwashiekuma	Lentic	5.71583	-0.36531	1		7	28			1	3	1	17	3		**61**
64	Kpandu	Anfoega Woademaxe	Lentic	6.89704	0.29705				2		1				3			**6**
65	Amansie West	Groso	Lotic	6.29844	-2.01478				1	2	1			8				**12**
66	Asante Akim North	Agogo	Lotic	6.79781	-1.07714			2	23									**25**
67	Kpandu	Gbefi	Lotic	7.00388	0.37607	1				3				2				**6**
68	Ga West	Ashalaja	Lotic	5.66613	-0.36466											1		**1**
69	Ga East	Otinibi	Lentic	5.78889	-0.14359			1	4	1				1	1			**8**
70	Kpandu	Anfoega Ando	Lotic	6.90363	0.31125			2	8		2		5	2		4		**23**
71	Ho	Gavorkpo	Lentic	6.52854	0.63203				1	1		1		1	4			**8**
72	Atwima Mponuah	Baakoniaba	Lotic	6.63783	-2.08811			6	5	1			1	2	1	1		**17**
73	Asante Akim North	Serebuoso	Lotic	6.94313	-1.02683					1					1			**2**
74	Cote d'Ivoire	Zaibo	Lotic	6.95084	-6.60888				4	1					1			**6**
75	Amansie Central	Wromanso	Lentic	6.03256	-1.89761			5	9			3			7			**24**

Appendix 6 Table Cont'

SITE No.	DISTRICT	COMMUNITY	TYPE OF FLOW	Latitude	Longitude	Hyrometridae	Nepidae	Naucoridae	Belostomatidae	Gerridae	Unidentified*	Ranatridae	Pleidae	Veliidae	Notonectidae	Corixidae	Macroveliidae	TOTAL
76	Cote d'Ivoire	Gorodi	Lentic	6.67899	-6.91062	2			12	2				1				**17**
77	Cote d'Ivoire	Ahondo	Lentic	6.30305	-5.15112			1	6		1	2		3	11			**24**
78	Cote d'Ivoire	Zaibo	Lentic	6.99083	-6.67347				6	2		1						**9**
79	Cote d'Ivoire	Sokrogbo	Lentic	6.04632	-4.96427			12	26	3		10		1	4			**56**
80	Amansie Central	Monia Gyaman	Lotic	6.05392	-1.94514			1	1	2		1		4	6			**15**
81	Amansie Central	Monia Gyaman	Lotic	6.05472	-1.93972				1	3	1			3				**8**
82	Amansie Central	Monia Gyaman	Lotic	6.04861	-1.94667					3								**3**
83	Amansie Central	Bepotenten	Lotic	6.09194	-1.96883					19								**19**
84	Amansie Central	Sukuumu	Lotic	6.05916	-1.94250			14	12	1		7			1			**35**
85	Amansie Central	Sukuumu	Lotic	6.05222	-1.95778					34								**34**
86	Amansie Central	Sukuumu	Lotic	6.07083	-1.95194	2			14	4		1	1	1	6			**29**
87	Amansie Central	Sukuumu	Lotic	6.07639	-1.95000	1		2	6	2		1						**12**
88	Cote d'Ivoire	Leleble	Lentic	6.24138	-5.21960		2	29	47	1		12			15			**106**
89	Cote d'Ivoire	Leleble	Lentic	6.23055	-5.22492				14						4			**18**
TOTAL						**23**	**15**	**262**	**891**	**137**	**33**	**68**	**335**	**76**	**218**	**40**	**15**	**2113**

Appendix 7: Distribution of Genera of Belostomatidae Identified

SITE No.	DISTRICT	COMMUNITY	TYPE OF FLOW	*Abedus* sp	*Diplonychis* sp	*Lethocerus* sp	*Benacus* sp	TOTAL
1	Amansie Central	Monia Gyaman	Lotic	1				1
2	Amansie Central	Monia Gyaman	Lotic				1	1
3	Amansie Central	Wromanso	Lentic			1		1
6	Atwima Mponuah	Bofaso	Lotic	11	8	2		21
7	Amansie Central	Abuakwaa	Lotic	1	2	2	3	8
8	Ho	Adaklu Amedivie	Lentic	4	12			16
9	Ga East	Adigon	Lentic		1			1
10	Amansie East	Senfi	Lentic	2	4			6
13	Atwima Mponuah	Akentensu	Lotic	4	3			7
14	Kwabre	Achiase	Lentic	2	2			4
15	Jasikan	Nkonya Bumbula	Lotic	12	11			23
16	Akuapim South	Alafia	Lentic	4		10		14
17	Hohoe	Likpe Agbozume	Lotic	13		8		21
20	Suhum Kraboa Coaltar	Obotumpan	Lotic	3	10	3		16
21	Ga West	Olebu	Lentic	5	2	1		8
23	Kadjebi	Asato	Lotic			1		1
26	Ga West	Akotoshie	Lentic		1			1
27	Atwima Nwabiagya	Ataase	Lotic	1				1
29	Bekwai	Nsuta	Lotic	2	2	1		5
32	Jasikan	Tapa Bompaso	Lotic	1	1	3		5
34	Ho	Adaklu Dorkpo	Lentic	3		2		5
37	Ga East	Adoteiman	Lentic	6	5	6		17
39	Atwima Mponuah	Pamurusu	Lotic	9	5			14
40	Asante Akim North	Patrensa	Lotic	2	2	1		5
42	Kpandu	Kpandu Agudzi	Lentic	1	2			3
46	Ho	Wayanu	Lotic	1				1
47	Hohoe	Lolobi Hiyasem	Lentic	1	6			7
48	Jasikan	Bodada	Lotic	1	3			4
50	Jasikan	Takrabe	Lotic		5			5
51	Ga East	Oyarifa	Lentic			1		1
52	Kadjebi	Pampamwie	Lotic	1	2	3		6
53	Ga East	Danfa	Lentic			2		2

56	Upper Denkyira	Subin	Lotic	6				6
60	Kadjebi	Wawaso	Lotic			2		2
SITE No.	**DISTRICT**	**COMMUNITY**	**TYPE OF FLOW**	***Abedus* sp**	***Belostoma* sp**	***Lethocerus* sp**	***Benacus* sp**	**TOTAL**
61	Amansie East	Ofoasi Kokobeng	Lentic	42	23			65
75	Amansie Central	Wromanso	Lentic	9				9
80	Amansie Central	Monia Gyaman	Lotic			1		1
86	Amansie Central	Sukuumu	Lotic		1			1
87	Amansie Central	Sukuumu	Lotic	4	2			6
				152	**115**	**50**	**4**	**321**

Appendix 8: Distribution of Naucoridae Identified

SITE No.	DISTRICT	COMMUNITY	TYPE OF FLOW	*Ctenipocoris africanus*	*Aneorocoris insolitus*	*Laccocoris limicola*	*Laccocoris spurcus congoensis*	*Neomacrocoris bondelaufa*	*Neomacrocoris vuga*	*Naucoris obscuratus*	*Neomacrocoris usambaricus*	TOTAL
6	Atwima Mponuah	Bofaso	Lotic	10	1							11
7	Amansie Central	Abuakwaa	Lotic	4								4
8	Ho	Adaklu Amedivie	Lentic	6								6
10	Amansie East	Senfi	Lentic			1			1			2
13	Atwima Mponuah	Akentensu	Lotic		3							3
15	Jasikan	Nkonya Bumbula	Lotic	15							1	16
16	Akuapim South	Alafia	Lentic	5								5
17	Hohoe	Likpe Agbozume	Lotic	12								12
20	Suhum Kraboa Coaltar	Obotumpan	Lotic	6		2						8
21	Ga West	Olebu	Lentic	3								3
29	Bekwai	Nsuta	Lotic	1								1
32	Jasikan	Tapa Bompaso	Lotic							1		1
34	Ho	Adaklu Dorkpo	Lentic								1	1
37	Ga East	Adoteiman	Lentic	4	1					5		10
39	Atwima Mponuah	Pamurusu	Lotic	2	1						3	6
40	Asante Akim North	Patrensa	Lotic		1							1
47	Hohoe	Lolobi Hiyasem	Lentic		2					1		3
48	Jasikan	Bodada	Lotic					1				1
50	Jasikan	Takrabe	Lotic	2								2
52	Kadjebi	Pampamwie	Lotic					1		1		2
56	Upper Denkyira	Subin	Lotic							2		2
61	Amansie East	Ofoasi Kokobeng	Lentic	4	14							18
75	Amansie Central	Wromanso	Lentic		5							5
87	Amansie Central	Sukuumu	Lotic				1				1	2
				74	**28**	**3**	**1**	**2**	**1**	**10**	**6**	**125**