THE ROLE OF MACROINVERTEBRATES IN BURULI ULCER DISEASE IN GHANA, WEST AFRICA

By

Ryan K. Kimbirauskas

A DISSERTATION

Submitted to

Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Entomology

2011

ABSTRACT

THE ROLE OF MACROINVERTEBRATES IN BURULI ULCER DISEASE IN GHANA, WEST AFRICA

By

Ryan K. Kimbirauskas

Buruli ulcer (BU) is an emerging, neglected, infectious disease most often associated with poor, rural communities within developing nations. To date, the disease has been reported from at least 32 countries, with the highest frequency of new cases being reported from the West African nations of Cote D' Ivoire, Benin and Ghana. It is known that *Mycobacterium ulcerans* is the pathogen responsible for causing BU disease; however, researchers have yet to conclusively identify the extent of the pathogen's distribution in the environment, the reservoir(s) of the pathogen in nature, or the mode(s) of transmission to humans. It is widely accepted that BU disease is in some way related to exposure to freshwater environments, and furthermore, it has been suggested that human activities leading to environmental disturbance increase risk of BU infection. Aquatic macroinvertebrates have been implicated as both potential reservoirs and vectors *M. ulcerans* infection to humans; however, field-based ecological studies to investigate the role of macroinvertebrates in BU disease have not been conducted. The purpose of this study was to: 1) characterize and compare overall macroinvertebrate communities from aquatic environments in Ghana, West Africa: 2) identify macroinvertebrate community associations with the presence and absence of *M. ulcerans* in aquatic environments: and 3) identify potential relationships between specific macroinvertebrates and *M.*

ulcerans. Results from this large survey of aquatic environments in Ghana suggest that macroinvertebrate communities and individual taxa may be useful sentinels for initial identification of pathogen presence or habitat conditions associated with disease agent transmission; however, further studies are needed to elucidate the exact role of macroinvertebrates as reservoirs of *M. ulcerans* and potential vectors of BU.

This dissertation is dedicated to my parents and sister: Paul, Cinda and Paula Kimbirauskas, respectively.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Richard Merritt. Throughout my graduate career he provided endless support, encouragement, friendship, and new opportunities that have made me a better scientist, person and future mentor. I must also thank Dr. Michael Kaufman, Dr. Eric Benbow, and Dr. Mollie McIntosh, who not only served on my committee, but also helped with field logistics, data management and analyses for this project. I would like to acknowledge collaborators on this project: Charles Quaye, Charles Yeboah, Lydia Mosi, and Daniel Boyake from the University of Ghana; and Dr. Heather Williamson and Dr. Pamela Small from the University of Tennessee. There are numerous individuals at MSU I would also like to thank who played a significant role in my development during my graduate years. The administrative and support staff for the Department of Entomology is the best around and not only made my work easier, but were always pleasant to visit. I would like to recognize and thank the many people who have helped either in the field or laboratory processing samples: Todd White, Rebecca Kolar, Tom Alwin, Bethany Coggins, Alvin Makhon-Moore, and Sanjeev Mahabir. Gary Parsons should also be recognized for his help and guidance in identifying insect specimens. Finally I would like to thank the members of the Merritt Lab, including: Jaree Johnson, Kristi Zurwaski, Osvaldo Hernandez, Mollie McIntosh, Eric Benbow, Christian Lesage, Todd White, Emily Campbell, and Matt Wessner; for helping make this a lot of fun and more than just the process of obtaining a degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION TO THE ASSOCIATIONS BETWEEN AQUATIC INSECTS AND THE ECOLOGY OF BURULI ULCER DISEASE

Buruli ulcer (BU) is an emerging neglected disease caused by infection of *Mycobacterium ulcerans* (Walsh et al. 2008, Duker et al. 2006, Wansbrough-Jones and Philips 2006, van der Werf et al. 2005). The disease can inflict people of any age or gender, although nearly 70% of the cases occur in children under the age of 15 years and in some communities more females are infected than males (WHO 2000, 2008; Duker et al. 2004). Confirmed cases of BU have been reported from 32 countries mainly in Africa, Australia, southeast Asia, China, Central and South America, and the Western Pacific (Johnson et al. 1999; WHO 2000, 2008, Guerra et al. 2008, Walsh et al. 2009) (Fig. 1.1). Endemism is primarily confined to tropical and subtropical climates (WHO 2000; Duker 2006); however, outbreaks have occurred in a few isolated regions in temperate Australia (Hayman 1991; Veitch et al. 1997; Johnson et al. 2009). Infection rates are more severe in rural and remote areas of developing nations (WHO 2008), and the highest numbers of new cases come from the west African nations of Cote d'Ivoire, Ghana, and Benin, where BU is now the second most frequent mycobacterial disease in humans after tuberculosis (Debecker et al. 2004, Amofah et al. 2002, Sopoh et al. 2007). Cases of BU appear to be on the rise throughout endemic regions, however, true incidence is difficult to determine due to poor case confirmation and surveillance measures (WHO 2008).

The genus *Mycobacterium* comprises more than 50 species, most of

which are nonpathogenic environmental bacteria closely related to the soil bacteria *Streptomyces* and *Actinomyces* (Cosma et al. 2003). *M. ulcerans*, however, is a facultative environmental pathogen belonging to the *M. marinum* complex and is closely related to *M. tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy, respectively (Chemlal et al. 2002; Kaser et al. 2009; Stinear et al. 2000a; Stinear et al. 2004; Yip et al. 2007). The major virulence determinant of *M. ulcerans* is an immunosuppressant toxin called mycolactone, which is a polyketide-derived macrolide secreted by *M. ulcerans* causing cell necroses and tissue damage in infected individuals (George et al. 1999; Gunawardana et al. 1999; Demangel, et al, 2009). *Mycobacterium ulcerans* is characterized as a slow-growing mycobacteria, sensitive to UV light (Stinear et al. 2004), and has optimal growth under a narrow range of temperatures (WHO 2000; Yeboah-Manu et al. 2004; Boisvert and Schroder 1977; Garrity et al. 2001) and in oxygen deprived environments (Palomino et al. 1998). The combination of these characteristics suggests *M. ulcerans* has adapted to a specific niche and does not live freely in the environment (Stinear et al. 2000a, Stinear et al. 2004, Stinear et al. 2007). Improved PCR techniques have allowed for more accurate testing for *M. ulcerans* in environmental samples (Ross et al. 1997; Stinear et al. 1999; Stinear et al. 2000b,c, 2004, Johnson et al. 2005, Fyfe et al. 2007, Lavender et al. 2008; Williamson et al. 2008), and have contributed to the detection of *M. ulcerans* DNA from soil and mud, detritus, biofilms, filtered water, fish, frogs, snails, spiders, several insect groups and other invertebrates (Williamson et al. 2008, Benbow et al. 2008, Marsollier et al. 2002b,

2004a, b, Eddyani et al. 2004, Johnson et al. 2007, Portaels et al. 1999, 2001, 2008, Stinear et al. 2000b, Kotlowski et al. 2004, Fyfe et al. 2007, Trott et al. 2004). These findings have provided more insight into the distribution of *M. ulcerans* throughout aquatic environments; however, a thorough understanding of the ecology of *M. ulcerans* is lacking and remains understudied.

Buruli ulcer has been referred to as the “mystery disease”, in part, because researchers still do not know how the disease is spread or where the primary source of *M. ulcerans* is in the environment. Most epidemiological studies have associated cases of BU with proximity and prolonged exposure to freshwater environments (Lunn et al. 1965, Revill and Barker 1972, Barker and Carswell 1973, Duker et al. 2006, Marston et al. 1995, Walsh et al. 2008, Portaels 1995, Debacker et al. 2006, Noeske et al. 2004, Johnson et al. 2007, Wagner et al. 2008a, WHO 2000). It has further been suggested that people living in areas prone to flooding are at higher risk of infection (Barker and Carswell 1973, Wagner et al. 2008a; Radford 1974b, Barker 1972 Meyers et al. 1996, Portaels 1995, Hayman 1991). Anthropogenic disturbances to waterbodies and adjacent landscapes have also been linked to higher disease incidence. In particular, the damming of streams and rivers, modification of wetlands, deforestation practices, increased agriculture development, and sand mining operations are believed to promote proliferation of *M. ulcerans* in the environment and therefore increase risk of becoming infected (Hayman 1991b; Marston et al. 1995; Meyers et al. 1996; Johnson et al. 1999; Portaels et al. 2001, Wagner et al. 2008a, Merritt et al. 2005, Duker et al. 2006, Kibadi et al.

2008). Although nearly all the epidemiological studies on BU have associated disease outbreaks with communities in close proximity to disturbed aquatic environments, the source of infection and mode of transmission still remains a mystery (Merritt et al. 2010).

Two hypotheses explaining potential pathways for *M. ulcerans* infection have been proposed. The first hypothesis suggested *M. ulcerans* could be inhaled or ingested as an aerosol (Connor and Lunn, 1965; Hayman, 1991; Veitch et al. 1997; Johnson et al., 1999); however, this hypothesis has since been considered unlikely as a primary mode of transmission. The second hypothesis, which is more widely accepted, is mechanical transmission where *M. ulcerans* enters an individual through direct contact with the pathogen from contaminated soils, water, plant biofilms and aquatic insects (Barker 1971; Radford, 1974; Hayman, 1991; Johnson et al., 1999; Portaels, 1995; Portaels, 1999; Portaels, 2001; Merritt et al. 2005). Portaels et al. (1999) first hypothesized that predacious aquatic insects infected with *M. ulcerans* mechanically transmit the bacteria to humans through bites and offered a model describing the movement of *M. ulcerans* through trophic pathways. Merritt et al. (2005) elaborated on the role of aquatic invertebrates in maintaining *M. ulcerans* in aquatic food webs and expanded on this model with the addition of potential pathways for the dissemination of *M. ulcerans* between waterbodies (Fig. 1.2). The work by Portaels and colleagues prompted researchers to more closely investigate the role of aquatic insects, particularly aquatic hemipterans, as potential vectors and environmental reservoirs of *M. ulcerans.* Most of the

subsequent field research initiatives to investigate these relationships have been conducted in Africa and Australia, where BU cases are most prevalent. The following section presents a brief overview of published studies examining the associations between BU and aquatic invertebrates on these two continents.

Most of the research investigating associations between aquatic insects and BU in Africa has taken place in west Africa where disease incidence is greatest. Portaels et al. (1999) first suspected that aquatic insects might be reservoirs of *M. ulcerans* following detection of the pathogen in water bugs (Hemiptera: Gerridae) collected from wetlands in endemic areas of Benin. Their results led to the development of the first transmission model involving an aquatic insect and initiated a series of studies placing biting aquatic hemipterans, particularly Belsotomatidae and Naucoridae, as potential vectors and reservoirs of *M. ulcerans*. More recently, Portaels et al. (2008) cultured *M. ulcerans* from a water strider (*Gerris* sp.) and became the first to successfully culture *M. ulcerans* from the environment. Benbow et al. (2008) were the first to conduct a large-scale survey of aquatic invertebrates associated with waterbodies in both BU endemic and non-endemic regions. From their research in Ghana they concluded that aquatic insects were unlikely vectors, in part due to relatively low numbers of biting hemipterans (Belostomatidae, Naucoridae) compared to reported disease occurrence (Benbow et al. 2008). In addition, they found that *M. ulcerans* was more widespread in the environment than previously believed and reported several aquatic insect taxa that tested positive for *M. ulcerans* (Williamson et al. 2008). A number of studies have provided similar results and

identified *M. ulcerans* in association with aquatic insects, as well as snails, tadpoles, and fish (Kotlowski et al. 2004; Marrion et al. 2010; Morsolier et al. 2004a; Portaels et al. 2001, 2008).

A series of laboratory experiments by Marsollier and colleagues demonstrated that naucorid water bugs (*Naucoris cimicoides* sp.) could become infected by feeding on inoculated prey and then transmit *M. ulcerans* to uninfected mice (2002b). They also reported that *M. ulcerans* could survive and multiply within the salivary glands of *N. cimicoides* sp. (2002a, 2003, 2004a, 2005). Results from these studies reinforced the hypothesis of an insect vector and environmental reservoir, however, these results have been scrutinized because African naucorids were not used, the amount of innocula present in the naucorids was much higher than what would be found in nature, and the results only showed indirect transmission (Benbow et al. 2008). Mosi et al. (2008) investigated the trophic movement of *M. ulcerans* experimentally using belostomatids (*Appasus* sp.) collected from Ghana and found that water bugs can become infected after feeding on inoculated prey; however, they concluded that replication of *M. ulcerans* did not occur in the salivary complex and was most likely restricted to the exoskeleton. Wallace et al. (2010) found that *M. ulcerans* could become concentrated in filter-feeding mosquito larvae and then acquired by predaceous mosquito larvae up a food chain. However, the bacteria was found to not pass through all instars nor survive metamorphosis to the adult stage. Together, these results provide evidence that *M. ulcerans* can become concentrated and passed trophically up an aquatic food chain and support the

hypothesis of an aquatic invertebrate reservoir; however, the role of an aquatic insect as a vector involved in actual transmission of *M. ulcerans* requires further investigation (Merritt et al. 2010).

Outbreaks of BU in Australia have occurred in confined areas and most of the research efforts have been directed towards associating mosquitoes with disease incidence. Johnson et al. (2007) sampled salt marsh mosquitoes following an outbreak of BU and found *M. ulcerans* positive adult mosquitoes in pooled samples collected from the outbreak area. Further epidemiological work has supported the mosquito vector hypothesis (Fyfe et al. 2007; Lavender et al. 2008); however, conclusive evidence demonstrating transmission by adult mosquitoes is lacking. Tobias et al. (2009) conducted feeding experiments in an attempt to connect a potential environmental source of *M. ulcerans* with adult mosquitoes and found that mosquito larvae could consume and concentrate *M. ulcerans*, but they were unable to demonstrate that the bacteria can persist beyond the fourth instar. These results were consistent with findings by Wallace et al. (2010), which collectively are significant in that they showed that *M. ulcerans* can be maintained in aquatic food webs. It should be further noted that despite the literature from both Australia and Africa showing an association of aquatic insects in the transmission of this disease, major scientific criteria are lacking for implicating the roles of living agents as biologically significant reservoirs and/or vectors of pathogens (Merritt et al. 2010).

In order to better understand associations between aquatic macroinvertebrates and BU disease, more quantitative studies evaluating the

ecology of *M. ulcerans* are needed. The major objective of this research was to systematically assess and characterize the macroinvertebrate communities within aquatic habitats of disease endemic and non-endemic areas in Ghana, West Africa. The specific objectives of this study were divided into the following three chapters: 1) Associations between *M. ulcerans* and benthic macroinvertebrate assemblages in aquatic environments of Ghana, West Arica, 2) Seasonal differences in aquatic macroinvertebrate assemblages in relation to the presence of *M. ulcerans* in waterbodies of Ghana, West Africa, and 3) Gut content analysis of Naucoridae and trophic relationships of benthic macroinvertebrates in waterbodies of Ghana, West Africa.

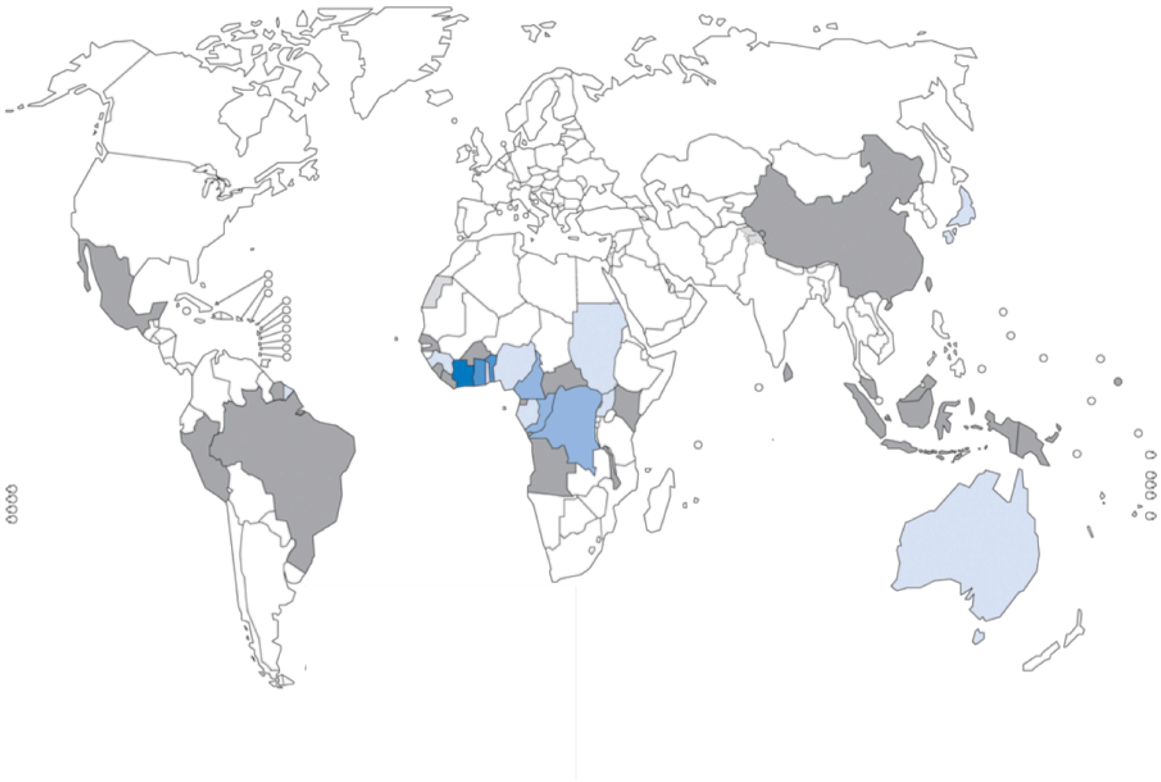


Figure 1.1. Global map showing countries where Buruli ulcer disease has been confirmed and the number of cases reported in 2009 for each country. Map provided by World Health Organization (2009) and modified to fit formatting requirements.

“For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis (or dissertation).”

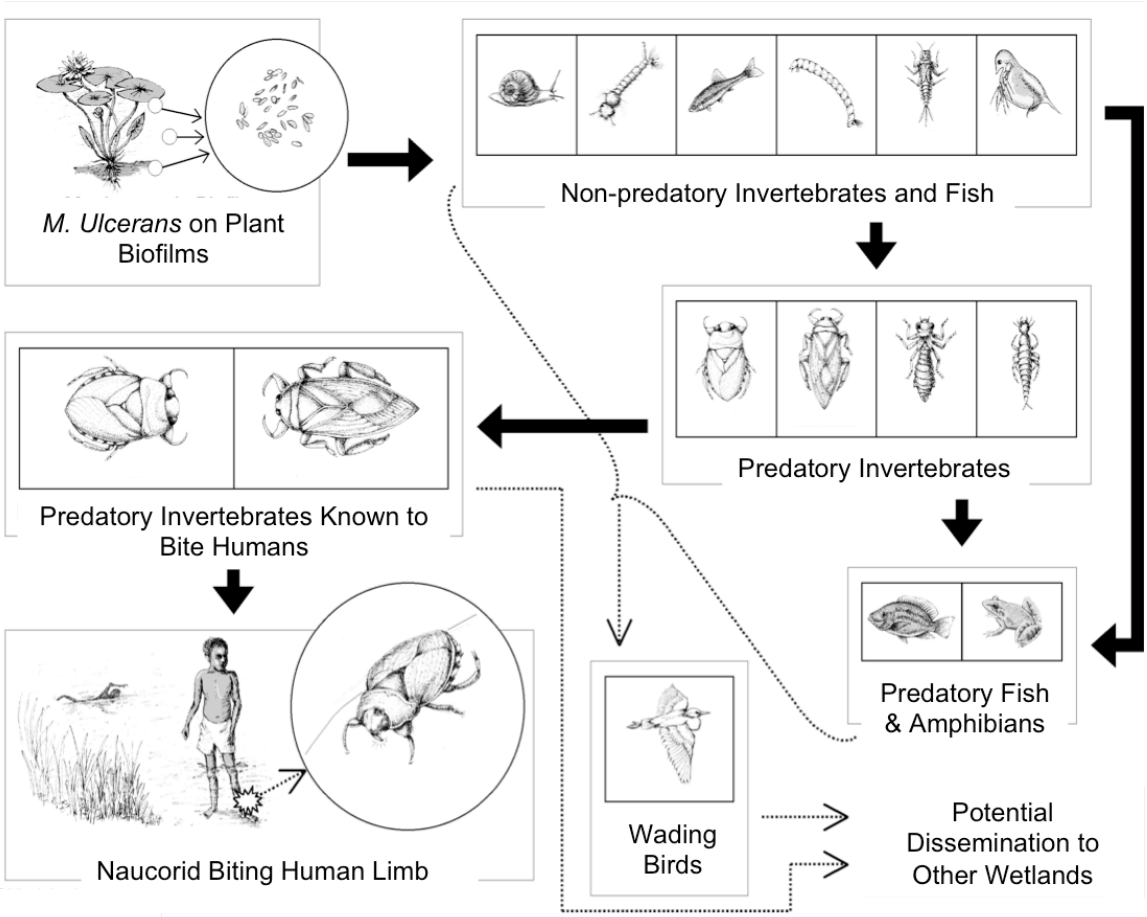


Figure 1.2. Conceptual model illustrating potential reservoirs and movement of *Mycobacterium ulcerans* within and among aquatic environments. Dark arrows indicate potential movement within a water body; dashed lines and arrows represent potential dissemination pathways to other water bodies. This diagram was published in Merritt et al. (2005) and modified to fit formatting requirements. All drawings made by RA MacKarrall.

LITERATURE CITED

Literature Cited

Aiga H, Amano T, Cairncross S, Domako JA, Nanas OK, et al. (2004) Assessing water-related risk factors for Buruli ulcer: A case-control study in Ghana. Am J Trop Med Hyg 71: 387–392.

Amofah GK, Bonsu F, Tetteh C, Okrah J, Asamoa K, et al. (2002) Buruli ulcer in Ghana: results of a national case search. Emerging Infectious Diseases 8: 167–170.

Asiedu K, Etuaful S (1998) Socioeconmoic implications of Buruli ulcer in Ghana: a three-year review. Trans R Soc Trop Med & Hyg 59: 1015–1022.
18. Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, et al. (2001) Mycobacterium ulcerans in wild animals. Rev sci tech Off int Epiz 20: 252–264.

Barker, DJP (1971) Buruli disease in a district of Uganda. J. Trop. Med. Hyg. 74: 260-264.

Barker DJP (1972) The distribution of Buruli disease in Uganda. Trans R Soc Trop Med Hyg. 66:867-74.

Barker DJP, Carswell JW (1973) Mycobacterium ulcerans infection among Tsetse control workers in Uganda. International Journal of Epidemiology 2: 161–165.

Benbow M, Williamson H, Kimbirauskus R, McIntosh M, Kolar R, et al. (2008) Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis 14: 1247–1254.

Boisvert H, Schroder KH (1977) Skin ulcer caused by Mycobacterium ulcerans in Cameroon II: Bacteriologic study. Bull Soc Pathol Exot Filiales 70: 125–131.

Chemlal, K, Huys, G, Laval, F, et al. (2002) Characterization of an unusual Mycobacterium: a possible missing link between Mycobacterium marinum and Mycobacterium ulcerans. Jornal of Clinical Microbiology 40:2370.

Connor DH, and Lunn F (1965) *Mycobacterium ulcerans infection. Int. J. Leprosy 33*:*698*.

Cosma CL, Sherman DR, and Ramakrishnan L (2003) The Secret Lives of the Pathogenic Mycobacteria. Annu. Rev. Microbiol. 57:641–76.

Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, et al. (2004) Mycobacterium ulcerans disease (Buruli ulcer) in rural hospital, Southern Benin, 1997-2001. Emerg Infect Dis 10: 1391–1398.

Demangel C, Stinear TP, Cole ST (2009) Buruli ulcer: reductive evolution enhances pathogenicity of Mycobacterium ulcerans. Nat Rev Microbiol 7: 50–60.

Duker AA, Carranza JM, Hale M (2004) Spatial dependency of Buruli ulcer prevalence on arsenic-enriched domains in Amansie West District, Ghana: implications for arsenic mediation in *Mycobacterium ulcerans* infection. Int J Health Geogr. 3:19.

Duker AA, Portaels F, Hale M (2006) Pathways of Mycobacterium ulcerans infection: A review. Environment International 32: 567–573.

Eddyani M, Ofori-Adjei D, Teugels G, De Weirdt D, Boakye D, et al. (2004) Potential role for fish in transmission of Mycobacterium ulcerans disease (Buruli Ulcer): an environmental study. Appl Environ Microbiol 70: 5679–5681.

Fyfe JAM, Lavender CJ, Johnson P, Globan M, Sievers A, et al. (2007) Development and Application of Two Multiplex Real-Time PCR Assays for the Detection of Mycobacterium ulcerans in Clinical and Environmental Samples. Appl Environ Microbiol 73: 4733–4740.

Garrity GM (2001) Bergey's Manual of Systematic Bacteriology; In: Garrity GM, ed. New York: Springer-Verlag.

George KM, Chatterjee D, Gunawardana G, Welty D, Lee T, et al. (1999) Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science 283: 854–857.

Gunawardana G, Chatterjee D, George KM, Brennan P, Whittern D, et al. (1999) Mycolactone A and B: toxins of Mycobacterium ulcerans. J Amer Chem Soc 121: 6092–6093.

Guerra H, Palomino JC, Falconi E, Bravo F, Donaires N, et al. (2008) Mycobacterium ulcerans Disease, Peru. Emerging Infectious Diseases 14: 373–377.

Hayman J. (1991) Postulated epidemiology of Mycobacterium ulcerans infection. International Journal of Epidemiology. 20(4): 1093-8.

Hayman J (1991) Mycobacterium ulcerans infection. The Lancet 337: 124.

Johnson PDR, Stinear TP, Hayman JA (1999) Mycobacterium ulcerans — a minireview. J Med Microbiol 48: 511–513.

Johnson PDR, Stinear TP, Small PLC, Pluschke G, Merritt RW, et al. (2005) Buruli ulcer (M. ulcerans Infection): new insights, new hope for disease

control.PLoS Med 2(4): e108.

Johnson PDR, Hayman JA, Quek TY, Fyfe JAM, Jenkin GA, et al. (2007) Consensus recommendations for the diagnosis, treatment and control of Mycobacterium ulcerans infection (Bairnsdale or Buruli ulcer) in Victoria, Australia. Medical Journal of Australia 186: 64–68.

Johnson PDR, Lavender CJ (2009) Correlation between Buruli ulcer and vector-borne notifiable diseases, Victoria, Australia. Emerging Infectious Diseases 15: 614–615.

Kaser, M., Hauser, J., Small, P., Pluschke, G. (2009). Large Sequence Polymorphisms Unveil the Phylogenetic Relationship of Environmental and Pathogenic Mycobacteria Related to Mycobacterium ulcerans. *Appl. Environ. Microbiol.* 75: 5667-5675.

Kibadi K, Panda M, Tamfum JM, Fraga AG, Filho AL, et al. (2008) New foci of Buruli ulcer, Angola and Democratic Republic of Congo. Emerging Infectious Diseases 14: 1790–1792.

Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne P, et al. (2004) Onetube cell lysis and DNA extraction procedure for PCR-based detection of Mycobacterium ulcerans in aquatic insects, molluscs and fish. Journal of Medical Microbiology 53: 927–933.

Lavender CJ, Stinear TP, Johnson PDR, Azuolas J, Benbow ME, et al. (2008) Evaluation of VNTR typing for the identification of Mycobacterium ulcerans in environmental samples from Victoria, Australia. FEMS Microbiology Letters 287: 250–255.

Lunn HF, Connor DH, Wilks NE, Barnley GR, Kamunvi F, et al. (1965) Buruli (Mycobacterial) ulceration in Uganda. East African Medical Journal 42: 275–288.

Marion E, Eyangoh S, Yeramian E, Doannio J, Landier J Aubry J et al. (2010) Seasonal and regional dynamics of M. ulcerans transmission in environmental context: deciphering the role of water bugs as hosts and vectors. PLoS Negl Trop Dis. 6:4(7):e731.

Marsollier L, Robert R, Aubry J, Andre JS, Kouakou H, et al. (2002) Aquatic insects as a vector for Mycobacterium ulcerans. Applied and Environmental Microbiology 68: 4623–4628.

Marsollier L, Legras P, Manceau AL, Saint-Andre´ JP, Aubry J, et al. (2002) Role des punaises d'eau dans la transmission de M. ulcerans. BULL ALLF or Bulletin de l'ALLF 10: 23–25.

Marsollier L, Aubry J, Saint-Andre JP, Robert R, Legras P, et al. (2003)

Ecology and transmission of Mycobacterium ulcerans. Pathologie Biologie 51: 490–495.

Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, et al. (2004) Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl Environ Microbiol 70: 6296–6298.

Marsollier L, Stinear TP, Aubry J, Saint-Andre J-P, Robert R, et al. (2004) Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Applied and Environmental Microbiology 70: 1097–1103.

Marsollier L, Aubry J, Coutanceau E, Andre JPS, Small PL, et al. (2005) Colonization of the salivary glands of Naucoris cimicoides by Mycobacterium ulcerans requires host plasmatocytes and a macrolide toxin, mycolactone. Cellular Microbiology 7: 935–943.

Marsollier L, Andre J, Frigui W, Reysset G, Milon G, et al. (2006) Early trafficking events of Mycobacterium ulcerans within Naucoris cimicoides. Cellular Microbiology.

Marsollier L, Brodin P, Jackson M, Kordulakova J, Tafelmeyer P, et al. (2007) Impact of Mycobacterium ulcerans biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. PLoS Pathogens 3: e62.

Marston BJ, Diallo MO, Horsburgh CR Jr., Diomande I, Saki MZ, et al. (1995) Emergence of Buruli ulcer disease in the Daloa region of Cote D'ivoire. Am J Trop Med Hyg 52: 219–224.

Merritt RW, Benbow ME, Small PLC (2005) Unraveling an Emerging Disease Associated with Disturbed Aquatic Environments: The Case of Buruli Ulcer. Frontiers in Ecology and the Environment 3: 323–331.

Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review. PLoS Negl Trop Dis 4(12): 1-15.

Meyers WM, Tignokpa WM, Priuli GB, Portaels F (1996) Mycobacterium ulcerans infection (Buruli ulcer): first reported patients in Togo. British Journal of Dermatology 134: 1116–1121.

Mosi L, Williamson H, Wallace JR, Merritt RW, Small PLC (2008) Persistent association of Mycobacterium ulcerans with West African predaceous insects of the family Belostomatidae. Applied and Environmental Microbiology 74: 7036–7042.

Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, et al. (2004) Buruli ulcer disease in Cameroon rediscovered. Am J Trop Med Hyg 70: 520–526.

Palomino JC, Obiang AM, Realini L, Meyers WM, and Portaels F (1998) Effect of Oxygen on Growth of *Mycobacterium ulcerans* in the BACTEC System. Journal of Clinical Microbiology. Vol. 36, No. 11, p. 3420-3422,

Phanzu DM, Bafende EA, Dunda BK, Imposo DB, Kibadi AK, et al. (2006) Mycobacterium ulcerans disease (Buruli Ulcer) in a rural hospital in Bas-Congo, Democratic Republic of Congo, 2002-2004. American J Tropical Medicine and Hygiene 75: 311–314.

Portaels, F. (1995) Epidemiology of mycobacterial diseases. Clin. Dermatol. 13:207-222.

Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P, Meyers WM (1999) Insects in the transmission of Mycobacterium ulcerans infection. The Lancet 353: 986.

Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, et al. (2001) Mycobacterium ulcerans in wild animals. Rev sci tech Off int Epiz 20: 252–264.

Portaels F, Manuel S, Meyers W (2009) Buruli ulcer. Clinics in dermatology 27: 291–305.

Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, et al. (2008) First Cultivation and Characterization of Mycobacterium ulcerans from the Environment. PLoS Neglected Tropical Diseases 2: e178.

Radford AJ (1974) Mycobacterium ulcerans: a review, I: Epidemiology. Papua New Guinea Medical Journal 17: 129–133.

Revill WDL, Barker DJP (1972) Seasonal distribution of mycobacterial skin ulcers. Brit J prev soc Med 26: 23–27.

Ross BC, Marino L, Oppedisano F, Edwards R, Robins-Browne RM, et al. (1997) Development of a PCR assay for rapid diagnosis of Mycobacterium ulcerans infection. Journal of Clinical Microbiology 35: 1696–1700.

Silva MT, Portaels F, Pedrosa J (2007) Aquatic Insects and Mycobacterium ulcerans: an association relevant to Buruli ulcer control? PLoS Medicine 4: e63.

Sopoh GE, Johnson RC, Chauty A, Dossou AD, Aguiar J, et al. (2007) Buruli ulcer surveillance, Benin, 2003-2005. Emerg Infect Dis 13: 1374–1376.

Stinear T, Ross, BC, Davies JK, Marino L, Robins-Browne RM, Oppedisano F,

Grant A, Jenkin GA, Potaels, F, Ross, BC, Oppedisano F, Sievers A, Johnson PDR (1999) Identification and Characterization of IS*2404* and IS*2606*: Two Distinct Repeated Sequences for Detection of *Mycobacterium ulcerans* by PCR. American Society for Microbiology vol. 37, No. 4.

Stinear T, Jenkin GA, Johnson PDR, Davies JK (2000) Comparative Genetic Analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* Reveals Evidence of Recent Divergence. Journal of Clinical Microbiology vol. 182, No. 22. pp-6322-6330.

Stinear T, Davies JK, Jenkin GA, Hayman JA, Oppedisano F, et al. (2000) Identification of Mycobacterium ulcerans in the environment from regions in Southeast Australia in which it is endemic with sequence Capture-PCR. Appl Environ Microbiol 66: 3206–3213.

Stinear T, Davies JK, Grant A, Jenkin GA, Potaels, F, Ross, BC, Oppedisano F, Purcell M, Hayman JA, Johnson PDR (2000) A Simple PCR Method for Rapid Genotype Analysis of *Mycobacterium ulcerans.* Journal of Clinical Microbiology vol. 38, No. 4. pp-1482-1487.

Stinear TP, Mve Obiang A, Small PL, Frigui W, Pryor MJ, et al. (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proc Natl Acad Sci U S A 101: 1345–1349.

Stinear T, Johnson PDR (2007) From marinum to ulcerans: a mycobacterial human pathogen emerges. Microbe 2: 187–194.

Tobias N, Seemann T, Pidot S, Porter J, Marsollier L, et al. (2009) Mycolactone gene expression is controlled by strong SigA-like promoters with utility in studies of Mycobacterium ulcerans and buruli ulcer. PLoS Negl Trop Dis 3: e553.

Trott KA, Stacy BA, Lifland BD, Diggs HE, Harland RM, et al. (2004) Characterization of a Mycobacterium ulcerans-like infection in a colony of African tropical clawed frogs (Xenopus tropicalis). Comp Med 54: 309–317.

van der Werf TS, Stienstra Y, Johnson C, Phillips R, Adjei O, et al. (2005) Mycobacterium ulcerans disease. Bulletin of the World Health Organization 83: 785–791.

Veitch MGK, Johnson PDR, Flood PE, Leslie D, Street AC, et al. (1997) A large localized outbreak of Mycobacterium ulcerans infection on a temperate southern Australian island. Epidemiol Infect 119: 313–318.

Wagner T, Benbow ME, Burns M, Johnson RC, Merritt R, et al. (2008) A Landscape-based Model for Predicting Mycobacterium ulcerans Infection (Buruli

Ulcer Disease) Presence in Benin, West Africa. EcoHealth 5: 69–79.

Wagner T, Benbow ME, Brenden T, Qi J, Johnson RC (2008) Buruli ulcer disease prevalence in Benin, West Africa: associations with land use/cover and the identification of disease clusters. International Journal of Health Geographics 7: 25.

Wallace J, Gordon M, Hartsell L, Mosi L, Benbow M, et al. (2010) Interaction of Mycobacterium ulcerans with mosquito species: Implications for transmission and trophic relationships. Applied Environ Microbiol 76: 6215–6222.

Walsh D, Portaels F, Meyers W (2008) Buruli ulcer (Mycobacterium ulcerans infection). Transactions of the Royal Society of Tropical Medicine and Hygiene 102: 969–978.

Walsh D, Eyase F, Onyango D, Odindo A, Waitumbi JN et al. (2010) Short Report: Clinical and Molecular Evidence for a Case of Buruli Ulcer (Mycobacterium ulcerans Infection) in Kenya. Am. J. Trop. Med. Hyg., 81(6), pp. 1110–1113

Wansbrough-Jones M, Phillips R (2006) Buruli ulcer: emerging from obscurity. Lancet 367: 1849–1858.

Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, et al. (2008) Distribution of Mycobacterium ulcerans in Buruli Ulcer Endemic and Non-Endemic Aquatic Sites in Ghana. PLoS Neglected Tropical Diseases 2: e205.

WHO, ed (2000) Buruli ulcer. Mycobacterium ulcerans infection. Geneva, Switzerland: WHO. 118 p.

WHO (2008) Buruli ulcer: progress report, 2004–2008. In: WHO, ed. Weekly epidemiological record. Geneva, Switzerland: World Health Organization 83: 145–156.

Yeboah-Manu D, Bodmer T, Mensah-Quainoo E, Owusu S, Ofori-Adjei D, et al. (2004) Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. J Clin Microbiol 42: 5875–5876.

Yip MJ, Porter JL, Fyfe JA, Lavender CJ, Portaels F, et al. (2007) Evolution of Mycobacterium ulcerans and other mycolactone-producing mycobacteria from a common Mycobacterium marinum progenitor. J Bacteriol 189: 2021–9.

CHAPTER 2

ASSOCIATIONS BETWEEN MYCOBACTERIUM ULCERANS AND MACROINVERTEBRATE ASSEMBLAGES IN AQUATIC ENVIRONMENTS OF GHANA, WEST AFRICA

INTRODUCTION

Buruli ulcer (BU) is an emerging skin disease caused by an infection of *Mycobacterium ulcerans* (Walsh et al. 2008, Duker et al. 2006, Wansbrough-Jones and Philips 2006, van der Werf et al. 2005). This disease is generally considered non-fatal; however, infections often result in cell necroses, which may lead to severe ulcerations, disfigurement, and disability in humans (Asiedu and Etuaful 1998, Amofah et al. 2002, Johnson et al. 2005, van der Werf et al. 1999, 2005, Wansbrough-Jones and Philips 2006). Johnson et al. (1996) reported incidence of BU in isolated temperate regions of Australia, but BU is most prevalent in tropical and subtropical climates, with the highest number of new cases being reported from sub-Saharan West Africa (WHO 2008)(Fig. 2.1). It is widely accepted that BU incidence is associated with exposure and proximity to freshwater habitats (Barker & Carswell 1973, Radford 1975, Hayman and McQueen 1985, Hayman 1991, WHO 2003, Aiga et al. 2004, Porteals et al. 1999, Merritt et al. 2005, Debacker et al. 2006; Thangaraj et al. 1999), yet many questions regarding the ecology of *M. ulcerans* remain unanswered, including the pathogen's natural reservoir(s), environmental distribution, and method of transmission to humans (Merritt et al. 2005, 2010).

Epidemiological studies have linked cases of BU with aquatic environments that are both lentic (i.e. ponds, lakes) and lotic (i.e. streams,

rivers)(Merritt et al. 2010). Field studies have also associated BU endemicity with disturbance to waterbodies through modification of freshwater habitats and their adjacent landscapes (Hayman 1991b; Marston et al. 1995; Meyers et al. 1996; Johnson et al. 1999; Portaels et al. 2001, Wagner et al. 2008a, Merritt et al. 2005, Duker et al. 2006, Kibadi et al. 2008). Merritt et al. (2010) provided a thorough review of published work on the transmission and ecology of BU disease and reported that anthropogenic influences; such as, mining activity, damming of waterbodies, deforestation practices, and agricultural development were among the most commonly cited factors attributed to environmental disturbance and BU disease incidence. Natural disturbances, such as flooding events, also have been proposed to be factors that could lead to potential increased risk of MU infection (Barker and Carswell 1973, Wagner et al. 2008a; Radford 1974b, Barker 1972 Meyers et al. 1996, Portaels 1995, Hayman 1991). Merritt et al. (2005) proposed a model describing how anthropogenic and natural disturbances may lead to the proliferation of MU in aquatic environments; however, this model has not yet been field-tested and the source of *M. ulcerans* in the environment remains unknown.

Aquatic macroinvertebrates have received the most attention as potential reservoirs and biological vectors of *M. ulcerans*. The isolation and successful culturing of *M. ulcerans* from a water strider (Gerris sp.) in Benin, West Africa (Portaels et al. 2008) and laboratory experiments demonstrating water bugs (Naucoris sp.) can transmit *M. ulcerans* to a mammal model (Marsollier et al. 2004) have supported the role of aquatic insects as reservoirs and vectors. While

these findings demonstrate a direct association of macroinvertebrates with *M. ulcerans* and BU disease, aquatic invertebrates may also provide valuable information into the ecology of this disease through indirect associations with *M. ulcerans* in the environment. Aquatic macroinvertebrates are often used as biological indicators of water quality and analysis of macroinvertebrate communities can identify short and long term disturbances to aquatic environments (Merritt and Cummins 1996). If *M. ulcerans* proliferation is associated with disturbed aquatic environments, then aquatic insects could possibly be useful indicators of environmental conditions suitable for the establishment of *M. ulcerans* and an increased risk of BU infection.

The role of aquatic macroinvertebrates in the transmission of BU has been proposed by several authors (Johnson et al. 2005, Portaels et al., 1999, Marsollier 2004, Merritt et al. 2005, Wansbrough-Jones and Phillips 2006); however, field based ecological studies to specifically address the association between macroinvertebrate communities and *M. ulcerans* are limited (Benbow et al. 2007). An initial step in understanding the potential role of macroinvertebrates in the ecology of BU, whether direct or indirect, is identifying the relative abundances and composition of the aquatic macroinvertebrate communities in relation to the disease pathogen. As part of a large-scale systematic study, I surveyed 98 water bodies from BU endemic and non-endemic regions, in Ghana, West Africa to: 1) characterize and compare overall macroinvertebrate communities from aquatic environments in Ghana: 2) identify macroinvertebrate community metrics associated with the presence and absence of *M. ulcerans* in

aquatic environments: and 3) identify potential relationships between specific macroinvertebrates and pathogen presence.

MATERIALS AND METHODS

Study Location and Scale. A large-scale, standardized assessment of aquatic habitats was conducted to characterize benthic macroinvertebrate communities in Ghana, West Africa. In this study water bodies (n=98) were selected from individual villages located within three distinct regions of southern Ghana: the Greater Accra (n=29) and Ashanti regions (n=39), which are endemic for the disease, and the Volta region (n = 30), which is non-endemic. Villages were randomly selected within each region and water bodies were selected within each village, based on location (<100-200m from community housing structures) and human use (daily domestic activities) to reflect aquatic environments with potential human exposure to Buruli ulcer. Community discussions on water body selection were conducted in each village as described by Benbow et al. (2005). Various types of water bodies were selected from all regions, including streams, rivers, wetlands, ponds, fetches and reservoirs. Water bodies and thus aquatic macroinvertebrate communities, were surveyed on a single sampling date in 2005 (6 July to 15 August), 2006 (7 July to 15 August), or 2007 (15 August to 7 September).

Macroinvertebrate sample collections. All samples were collected from the littoral margins of water bodies. Within each water body, two 10–20-m transects were measured parallel to the shoreline and positioned through the dominant macrophyte community. Along each transect, two floating 1-m2

polyvinyl chloride (PVC) quadrats were randomly placed and invertebrates were collected by sweeping within the quadrat with a 500-µm mesh dip net. The quadrats floated on top of the water and delineated 1 m2 of area to be sampled. Three sweeps of the dip net were performed from the water surface to the bottom substrate for comprehensive sampling of specimens in the water column, and all samples were collected using the same technique. Contents within each net were washed through a 500-µm sieve, preserved in 99% ethanol, and transported to the laboratory for identification. All specimens were enumerated and identified to lowest possible taxon under dissecting microscope using African regional keys (Durand and Leveque 1981; Invertebrates of South Africa - Identification Keys, vols. 2-10, 1999-2007), and keys from elsewhere (Merritt et al. 2008).

Detection of *Mycobacterium ulcerans*. To identify potential relationships between macroinvertebrate communities and *M. ulcerans*, biofilms and water samples were collected at each waterbody. Biofilms were collected from the surfaces of dominant macrophytes and detritus (n=3), and a composite water sample was collected from open water areas within the water body at the mid-water column depth. From the composite, five 100-200ml sub-samples were filtered through a 1.6 micron fiberglass filter followed by a 0.2 micron nitrocellulose filter (Whatman Inc). Filters were sealed inside aluminum foil packets for later laboratory analysis. In addition, a 500 µl of sample liquid was used for DNA extraction. Extracted DNA was also collected from *M. ulcerans* agy99, *Mycobacterium marinum* 1218, or water for use as positive and negative

controls. All samples were processed at the University of Tennessee, Knoxville, Tennessee and follow methods as previously described by Williamson et al. (2008).

Primers, PCR conditions and sequencing. A tiered PCR detection method was used for the identification of *M. ulcerans* in which DNA was subjected to amplification of the enoyl reductase (ER) domain and variable tandem repeat (VNTR) sequences. The enoyl reductase domain is partially responsible for production of the toxin mycolactone, and was used as a presumptive identification for mycolactone producing mycobacteria (MPMs) including *M. ulcerans* (George et al. 1999; Sizaire et al. 2002). ER-PCR positive samples were then subjected to VNTR analysis to identify *M. ulcerans* (MU) from other MPMs, and to match sample profiles to known VNTR profiles obtained from patients (Ablordey et al. 2005; George et al. 1999; Johnson et al. 2007; Portaels et al. 2001; Sizaire et al. 2002). Primers and PCR conditions for amplification of the enoyl reductase domain as well as VNTR loci, including BNTR MIRU 1, locus 6, ST 1 and locus 19, were used as described by Williamson et al. (2008).

Site Classification. Sites were classified into pre-defined groups based on the overall presence or absence of *M. ulcerans*. Due to the complexity of *M. ulcerans* detection in environmental samples, the presumptive presence of *M. ulcerans* was detected using PCR for the ER domain and thus represents the presence of *M. ulcerans* and any additional mycolactone producing microorganisms (MPMs). Although this provides an overestimate of *M. ulcerans*, it provides a maximum estimate for toxin-producing mycobacteria. Thus, sites

were classified into the pre-defined groups ER+ or ER- to identify potential relationships between macroinvertebrate communities and *M. ulcerans.*

Data Analyses. Multivariate tests were performed to characterize overall macroinvertebrate community structure among sites. For these procedures, a total of 98 sites were analyzed and all macroinvertebrate data (relative abundance as a proportion) were transformed using the arcsin square-root calculation (Mielke 1991). Rare taxa were determined as those taxa occurring in fewer than 5% of all sites, and were eliminated from analyses to improve the detection of potential relationships (McCune and Grace 2002). Nonmetric multi-dimensional scaling (NMDS) and multi-response permutation procedure (MRPP) were used to analyze differences in the overall macroinvertebrate community structure among pre-defined groups (ER+/-). Since multiple MRPP tests were completed, it was necessary to calculate a Bonferroni adjusted α (and corresponding p value) of 0.008 to assist in interpreting statistically significant differences. Indicator species analyses (ISA) were performed to identify specific macroinvertebrate taxa that best characterize or represent our predefined groups. Monte Carlo randomization tests were used to assess indicator significance (McCune and Grace 2002). Multivariate analyses were repeated for subsets of data based on water body flow, with 50 lotic sites (e.g., rivers, streams) and 48 lentic sites (e.g., ponds, wetlands, reservoirs). Bonferroni adjusted α (and corresponding p value) of 0.025 were used to analyze subset data. All multivariate statistical analyses were conducted in PC-ORD (Version 5).

Paired sample t-tests were used to compare macroinvertebrate

community metrics between the pre-defined groups (ER+/-). For all sites community diversity and similarity indices (Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness, Pielou's Eveness, % Dominant taxa, % Diptera taxa, and Total taxa), percent functional-feeding group abundances (Filter-Collector, Gather-Collector, Engulfing-Predator, Piercing-Predator, Scraper-Grazer, Shredder, and ratio of Scraper to Filter-Collector) (Merritt and Cummins 2006), and certain relative taxa abundances (Belostomatidae, Naucoridae, Nepidae, and Culicidae) were calculated and compared between ER+ and ER- water bodies. For lotic sites, EPT taxa richness (Ephemeroptera-Plecoptera-Trichoptera taxa) and the ratio EPT taxa to total organisms were calculated (Rosenberg and Resh 1993). For lentic sites, EOT taxa richness (Ephemeroptera-Odonata-Trichoptera taxa), ESTD taxa richness (Ephemeroptera-Sphaeriidae-Trichoptera-Odonata taxa), and percent Corixidae abundances were calculated and compared between ER+ and ER- water bodies (Radar 2001, USEPA 2001, Helgen and Gernes 2002). To meet the assumptions of normality and equal variances data were log + 1 transformed. For percentage composition differences, data were arc-sine square root transformed. The nonparametric Wilcoxon/Kruskal-Wallis rank sum test was used when appropriate. The t-tests were analyzed using SAS software (8.2 2001).

RESULTS

Aquatic macroinvertebrates were analyzed from 98 sites in southern Ghana to assess overall community differences based on the presence of environmental *M. ulcerans.* A total of 73,892 invertebrates from 77 unique taxa

were identified in this study (Table 2.1). A three-axis NMDS solution explained 70% of the total variation in the macroinvertebrate community (stress: 16.9, p=0.004), with 1% on axis 1, 42% on axis 2, and 27% on axis 3. No significant differences in the overall macroinvertebrate community structure between sites that were ER+ and ER- (MRPP: A=0.001, p = 0.23) were observed. The NMDS ordination did identify differences in macroinvertebrate community structure based on water body flow (MRPP: A=0.046, p < 0.000; Fig. 2.2), indicating that sites with flowing water were characterized by a different macroinvertebrate community compared to sites with standing water. To eliminate variation in the macroinvertebrate community due to flow, we classified all sites into two separate flow groups (lotic and lentic) and repeated all multivariate and univariate analyses; this allowed for comparisons of macroinvertebrate and bacterial communities controlling for variation due to flow regime.

Benthic macroinvertebrates were surveyed from 48 lentic water bodies in southern Ghana to assess differences in macroinvertebrate community structure between sites based on the presence or absence of environmental *M. ulcerans*. Among the 48 lentic sites, 34 were identified as ER+ and 14 ER-. A total of 42,498 invertebrates from 69 unique taxa were identified and used for final analysis of lentic water bodies. A three-axis NMDS solution explained 71% of the total variation in the macroinvertebrate community (stress: 17.7, p=0.004), with 21% on axis 1, 30% on axis 2, and 20% on axis 3. No significant differences were detected in the overall macroinvertebrate community between sites that were ER+ and ER- (MRPP: A=0.000, p = 0.358), and there were also no

significant differences in macroinvertebrate community metrics between lentic sites that were ER+ and ER- (a=0.05).

Aquatic macroinvertebrates were surveyed from 50 lotic water bodies in southern Ghana to assess differences in macroinvertebrate community structure between sites based on the presence or absence of environmental *M. ulcerans*. Among the 50 lotic sites, 38 were identified as ER+ and 12 ER-. A total of 31,394 invertebrates from 76 unique taxa were identified and used for final analysis of lotic water bodies. A three-axis NMDS solution explained 80% of the total variation in the macroinvertebrate community (stress: 15.4, p=0.004), with 38% on axis 1, 14% on axis 2, and 28% on axis 3. We found significant differences between lotic sites that were ER+ and ER- (MRPP: A=0.01, p = 0.02; Fig. 2.3), and 7 macroinvertebrate taxa were identified as significant indicators of ER+ or ER- lotic waterbodies. Indicators of ER+ were Pleidae (ISA: $p < 0.010$), Gerridae (ISA: $p<0.019$) (Hemiptera), Hydroacari (ISA: $p < 0.021$) (Acarina), and Libellulidae (ISA: $p < 0.042$) (Odonata). Indicators of ER- were Elmidae (ISA: $p < 0.026$) (Coleoptera). Simuliidae (ISA: $p < 0.035$) (Diptera), and Calopterygidae (ISA: $p < 0.044$) (Odonata).

There were also significant differences in commonly used macroinvertebrate community metrics between lotic sites that were ER+ and ER- (A=0.05). Total taxa counts were higher in ER+ water bodies (29.14, std 6.5) than in ER- water bodies (24.0, std 6.1)(p0.02), as was mean taxa richness (Margalef's) (4.49, std 0.81)(3.91, std 0.71)(p= 0.0323). Percent dominance of the top three taxa was lower in ER+ sites (60.2, std 10.1) than in ER- sites (68.8,

std 10.1)(p0.0137). The functional-feeding group consisting of piercing-predators had a higher mean percent in ER+ sites (0.066, std 0.061) compared to ER- sites (0.024, std 0.0237, p0.038), and the ratio of scrapers to collector-filterers was lower in ER+ sites (6.13, std 16.22) compared to ER- sites (6.32, std 8.10)(p= 0.041).

DISCUSSION

The role of aquatic macroinvertebrates in the transmission of BU has been proposed by several authors (Johnson et al. 2005, Portaels et al., 1999, Marsollier 2004, Merritt et al. 2005, Wansbrough-Jones and Phillips 2006); however, field based ecological studies to specifically address the association between macroinvertebrate communities and *M. ulcerans* are limited (Benbow et al. 2007). An initial step in understanding the potential role of macroinvertebrates in the ecology of BU is identifying the relative abundances and composition of the aquatic macroinvertebrate communities in relation to the disease pathogen. As part of a large-scale systematic study, 98 water bodies were surveyed from BU endemic and non-endemic regions, in Ghana, West Africa to: 1) characterize and compare overall macroinvertebrate communities from aquatic environments in Ghana; 2) identify macroinvertebrate community metrics associated with the presence and absence of *M. ulcerans* in aquatic environments; and 3) identify potential relationships between specific macroinvertebrates and pathogen presence. When water bodies were separated by flow regime, I found differences in macroinvertebrate community structure and function in relation to

the presence of *M. ulcerans*, and also identified specific taxa that may potentially be used as biological indicators of *M. ulcerans* in aquatic environments.

Several studies have been conducted on aquatic invertebrates in West Africa; however, most of these have focused on specific taxa of medical importance (i.e. *Anopheles sp.*, *Simulium sp.*, *Bulinus sp.*) and rarely have provided data on entire macroinvertebrate communities (Resh et al. 2004, Hynes 1975a,b, Thorne et al. 1997, Thorne et al. 2000). Hynes et al. (1975a,b) conducted studies in rivers of Ghana and sampled the riffle habitat community to examine annual life-cycles and drift behavior of benthic macroinvertebrates. Thorne et al. (1997, 2000) anchored artificial substrates to streambeds in southern Ghana to sample benthic communities in an examination of the responses of macroinvertebrates to gradients of pollution. They found similar community responses to pollution observed in studies from temperate areas, and concluded that established macroinvertebrate community metrics can be used to characterize sites of differing water qualities in the tropics. In 1974, an independent ecological oversight committee initiated a long-term monitoring program in West Africa to evaluate the effects of insecticides used to control black flies and Onchocerciasis. In these studies, riverine benthic communities that occupy the same habitats as black flies were sampled to evaluate changes in aquatic fauna studies in relation to insecticide treatments. Resh et al. (2004) summarized results from this 29 year study and concluded that permanent damage to non-target invertebrate communities due to insecticides was unlikely, but also reported that clear associations with macroinvertebrate communities and

treatment effect were difficult to analyze due to seasonal variations and lack of pre-treatment community data. Direct comparisons of my work in Ghana to these studies, and others in West Africa, are difficult due to differences in specific research objectives and sampling strategies. Therefore, information used in the interpretation of my results was largely drawn from research on macroinvertebrate communities conducted elsewhere.

Rapid bioassessment techniques that use macroinvertebrates to assess water quality incorporate the use of metrics to assess environmental degradation by measuring changes in the macroinvertebrate community and comparing them to a predicted response in relation to increased disturbance (Metcalfe-Smith 1994, Resh and Jackson 1993, USEPA 1996, Barbour et al. 1995, 1999). My analysis of lotic water bodies revealed that macroinvertebrate total taxa and taxa richness (Margalef's) were significantly greater when *M. ulcerans* was detected. I also found that percent taxa dominance was higher in waterbodies with *M. ulcerans*. The predicated responses of these measurements are that total taxa and taxa richness decrease with increased environmental perturbation, and percent dominance increases with increased environmental perturbation (Plafkin et al 1989). Proliferation of *M. ulcerans* in the aquatic environment is believed to be associated with natural and anthropogenic disturbances (Hayman 1991b; Marston et al. 1995; Meyers et al. 1996; Johnson et al. 1999; Portaels et al. 2001, Wagner et al. 2008a, Merritt et al. 2005, Duker et al. 2006, Kibadi et al. 2008). My data indicated that the macroinvertebrate communities of waterbodies without *M. ulcerans* were more characteristic of disturbed habitats. While these

community metrics allow for general statements to be made regarding water quality and overall community health within and between waterbodies, direct associations between my results and the ecology of *M. ulcerans* should be made with caution.

Functional-feeding groups were investigated to better understand potential ecological associations between macroinvertebrate communities and *M. ulcerans*. Where other metrics rely strictly on taxonomic groupings, functional-feeding group classifications are based on morpho-behavioral mechanisms of food acquisition and provide insight into the balance between food resource availability and the predictable response of aquatic insect assemblages (Cummins and Klug 1979, Merritt and Cummins 1984, Merritt and Cummins 2006). My analysis revealed that the ratio of scraper-grazers (i.e. snails) to collector-filterers (i.e. mosquitoes, black flies) was greater in waterbodies when *M. ulcerans* was detected. A shift in the dominance of the scraper-grazer community can be an indication of increased periphyton (i.e. attached algae, diatoms)(Merritt and Cummins 2006), and periphyton assemblages have been linked to the presence and absence of *M. ulcerans* in waterbodies of Ghana (Miller et al. unpublished data). Periphyton enrichment in aquatic habitats has also been associated with eutrophication (Davis 1994, McCormick and Stevenson 1998, Gaiser et al. 2005), which has been proposed by several authors to play a significant role in the establishment of *M. ulcerans* in the environment (Hayman 1991b; Marston et al. 1995; Meyers et al. 1996; Palomino et al. 1998, Johnson et al. 1999; Portaels et al. 2001, Merritt et al. 2005, Duker et

al. 2006, Kibadi et al. 2008). The observed increase of the scraper-grazer group in relation to *M. ulcerans* in my study supports an association of the pathogen with nutrient enrichment and eutrophication of aquatic habitats, and suggests that macroinvertebrate feeding-group analyses may be a viable way to identify environmental conditions favorable for the establishment of *M. ulcerans*.

The feeding group comprised of piercing-predators, which includes families of biting hemipterans implicated as vectors of BU (i.e. Belostomatidae, Naucoridae), also was greater in waterbodies when *M. ulcerans* was detected. Upon further examination of this community, it was an abundance of the pygmy backswimmer (Hemiptera: Pleidae) that was responsible for the significant difference among these waterbodies. Pleidae are predators of micro-crustaceans (i.e. Cladocera, Copepoda, Ostracoda) and to date have not been formerly associated with the ecology of *M. ulcerans* or BU disease. Williamson et al. (2008) processed more than 100 pleid specimens collected from field samples and found no direct associations with *M. ulcerans* and this group, but they did identify *M. ulcerans* associated with micro-crustaceans. Considering the feeding behavior of Pleidae and the growing body of work indicating that *M. ulcerans* is transferred trophically (Eddyani et al. 2004, Marsollier et al. 2004a,b, Duker et al. 2006, Mosi et al. 2008, Wallace et al. 2010), a closer look into the potential role of this group as an environmental reservoir of *M. ulcerans* may be warranted. It should be noted that although the piercing-predator community was greater in waterbodies with *M. ulcerans*, an investigation of Belostomatidae (giant water bugs) and Naucoridae (creeping water bugs) yielded no identifiable differences

between waterbodies. In addition, the overall relative abundances of these two families were low among all waterbodies sampled in this survey. These data were consistent with reports by Benbow et al. (2008), whom suggested a possible role as reservoirs of *M. ulcerans* for biting Hemiptera, but that caution should be taken when describing the role of biting hemipterans in BU transmission.

An indicator species analysis was used to detect relationships between specific macroinvertebrates and *M. ulcerans.* As a result, I found 4 taxa that were identified as indicators of the pathogen in the environment. These taxa, all of which are predators, included: Hemiptera (Pleidae, Gerridae), Odonata (Libellulidae), and Hydroacrines (water mites). Overall, these taxa are considered to be tolerant of moderate levels of pollution and physical disturbance and, as a result, not strong indicators of a particular environmental condition (Bode *et al*. 1996; Hauer and Lamberti 1996; Hilsenhoff 1988; Plafkin *et al*. 1989). In lotic systems, an increase in the abundance of predators can indicate a healthy biological community (Karr et al. 1986; Morley 2000); however, these particular macroinvertebrates are more characteristic of lentic habitats and this generalization does not relate due to my sampling strategy which focused on the marginal zones. The association of these taxa with *M. ulcerans* does suggest an ecological connection between pathogen and waterbodies with riparian margins subject to prolonged periods of inundation. This is consistent with field research and epidemiological data that have associated BU disease with areas prone to flooding (Lunn et al. 1965, Revill and Barker 1972, Barker and Carswell 1973,

Duker et al. 2006, Marston et al. 1995, Walsh et al. 2008, Portaels 1995, Debacker et al. 2006, Noeske et al. 2004, Johnson et al. 2007, Wagner et al. 2008a, WHO 2000). Additional field collections, particularly to address seasonal variation, will provide more insight as to whether these taxa have a more specific connection with the ecology of *M. ulcerans*.

In conclusion, my investigation of macroinvertebrate community associations with *M. ulcerans* did produce results that suggest potential use for these communities to be useful as indicators of environmental conditions preferable to the proliferation of the pathogen in the environment; however these data should be treated with caution. First, while there were a few metrics and taxa associated with the presence of *M. ulcerans*, there were several more that were not. This could be the result of sampling strategy, taxonomic resolution used for macroinvertebrate identifications, inaccurate estimate of the presence or absence of *M. ulcerans* within sites, or simply that there aren't true associations between this bacteria and the macroinvertebrate community that can be measured with the standard biomontoring techniques. Second, the sampling strategy used in this study was aimed to standardize collections among all sites by targeting the marginal habitats where it is believed *M. ulcerans* is most likely to flourish and where people most likely contact the pathogen in the environment. While sampling this habitat allowed for comparison to be made between sites, the true macroinvertebrate community profile within individual waterbodies may have been missed by not sampling additional habitats. For example, analyses of lotic waterbodies are most often based on collections made from riffles, pools

and stream runs, whereas our collections were based on marginal habitats typically out of the current. One suggestion for future studies would be to sample lotic habitats in a way that incorporates collections of benthic communities from habitats apart from the vegetative and marginal zone. This might produce a different community assemblage more characteristic of stream and river systems and would allow for more accurate comparisons of data with common biomontoring practices and previous studies conducted in West Africa. Third, the use of ER positivity as an indication of the presence and absence of *M. ulcerans* may overestimate the true distribution of the pathogen in the environment. There also was the potential for the pathogen to have been present at a waterbody but not collected or possibly not enough DNA to have been collected to generate an adequate positive confirmation in the laboratory. If either of these conditions were true, then comparisons of macroinvertebrate communities between and among waterbodies with and without the pathogen would likely produce different results than what were observed. The methods used to confirm positive detection of *M. ulcerans* were up to date at the time of this study, however increasing the number of samples collected per site to determine pathogen presence or absence might have led to more positive waterbodies than what were observed during this study. This study did identify potential ecological relationships between macroinvertebrates and *M. ulcerans* in the environment, but further field-based studies are needed to more completely understand the specific role *M. ulcerans* may play on benthic macroinvertebrate communities.

Table 2.1. Total specimens and number of sites observed in lotic and lentic habitats, Ghana, W. Africa

Taxon (Higher, Lowest)		LOTIC				LENTIC			
		ER+ (N= 38)		ER- (N= 12)		ER+ (N= 34)		ER- (N= 14)	
		#Sites Obsrvd.	Total Spec.	#Sites Obsrvd.	Total Spec.	#Sites Obsrvd.	Total Spec.	#Sites Obsrvd.	Total Spec.
Annelida									
Clitellata, Hirudinea		14	68	3	9	18	129	7	43
Clitellata, Oligochaeta		36	572	10	189	32	1764	12	333
Arthropoda									
Arachnida, Araneae		33	280	6	20	31	344	12	89
Arachnida, Hydracarina		26	775	4	6	27	903	13	176
Crustacea, Branchiopoda	Cladocera	9	544	1	22	13	694	6	32
	Lynceidae	2	29	-	-	5	282	2	24
Crustacea, Decapoda	Atyidae	23	550	5	99	5	365	3	7
	Potamonautidae	4	7	4	6	-	-	1	3
Crustacea, Maxillopoda	Copepoda	10	470	1	4	20	2123	5	271
Crustacea, Ostracoda	Ostracoda	15	1669	7	41	26	4082	11	497
Insecta, Coleoptera	Curculionidae	4	18	-	-	6	24	1	2
	Dytiscidae	26	273	6	56	24	263	14	269
	Elmidae	22	880	11	285	3	3	2	5
	Gyrinidae	17	36	5	17	6	49	-	-
	Hydraenidae	27	435	8	48	21	578	8	294
	Hydrobiidae	3	4	-	-	2	33	-	-
	Hydrophilidae	24	227	8	13	30	673	13	220

Table 2.1 (cont'd). Total specimens and number of sites observed in lotic and lentic habitats, Ghana, W. Africa

Insecta, Coleoptera	Lampyridae	10	21	1	1	8	30	4	15
	Noteridae	8	290	1	1	27	498	10	40
	Scirtidae	17	125	3	4	13	144	6	73
Insecta, Collembola	Entomobryiidae	17	98	5	12	13	90	5	20
	Isotomidae	4	42	-	-	3	16	-	-
Insecta, Diptera	Athericidae	4	8	1	3	-	-	-	-
	Ceratopogonidae	30	364	11	109	30	392	10	62
	Chaoboridae	5	9	-	-	10	42	5	9
	Chironomidae	37	4911	12	1714	34	9121	14	1597
	Culicidae	24	941	4	10	26	1256	12	392
	Dixidae	4	15	3	5	-	-	1	13
	Empididae	4	11	-	-	1	1	1	1
	Ephydridae	8	15	-	-	5	7	1	1
	Psychodidae	10	42	6	29	2	8	-	-
	Sciomyzidae	-	-	-	-	4	13	1	1
	Simuliidae	11	122	7	134	2	2	1	1
	Stratiomyiidae	3	7	2	6	10	25	5	31
	Syrphidae	3	4	-	-	3	3	-	-
	Tipulidae	13	25	-	-	9	31	4	21
Insecta, Ephemeroptera	Baetidae	38	3714	12	615	34	3666	14	1623
	Caenidae	34	2529	11	454	24	565	10	117
	Heptageniidae	20	356	6	93	2	38	-	-
	Leptophlebiidae	17	254	6	27	1	2	-	-
	Polymitarcyidae	1	1	-	-	7	16	4	19

Table 2.1 (cont'd). Total specimens and number of sites observed in lotic and lentic habitats, Ghana, W. Africa

Insecta, Ephemeroptera	Tricorythidae	5	36	2	16	-	-	-	-
Insecta, Hemiptera	Belostomatidae	20	145	2	3	17	166	10	58
	Corixidae	7	28	1	3	7	111	5	34
	Gerridae	25	114	2	4	19	112	10	48
	Hebridae	6	9	2	8	1	1	4	4
	Hydrometridae	5	6	-	-	5	7	7	11
	Mesoveliidae	28	112	7	20	26	210	12	121
	Naucoridae	14	30	2	3	12	118	5	19
	Nepidae	6	10	1	1	10	14	7	16
	Notonectidae	19	147	3	17	28	651	12	218
	Pleidae	21	563	1	2	22	675	7	98
	Saldidae	1	1	-	-	2	2	2	3
	Veliidae	27	138	9	41	16	93	8	75
Insecta, Lepidoptera	Pyralidae	10	21	2	4	7	25	2	2
Insecta, Odonata	Calopterygidae	2	2	3	12	-	-	-	-
	Chlorocyphidae	2	9	1	1	-	-	-	-
	Coenagrionidae	25	401	4	12	24	242	8	79
	Corduliidae	13	77	7	74	9	141	4	61
	Gomphidae	11	31	4	10	-	-	-	-
	Libellulidae	24	394	4	6	29	449	9	189
	Protoneuridae	31	529	10	99	28	942	10	279
Insecta, Plecoptera	Perlidae	3	9	2	3	-	-	-	-

Table 2.1 (cont'd). Total specimens and number of sites observed in lotic and lentic habitats, Ghana, W. Africa

Insecta, Trichoptera	Ecnomidae	2	2	1	1	2	2	-	-
	Hydropsychidae	6	26	3	5	2	31	1	14
	Hydroptilidae	3	12	2	3	1	4	-	-
Insecta, Trichoptera	Leptoceridae	26	160	4	16	4	11	2	8
	Polycentropodidae	5	10	1	1	-	-	-	-
Mollusca									
Bivalvia, Veneroida	Sphaeriidae	5	60	-	-	1	10	-	-
Gastrpoda	Ancylidae	11	87	4	18	8	100	4	82
	Bithyniidae	3	9	1	2	1	1	-	-
	Lymnaeidae	3	20	-	-	8	36	1	24
	Physidae	9	20	1	4	5	44	-	-
	Pilidae	8	22	5	8	6	11	1	4
	Planorbidae	34	1603	9	152	32	1577	8	384
	Thiaridae	20	393	6	619	9	136	2	33

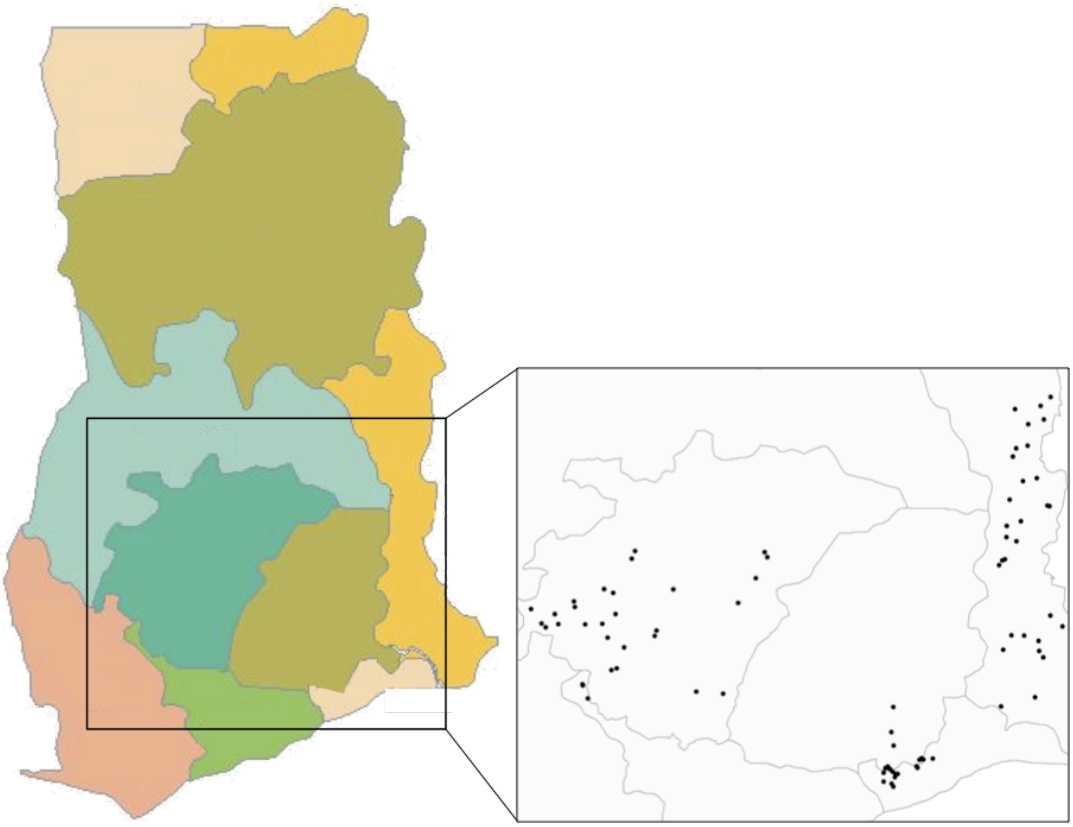


Figure 2.1. Map illustrating the locations of 98 water bodies sampled for macroinvertebrates from five geographic regions in southern Ghana, Africa. Villages were randomly selected within each region and water bodies were selected within each village, based on location (<100-200m from community housing structures) and human use (daily domestic activities) to reflect aquatic environments with potential human exposure to Buruli ulcer. Community discussions on water body selection were conducted in each village as described by Benbow et al. (2005).

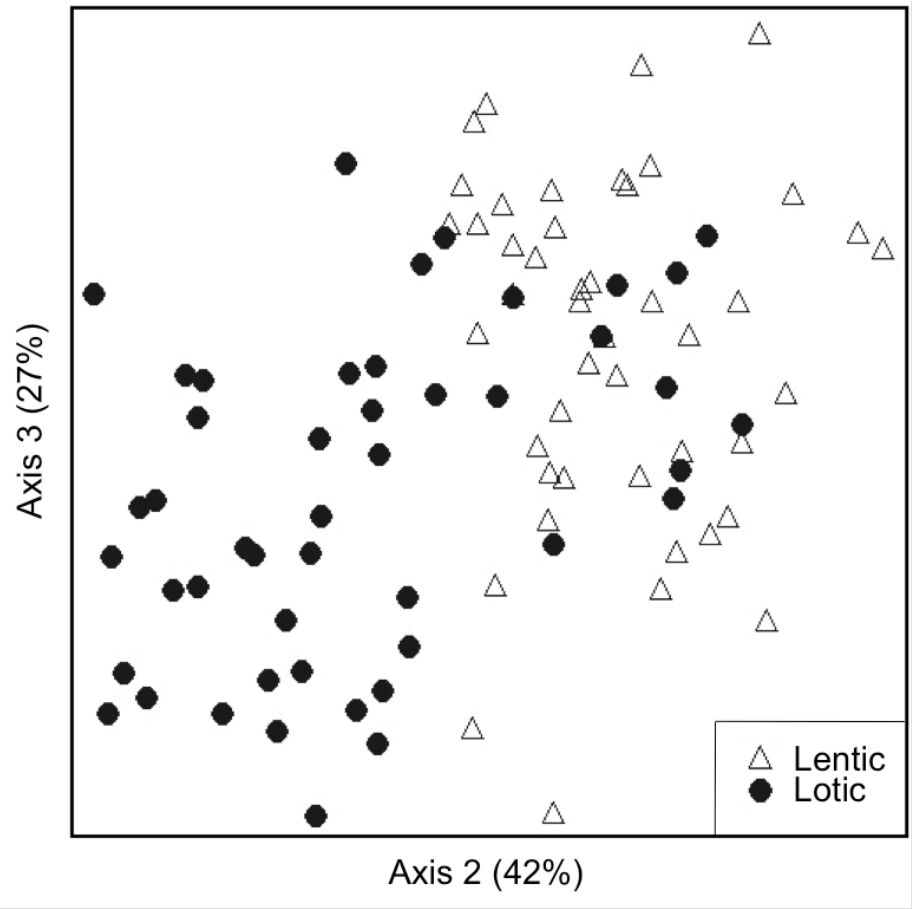


Figure 2.2. A non-metric multi-dimensional scaling (NMDS) ordination of macroinvertebrate communities collected from 98 waterbodies in Ghana, Africa. Each circle or triangle represents the overall macroinvertebrate community at each site and symbols closer together have more similar community structure while symbols further apart were more dissimilar communities. Closed circles represent lotic habitats and open triangles represent lentic habitats. A three-axis NMDS solution explained 70% of the total variation in the macroinvertebrate community (stress: 16.9, p=0.004), with 1% on axis 1, 42% on axis 2, and 27% on axis 3. No significant differences in the overall macroinvertebrate community structure between sites that were ER+ and ER- (MRPP: A=0.001, p = 0.23) were observed. The NMDS ordination did identify differences in macroinvertebrate community structure based on water body flow (MRPP: A=0.046, p < 0.000).

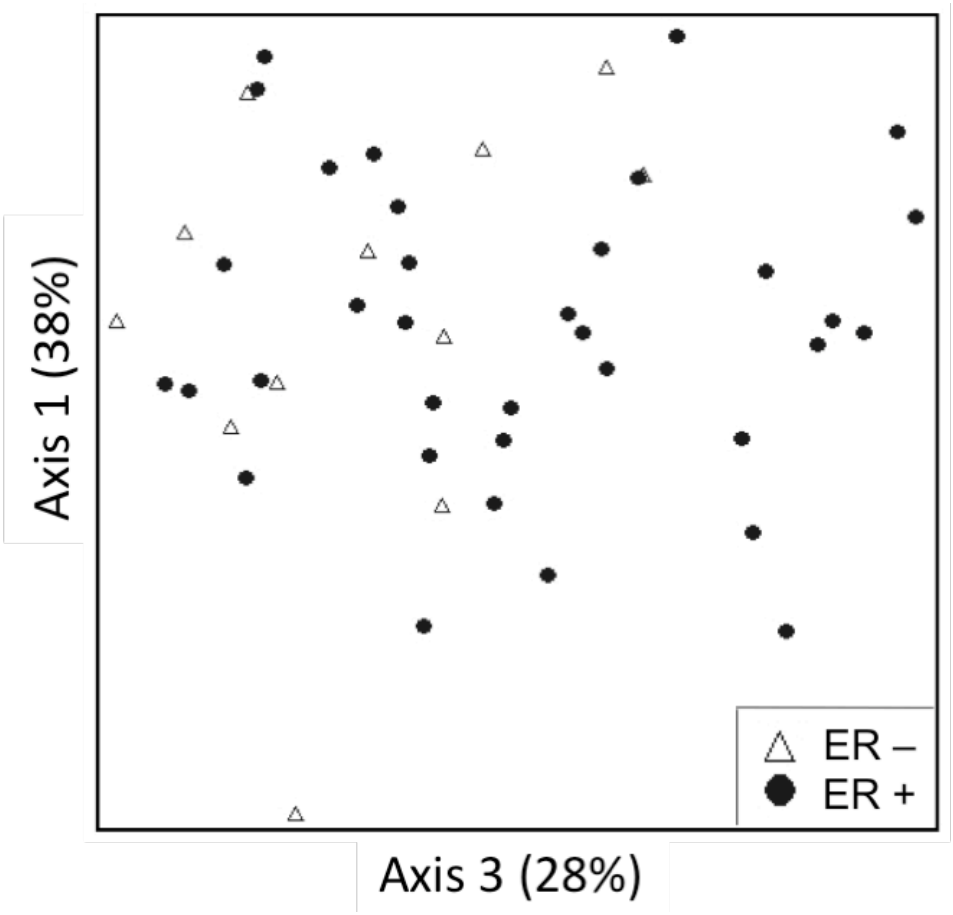


Figure 2.3. A non-metric multi-diminsional scaling (NMDS) ordination of macroinvertebrate communities collected from 98 waterbodies in Ghana, Africa. Each circle or triangle represents the overall macroinvertebrate community at each site and symbols closer together have more similar community structure while symbols further apart were more dissimilar communities. Closed circles represent ER+ and open triangles represent ER- habitats. A three-axis NMDS solution explained 80% of the total variation in the macroinvertebrate community (stress: 15.4, p=0.004), with 38% on axis 1, 14% on axis 2, and 28% on axis 3. We found significant differences between lotic sites that were ER+ and ER- (MRPP: A=0.01, p = 0.02).

LITERATURE CITED

Literature Cited

Ablordey, A, Swings, J, Hubans, C, Chemlal, K, Locht, C, Portaels, F, Supply, P (2005) Multilocus variable-number tandem repeat typing of *Mycobacterium ulcerans*. *J Clin Microbiol* 43, 1546–1551.

Aiga H, Amano T, Cairncross S, Domako JA, Nanas OK, et al. (2004) Assessing water-related risk factors for Buruli ulcer: A case-control study in Ghana. Am J Trop Med Hyg 71: 387–392.

Amofah GK, Bonsu F, Tetteh C, Okrah J, Asamoa K, et al. (2002) Buruli ulcer in Ghana: results of a national case search. Emerging Infectious Diseases 8: 167–170.

Asiedu K, Etuaful S (1998) Socioeconmoic implications of Buruli ulcer in Ghana: a three-year review. Trans R Soc Trop Med & Hyg 59: 1015–1022.
18. Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, et al. (2001) Mycobacterium ulcerans in wild animals. Rev sci tech Off int Epiz 20: 252–264.

Barbour, MT, JB Stribling, JR Karr (1995) Multimetric approach for establishing biocriteria and measuring biological condition. Pages 63-77 *in* WS Davis and TP Simon (editors*).* Biological assessment and criteria. Tools for water resource planning and decision making. Lewis Publishers, Boca Raton, Florida.

Barbour, MT, JM Diamond, CO Yoder (1996) Biological assessment strategies: Applications and Limitations. Pages 245-270 *in* DR Grothe, KL Dickson, and DK Reed-Judkins (editors). Whole effluent toxicity testing: An evaluation of methods and prediction of receiving system impacts, SETAC Press, Pensacola, Florida.

Barker, DJP (1971) Buruli disease in a district of Uganda. J. Trop. Med. Hyg. 74: 260-264.

Barker DJP (1972) The distribution of Buruli disease in Uganda. Trans R Soc Trop Med Hyg. 66:867-74.

Barker DJP, Carswell JW (1973) Mycobacterium ulcerans infection among Tsetse control workers in Uganda. International Journal of Epidemiology 2: 161–165.

Benbow M, Williamson H, Kimbirauskus R, McIntosh M, Kolar R, et al. (2008) Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis 14: 1247–1254.

Bode, RW, MA Novak, LA Abele (1996) Quality assurance work plan for biological stream monitoring in New York State. NYS Department of

Environmental Protection; Division of Water; Bureau of Monitoring and Assessment; Stream Biomonitoring Unit; Albany, NY.

Boisvert H, Schroder KH (1977) Skin ulcer caused by Mycobacterium ulcerans in Cameroon II: Bacteriologic study. Bull Soc Pathol Exot Filiales 70: 125–131.

Chemlal, K, Huys, G, Laval, F, et al. (2002) Characterization of an unusual Mycobacterium: a possible missing link between Mycobacterium marinum and Mycobacterium ulcerans. Journal of Clinical Microbiology 40:2370.

Chessman, BC (1995) Rapid assessment of rivers using macroinvertebrates: A procedure based on habitat-specific sampling, family level identification and a biotic index. Australian Journal of Ecology 20:122-129.

Connor DH, and Lunn F (1965) *Mycobacterium ulcerans infection. Int. J. Leprosy 33*:*698*.

Cosma CL, Sherman DR, and Ramakrishnan L (2003) The Secret Lives of the Pathogenic Mycobacteria. Annu. Rev. Microbiol. 57:641–76.

Cummins, KW, MJ Klug (1979) Feeding ecology of stream invertebrates. Annual Review of Ecology and Systematics 10: 147-172.

Cummins, KW, RW Merritt (1981) Application of invertebrate functional feeding groups to wetland ecosystem functions and biomonitoring. Pp. 85-11 in RB Rader, DP Batzer, SA Wissinger (editors). Bioassessment and Management of North American Freshwater Wetlands. John Wiley and Sons, New York.

Davis, SM (1994) Phosphorus inputs and vegetation sensitivity in the Everglades. In Davis, S. M. & J. C. Ogden (eds), 355 Everglades: The Ecosystem and its Restoration. St. Lucie Press, Boca Raton, FL, USA, 357–378.

Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, et al. (2004) Mycobacterium ulcerans disease (Buruli ulcer) in rural hospital, Southern Benin, 1997-2001. Emerg Infect Dis 10: 1391–1398.

Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, et al. (2006) Risk factors for Buruli ulcer, Benin. Emerg Infect Dis 12: 1325–1331.

Demangel C, Stinear TP, Cole ST (2009) Buruli ulcer: reductive evolution enhances pathogenicity of Mycobacterium ulcerans. Nat Rev Microbiol 7: 50–60.

Duker AA, Carranza JM, Hale M (2004) Spatial dependency of Buruli ulcer prevalence on arsenic-enriched domains in Amansie West District, Ghana: implications for arsenic mediation in *Mycobacterium ulcerans* infection. Int J Health Geogr. 3:19.

Duker AA, Portaels F, Hale M (2006) Pathways of Mycobacterium ulcerans infection: A review. Environment International 32: 567–573.

Durand, JR, Leveque, C (1981) *Flore et Faune Aquatiques de l'Afrique Sahelo-Soudaniene,* 2 volumes. ORSTOM, Paris.

Eddyani M, Ofori-Adjei D, Teugels G, De Weirdt D, Boakye D, et al. (2004) Potential role for fish in transmission of Mycobacterium ulcerans disease (Buruli Ulcer): an environmental study. Appl Environ Microbiol 70: 5679–5681.

Fyfe JAM, Lavender CJ, Johnson P, Globan M, Sievers A, et al. (2007) Development and Application of Two Multiplex Real-Time PCR Assays for the Detection of Mycobacterium ulcerans in Clinical and Environmental Samples. Appl Environ Microbiol 73: 4733–4740.

Gaiser, EE, DL Childers, RD Jones, JH Richards, LJ, Scinto, JC Trexler (2005) Periphyton responses to eutrophication in the Florida Everglades: cross-system patterns of structural and compositional change. Limnology and Oceanography 50: 342–355.

Garrity GM (2001) Bergey's Manual of Systematic Bacteriology; In: Garrity GM, ed. New York: Springer-Verlag.

George KM, Chatterjee D, Gunawardana G, Welty D, Lee T, et al. (1999) Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science 283: 854–857.

Gunawardana G, Chatterjee D, George KM, Brennan P, Whittern D, et al. (1999) Mycolactone A and B: toxins of Mycobacterium ulcerans. J Amer Chem Soc 121: 6092–6093.

Guerra H, Palomino JC, Falconi E, Bravo F, Donaires N, et al. (2008) Mycobacterium ulcerans Disease, Peru. Emerging Infectious Diseases 14: 373–377.

Hauer, FR, GA Lamberti (editors) (1996) Methods in Stream Ecology. Academic Press. New York. p 674.

Hayman, J, A. McQueen (1985) The pathology of *Mycobacterium ulcerans* infection. Pathology 17:594-600.

Hayman J (1991a) Postulated epidemiology of Mycobacterium ulcerans infection. International Journal of Epidemiology. 20(4): 1093-8.

Hayman J (1991b) Mycobacterium ulcerans infection. The Lancet 337: 124.

Helgen, JC, MC Gernes (2001) Monitoring the condition of wetlands: indexes of biological integrity using invertebrates and vegetation. Pp. 167-185 IN: RB Rader, DP Batzer, and SA Wissinger (eds.), Bioassessment and Management of North American Freshwater Wetlands, John Wiley & Sons, New York, NY.

Hilsenhoff, WL (1988) Rapid field assessment of organic pollution with a family level biotic index. *Journal of the North American Benthological Society* 7(1):65-68.

Hynes, JD (1975a) Annual cycles of macro-invertebrates of a river in southern Ghana. Freshwater Biology (5): 71-83.

Hynes, JD (1975) Downstream Drift of Invertebrates in a River in Southern Ghana. Freshwater Biology 5(6): 515-531.

Johnson PDR, Veitch MGK, Leslie D, Flood PE, Hayman JA (1996) The emergence of *Mycobacterium ulcerans* infection near Melbourne. Medical Journal of Australia 164: 76–78.

Johnson PDR, Stinear TP, Hayman JA (1999) Mycobacterium ulcerans — a minireview. J Med Microbiol 48: 511–513.

Johnson PDR, Stinear TP, Small PLC, Pluschke G, Merritt RW, et al. (2005) Buruli ulcer (M. ulcerans Infection): new insights, new hope for disease control.PLoS Med 2(4): e108.

Johnson PDR, Hayman JA, Quek TY, Fyfe JAM, Jenkin GA, et al. (2007) Consensus recommendations for the diagnosis, treatment and control of Mycobacterium ulcerans infection (Bairnsdale or Buruli ulcer) in Victoria, Australia. Medical Journal of Australia 186: 64–68.

Johnson PDR, Lavender CJ (2009) Correlation between Buruli ulcer and vector-borne notifiable diseases, Victoria, Australia. Emerging Infectious Diseases 15: 614–615.

Karr, JR, KD Fausch, PL Angermeier, PR Yant, IJ Schlosser (1986) *Assessing biological integrity in running waters: A method and its rationale.* Special publication 5. Illinois Natural History Survey.

Kaser, M., Hauser, J., Small, P., Pluschke, G. (2009). Large Sequence Polymorphisms Unveil the Phylogenetic Relationship of Environmental and Pathogenic Mycobacteria Related to Mycobacterium ulcerans. *Appl. Environ. Microbiol.* 75: 5667-5675.

Kibadi K, Panda M, Tamfum JM, Fraga AG, Filho AL, et al. (2008) New foci of

Buruli ulcer, Angola and Democratic Republic of Congo. Emerging Infectious Diseases 14: 1790–1792.

Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne P, et al. (2004) Onetube cell lysis and DNA extraction procedure for PCR-based detection of Mycobacterium ulcerans in aquatic insects, molluscs and fish. Journal of Medical Microbiology 53: 927–933.

Lavender CJ, Stinear TP, Johnson PDR, Azuolas J, Benbow ME, et al. (2008) Evaluation of VNTR typing for the identification of Mycobacterium ulcerans in environmental samples from Victoria, Australia. FEMS Microbiology Letters 287: 250–255.

Lunn HF, Connor DH, Wilks NE, Barnley GR, Kamunvi F, et al. (1965) Buruli (Mycobacterial) ulceration in Uganda. East African Medical Journal 42: 275–288.

Marion E, Eyangoh S, Yeramian E, Doannio J, Landier J Aubry J et al. (2010) Seasonal and regional dynamics of M. ulcerans transmission in environmental context: deciphering the role of water bugs as hosts and vectors. PLoS Negl Trop Dis. 6:4(7):e731.

Marsollier L, Robert R, Aubry J, Andre JS, Kouakou H, et al. (2002) Aquatic insects as a vector for Mycobacterium ulcerans. Applied and Environmental Microbiology 68: 4623–4628.

Marsollier L, Aubry J, Saint-Andre JP, Robert R, Legras P, et al. (2003) Ecology and transmission of Mycobacterium ulcerans. Pathologie Biologie 51: 490–495.

Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, et al. (2004a) Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl Environ Microbiol 70: 6296–6298.

Marsollier L, Stinear TP, Aubry J, Saint-Andre J-P, Robert R, et al. (2004b) Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Applied and Environmental Microbiology 70: 1097–1103.

Marsollier L, Aubry J, Coutanceau E, Andre JPS, Small PL, et al. (2005) Colonization of the salivary glands of Naucoris cimicoides by Mycobacterium ulcerans requires host plasmatocytes and a macrolide toxin, mycolactone. Cellular Microbiology 7: 935–943.

Marsollier L, Andre J, Frigui W, Reysset G, Milon G, et al. (2006) Early trafficking events of Mycobacterium ulcerans within Naucoris cimicoides. Cellular Microbiology.

Marsollier L, Brodin P, Jackson M, Kordulakova J, Tafelmeyer P, et al. (2007) Impact of Mycobacterium ulcerans biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. PLoS Pathogens 3: e62.

Marston BJ, Diallo MO, Horsburgh CR Jr., Diomande I, Saki MZ, et al. (1995) Emergence of Buruli ulcer disease in the Daloa region of Cote D'ivoire. Am J Trop Med Hyg 52: 219–224.

McCormick, PV, RJ Stevenson (1998) Periphyton as a tool for ecological assessment and management in the Florida Everglades. Journal of Phycology 34: 726–733.

McCune, B, JB Grace (2002) Analysis of Ecological Communities, vol. MJM Software Designs, Glenden Beach, Oregon.

Merritt, RW, KW Cummins, TM Burton (1984) The role of aquatic insects in the processing and cycling of nutrients. pp. 134-163. In: Resh, VH and DN Rosenberg (eds.). The ecology of aquatic insects. Praeger Scient., N.Y. 438 p.

Merritt, RW, Cummins KW (1996) An introduction to the aquatic insects of North America. 3 ed. Kendall / Hunt, Iowa.

Merritt RW, Benbow ME, Small PLC (2005) Unraveling an Emerging Disease Associated with Disturbed Aquatic Environments: The Case of Buruli Ulcer. Frontiers in Ecology and the Environment 3: 323–331.

Merritt, RW, KW Cummins (2006) Trophic relationships., pp. 585-610. In: Hauer, F. R. and G. A. Lamberti (eds.). Methods in Stream Ecology (2nd ed.). Academic Press, San Diego, CA.

Merritt, RW, Cummins, KW, MB Berg (2008) An introduction to the aquatic insects of North America. Fourth Edition. Kendall/Hunt Publishing Co., Dubuque, Iowa. 1158pp.

Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review. PLoS Negl Trop Dis 4(12): 1-15.

Meyers WM, Tignokpa WM, Priuli GB, Portaels F (1996) Mycobacterium ulcerans infection (Buruli ulcer): first reported patients in Togo. British Journal of Dermatology 134: 1116–1121.

Mielke, PW (1991) The application of multivariate permutation methods based on distance functions in the earth sciences. Earth Science Review 31:55-71.

Morley, SA (2000) Effects of urbanization on the biological integrity of Puget Sound lowland streams: restoration with a biological focus. Seattle, University of Washington, M.S. Thesis.

Mosi L, Williamson H, Wallace JR, Merritt RW, Small PLC (2008) Persistent association of Mycobacterium ulcerans with West African predaceous insects of the family Belostomatidae. Applied and Environmental Microbiology 74: 7036–7042.

Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, et al. (2004) Buruli ulcer disease in Cameroon rediscovered. Am J Trop Med Hyg 70: 520–526.

Palomino JC, Obiang AM, Realini L, Meyers WM, and Portaels F (1998) Effect of Oxygen on Growth of *Mycobacterium ulcerans* in the BACTEC System. Journal of Clinical Microbiology. Vol. 36, No. 11, p. 3420-3422,

Phanzu DM, Bafende EA, Dunda BK, Imposo DB, Kibadi AK, et al. (2006) Mycobacterium ulcerans disease (Buruli Ulcer) in a rural hospital in Bas-Congo, Democratic Republic of Congo, 2002-2004. American J Tropical Medicine and Hygiene 75: 311–314.

Plafkin, JL, MT Barbour, KD Porter, SK Gross, RM Hughes (1989*)* Rapid bioassessment protocols for use in streams and rivers: Benthic macroinvertebrates and fish. U.S. Environmental Protection Agency, Office of Water Regulations and Standards, Washington, D.C. EPA 440-4-89-001.

Portaels, F. (1995) Epidemiology of mycobacterial diseases. Clin. Dermatol. 13:207-222.

Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P, Meyers WM (1999) Insects in the transmission of Mycobacterium ulcerans infection. The Lancet 353: 986.

Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, et al. (2001) Mycobacterium ulcerans in wild animals. Rev sci tech Off int Epiz 20: 252–264.

Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, et al. (2008) First Cultivation and Characterization of Mycobacterium ulcerans from the Environment. PLoS Neglected Tropical Diseases 2: e178.

Portaels F, Manuel S, Meyers W (2009) Buruli ulcer. Clinics in dermatology 27: 291–305.

Rader, RB (2001) An introduction to wetland bioassessment and management. Pages 1-9 in Bioassessment and Management of North American Freshwater Wetlands. John Wiley and Sons, New York.

Radford AJ (1974) Mycobacterium ulcerans: a review, I: Epidemiology. Papua New Guinea Medical Journal 17: 129–133.

Radford AJ (1975) *Mycobacterium ulcerans* in Australia. Aust NZ J Med 5: 162–169.

Resh, VH, JK Jackson (1993) Rapid assessment approaches to biomonitoring using benthic macroinvertebrates. Pages 195-*233 in* DM Rosenberg and VH Resh (editors). Freshwater biomonitoring and benthic macroinvertebrates. *Chapman and Hall, New York.*

Resh VH, Leveque C, Statzner B (2004) Long-Term, Large-Scale Biomonitoring Of The Unknown: Assessing the Effects of Insecticides to Control River Blindness (Onchocerciasis) in West Africa. Annu. Rev. Entomol. 2004. 49:115–39

Revill WDL, Barker DJP (1972) Seasonal distribution of mycobacterial skin ulcers. Brit J prev soc Med 26: 23–27.

Rosenberg, DM, VH Resh (eds.) (1993) Freshwater biomonitoring and benthic macroinvertebrates. Chapman and Hall, New York. 488 p.

Ross BC, Marino L, Oppedisano F, Edwards R, Robins-Browne RM, et al. (1997) Development of a PCR assay for rapid diagnosis of Mycobacterium ulcerans infection. Journal of Clinical Microbiology 35: 1696–1700.

Silva MT, Portaels F, Pedrosa J (2007) Aquatic Insects and Mycobacterium ulcerans: an association relevant to Buruli ulcer control? PLoS Medicine 4: e63.

Sizaire, V, Nackers, F, Comte, E, Portaels, F (2006) *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. *Lancet Infect Dis* 6, 288–296.

Sopoh GE, Johnson RC, Chauty A, Dossou AD, Aguiar J, et al. (2007) Buruli ulcer surveillance, Benin, 2003-2005. Emerg Infect Dis 13: 1374–1376.

Stinear T, Ross, BC, Davies JK, Marino L, Robins-Browne RM, Oppedisano F, Grant A, Jenkin GA, Potaels, F, Ross, BC, Oppedisano F, Sievers A, Johnson PDR (1999) Identification and Characterization of IS*2404* and IS*2606*: Two Distinct Repeated Sequences for Detection of *Mycobacterium ulcerans* by PCR. American Society for Microbiology vol. 37, No. 4.

Stinear T, Jenkin GA, Johnson PDR, Davies JK (2000) Comparative Genetic Analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* Reveals Evidence of Recent Divergence. Journal of Clinical Microbiology vol. 182, No. 22. pp-6322-6330.

Stinear T, Davies JK, Jenkin GA, Hayman JA, Oppedisano F, et al. (2000) Identification of Mycobacterium ulcerans in the environment from regions in Southeast Australia in which it is endemic with sequence Capture-PCR. Appl Environ Microbiol 66: 3206–3213.

Stinear T, Davies JK, Grant A, Jenkin GA, Potaels, F, Ross, BC, Oppedisano F, Purcell M, Hayman JA, Johnson PDR (2000) A Simple PCR Method for Rapid Genotype Analysis of *Mycobacterium ulcerans.* Journal of Clinical Microbiology vol. 38, No. 4. pp-1482-1487.

Stinear TP, Mve Obiang A, Small PL, Frigui W, Pryor MJ, et al. (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proc Natl Acad Sci U S A 101: 1345–1349.

Stinear T, Johnson PDR (2007) From marinum to ulcerans: a mycobacterial human pathogen emerges. Microbe 2: 187–194.

Thangaraj HS, Evans MRW, Wansbrough-Jones MH (1999) *Mycobacterium ulcerans*; Buruli ulcer. Transactions of the Royal Society of Tropical Medicine and Hygiene 93: 337–340.

Thorne, R, Williams WP (1997) The response of benthic macroinvertebrates to pollution in developing countries: a multimetric system of bioassessment. *Freshwater Biology* (1997) 37, 671–686

Thorne, R, Williams, WP, Gordon, C (2000) The macroinvertebrates of a polluted stream in Ghana. Journal of Freshwater Ecology 15(2): 209-217.

Tobias N, Seemann T, Pidot S, Porter J, Marsollier L, et al. (2009) Mycolactone gene expression is controlled by strong SigA-like promoters with utility in studies of Mycobacterium ulcerans and buruli ulcer. PLoS Negl Trop Dis 3: e553.

Trott KA, Stacy BA, Lifland BD, Diggs HE, Harland RM, et al. (2004) Characterization of a Mycobacterium ulcerans-like infection in a colony of African tropical clawed frogs (Xenopus tropicalis). Comp Med 54: 309–317.

U.S. Environmental Protection Agency (1996) The volunteer monitor's guide to quality assurance project plans. U.S. Environmental Protection Agency, Office of Wetlands, Oceans, and Watersheds, Washington, D.C. EPA 841-B-96-003.

U.S. Environmental Protection Agency (2001) Indicators for Monitoring Bilogical Integrity of Inland Freshwater Wetlands. U.S. Environmental Protection Agency, Office of Water, Wetlands Divison, Washington, D.C. EPA 843-R-01.

van der Werf TS, Stienstra Y, Johnson C, Phillips R, Adjei O, et al. (2005)

Mycobacterium ulcerans disease. Bulletin of the World Health Organization 83: 785–791.

Veitch MGK, Johnson PDR, Flood PE, Leslie D, Street AC, et al. (1997) A large localized outbreak of Mycobacterium ulcerans infection on a temperate southern Australian island. Epidemiol Infect 119: 313–318.

Wagner T, Benbow ME, Burns M, Johnson RC, Merritt R, et al. (2008a) A Landscape-based Model for Predicting Mycobacterium ulcerans Infection (Buruli Ulcer Disease) Presence in Benin, West Africa. EcoHealth 5: 69–79.

Wagner T, Benbow ME, Brenden T, Qi J, Johnson RC (2008b) Buruli ulcer disease prevalence in Benin, West Africa: associations with land use/cover and the identification of disease clusters. International Journal of Health Geographics 7: 25.

Wallace J, Gordon M, Hartsell L, Mosi L, Benbow M, et al. (2010) Interaction of Mycobacterium ulcerans with mosquito species: Implications for transmission and trophic relationships. Applied Environ Microbiol 76: 6215–6222.

Walsh D, Portaels F, Meyers W (2008) Buruli ulcer (Mycobacterium ulcerans infection). Transactions of the Royal Society of Tropical Medicine and Hygiene 102: 969–978.

Walsh D, Eyase F, Onyango D, Odindo A, Waitumbi JN et al. (2010) Short Report: Clinical and Molecular Evidence for a Case of Buruli Ulcer (Mycobacterium ulcerans Infection) in Kenya. Am. J. Trop. Med. Hyg., 81(6), pp. 1110–1113

Wansbrough-Jones M, Phillips R (2006) Buruli ulcer: emerging from obscurity. Lancet 367: 1849–1858.

Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, et al. (2008) Distribution of Mycobacterium ulcerans in Buruli Ulcer Endemic and Non-Endemic Aquatic Sites in Ghana. PLoS Neglected Tropical Diseases 2: e205.

WHO, ed (2000) Buruli ulcer. Mycobacterium ulcerans infection. Geneva, Switzerland: WHO. 118 p.

WHO (2008) Buruli ulcer: progress report, 2004–2008. In: WHO, ed. Weekly epidemiological record. Geneva, Switzerland: World Health Organization 83: 145–156.

Yeboah-Manu D, Bodmer T, Mensah-Quainoo E, Owusu S, Ofori-Adjei D, et al. (2004) Evaluation of decontamination methods and growth media for primary

isolation of Mycobacterium ulcerans from surgical specimens. J Clin Microbiol 42: 5875–5876.

Yip MJ, Porter JL, Fyfe JA, Lavender CJ, Portaels F, et al. (2007) Evolution of Mycobacterium ulcerans and other mycolactone-producing mycobacteria from a common Mycobacterium marinum progenitor. J Bacteriol 189: 2021–9.

CHAPTER 3

SEASONAL ASSOCIATIONS BETWEEN MYCOBACTERIUM ULCERANS AND MACROINVERTEBRATE ASSEMBLAGES IN AQUATIC ENVIRONMENTS OF GHANA, WEST AFRICA

INTRODUCTION

Buruli ulcer (BU) is an emerging, neglected, infectious disease most often associated with poor, rural communities within developing nations (WHO 2008). To date, the disease has been reported from at least 32 countries, with the highest frequency of new cases being reported from the West African nations of Cote D' Ivoire, Benin and Ghana (WHO 2008). It is known that *Mycobacterium ulcerans* is the pathogen responsible for causing BU disease (WHO 2000); however, researchers have yet to conclusively identify the extent of the pathogen's distribution in the environment, the reservoir(s) of the pathogen in nature, or the mode(s) of transmission to humans (WHO 2008; Merritt et al. 2010). For these reasons, in part, BU is referred to as the mysterious disease (WHO 2000).

It is widely accepted that BU disease is in some way related to exposure to freshwater environments (Aiga et al. 2004; Marston et al. 2005; Raghunathan et al. 2005; WHO 2008). Several epidemiological studies have associated cases of BU with proximity and prolonged exposure to disturbed aquatic habitats (Lunn et al. 1965; Revill and Barker 1972; Barker and Carswell 1973; Marston et al. 1995; Portaels 1995; Noeske et al. 2004; Debacker et al. 2006; Duker et al. 2006; Johnson et al. 2007; Wagner et al. 2008a; Walsh et al. 2008), and furthermore, it has been suggested that human activities, such as- surface

mining, damming of waterbodies, deforestation practices, and agricultural development are contributing factors leading to environmental disturbance and increased risk of BU infection (Hayman 1991b; Marston et al. 1995; Meyers et al. 1996; Johnson et al. 1999; Portaels et al. 2001; Merrit et al. 2005; Duker et al. 2006; Kibadi et al. 2008; Wagner et al. 2008a,b). These disturbances are believed to provide environmental conditions favorable for the establishment and proliferation of *M. ulcerans* in aquatic habitats (Hayman 1991b; Portaels 1999; Merritt et al. 2005; Williamson et al. 2008; McIntosh et al. submitted 2010). BU incidence also has been reported to increase during prolonged dry periods, after flooding events and in areas prone to flooding (Portaels 1989; Daire et al. 1993; Meyers et al. 1996; Dabacker et al. 2004; Merritt et al. 2005; Duker et al. 2006; Walsh et al. 2010), suggesting BU infection may be related to season.

It is hypothesized that *M. ulcerans* is acquired from the environment either through inoculation of the pathogen into skin lesions or from a biological vector (WHO 2000, 2008). There is a growing body of work that suggests aquatic macroinvertebrates may be vectors of *M. ulcerans* to humans (Portaels et al. 1999; Marsoilier et al. 2002b; Johnson et al. 2007; Marion et al. 2010), and also environmental reservoirs of the pathogen (Portaels et al. 1999, 2001, 2008; Marsoilier et al. 2002a; 2003, 2004a, 2005; Fyfe et al. 2007; Johnson et al. 2007; Lavender et al. 2008; Williamson et al. 2008; Tobias et al. 2009; Marion et al. 2010; Wallace et al. 2010). In Australia, mosquitoes are believed to play a role in BU transmission (Johnson et al. 1999, 2007; Fyfe et al. 2007; Lavender et al. 2008; WHO 2008) and disease outbreaks have been correlated with Ross River

virus and Barmah Forest virus, both of which are vectored by mosquitoes (Johnson et al. 2009). In West Africa, aquatic biting Hemiptera populations have been associated with BU infection and have been proposed to be both reservoirs and vectors of *M. ulcerans* to humans (Portaels et al. 1999; Marsoilier et al. 2002b, 2007; Marion et al. 2010). Despite the literature showing an association of aquatic insects in the transmission of this disease, major scientific criteria are lacking for implicating the roles of living agents as biologically significant reservoirs and/or vectors of pathogens (Merritt et al. 2010).

The role of aquatic macroinvertebrates as potential reservoirs or vectors of BU is well documented as reviewed by Merritt et al. (2010); however, field based ecological studies to specifically address these associations are few (Benbow et al. 2007, Merritt et al. 2010). An initial step in understanding the potential role of macroinvertebrates in the ecology of BU, whether direct or indirect, is identifying the relative abundances and composition of the aquatic macroinvertebrate communities in relation to the disease and disease pathogen. As part of a standardized assessment of the temporal patterns of macroinvertebrate communities, I surveyed 6 waterbodies selected from villages that were known to have reported cases of BU (n= 3) and villages with no previous record of BU (n= 3), in Ghana, West Africa to 1) characterize and compare seasonal variation in overall macroinvertebrate communities from aquatic environments in Ghana; 2) identify macroinvertebrate community metrics associated with the presence and absence of BU cases and *M. ulcerans* within these environments; and 3) identify potential relationships between macroinvertebrates, BU cases and *M. ulcerans.*

MATERIALS AND METHODS

Study location and site selection. A standardized, seasonal assessment of aquatic habitats was conducted to characterize seasonal variation in benthic macroinvertebrate communities in Ghana, West Africa. In this study, a total of 6 waterbodies were selected from villages located within the Greater Accra Region of southern Ghana. Villages were selected based on reported BU case data (Ghana Ministry of Health) and personal communication with local researchers familiar with BU infected and uninfected communities. Three villages from the Ga West District were identified as endemic for BU (Afieman, Kotoku, Nsakima) and 3 villages from the Ga East District were identified as BU non-endemic (Otinibi, Danfa, Teiman). Within each village, one waterbody was selected based on location (<100-200m from community housing structures), human use (daily domestic activities) to reflect aquatic environments with potential human exposure to BU, and community discussions as described by Benbow et al. (2005). Waterbodies varied in size and macrophyte community composition, but all were characterized by slow flowing water and identified as either modified ponds (MP, n= 3) or modified wetlands (MW, n= 3). Location, GPS coordinates and general description for each water body are provided in Table 3.1.

Seasonal sampling strategy. The climate of southern Ghana, where this study was conducted, is tropical and seasons are characterized by wet and dry periods. The dominant wet season occurs between September and November, followed by a dry season December to March. A wet season also occurs

between April and June, followed by another dry period between July and August. Aquatic macroinvertebrate communities in this study were surveyed on a single sampling date from each waterbody in June 2007 (wet), November 2007 (wet), February 2008 (dry), April 2008 (wet) and July 2008 (dry).

Macroinvertebrate sample collections. Aquatic macroinvertebrate communities were collected from the littoral margins of waterbodies. Within each waterbody, two 10–20-m transects were measured parallel to the shoreline and positioned through the dominant macrophyte community. Along each transect, two floating 1-m2 polyvinyl chloride (PVC) quadrats were randomly placed and macroinvertebrates were collected by sweeping within the quadrat with a 500-µm mesh aquatic dip net. The quadrats floated on top of the water and delineated 1 m2 of area to be sampled. Three sweeps of the dip net were performed from the water surface to the bottom substrate for comprehensive sampling of specimens in the water column and all samples were collected using the same technique. Contents within each net were then washed through a 500-µm sieve, preserved in 99% ethanol, and transported to the laboratory for identification. All specimens were enumerated and identified to lowest possible taxon under a dissecting microscope using African keys (Durand and Leveque 1981; Invertebrates of South Africa - Keys, vols. 2-10, 1999-2007), and keys from elsewhere (Merritt et al. 2008). Voucher specimens are maintained in the Entomological Collection Museum, Department of Entomology at Michigan State University.

Detection of *Mycobacterium ulcerans*. To identify potential relationships between macroinvertebrate communities and *M. ulcerans in the*

environment, biofilms and water samples were collected at each water body during each sampling event. Biofilms were collected from artificial substrates (glass slides), surfaces of dominant macrophytes, and detritus, and a composite water sample was collected from open water areas within the water body at the mid-water column depth. From the composite, five 100-200ml sub-samples were filtered through a 1.6 micron fiberglass filter followed by a 0.2 micron nitrocellulose filter (Whatman Inc.). Filters were sealed inside aluminum foil packets for later laboratory analysis. In addition, a 500 μl biofilm sample was used for DNA extraction. Extracted DNA was also collected from *M. ulcerans* agy99, *Mycobacterium marinum* 1218, or water for use as positive and negative controls. All samples were processed at the University of Tennessee, Knoxville, Tennessee and follow methods as previously described by Williamson et al. (2008).

Primers, PCR conditions and sequencing. A tiered PCR detection method was used for the identification of *M. ulcerans* in which DNA was first subjected to amplification of the enoyl reductase (ER) domain. The ER domain is partially responsible for production of the toxin mycolactone and was used for the presumptive identification of *M. ulcerans* (George et al. 1999; Sizaire et al. 2002). ER-PCR positive samples were then subjected to VNTR analysis to identify *M. ulcerans* from other mycolactone-producing mycobacteria, and to potentially match sample profiles to known VNTR profiles obtained from patients (George et al. 1999; Portaels et al. 2001; Sizaire et al. 2002; Ablordey et al. 2005; Johnson et al. 2007). Primers and PCR conditions for amplification of the

ER domain as well as VNTR loci, including BNTR MIRU 1, locus 6, ST 1 and locus 19, were used as described by Williamson et al. (2008). For this study, the presumptive PCR test for the ER domain was used for the determination of *M. ulcerans* from environmental samples. Although this presumptive identification potentially gives an overestimate of *M. ulcerans* in the environment, it also provides a maximum estimate for all mycolactone-producing mycobacteria.

Data Analyses. Descriptive and inferential statistics were used to test the relationships between macroinvertebrate communities and selected independent variables. The independent variables were: site, season, presence or absence of reported cases of BU (BU+/BU-), and presence or absence of the pathogen (ER+/ER-) within waterbodies. The macroinvertebrate community metrics evaluated in this study comprised both diversity and similarity indices (Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness). Prior to all analyses, data hygiene and data screening were undertaken to ensure the variables of interest met appropriate statistical assumptions. Thus, the following analyses follow a similar analytic strategy in that the dependent variables were first evaluated for normality, linearity and homoscedasticity. Subsequently, two methods were used to analyze macroinvertebrate metrics in relation to the independent variables. First, multiple linear regressions were run to investigate relationships between macroinvertebrate communities and the independent variables BU cases (BU+/BU-) and pathogen (ER+/ER-)(SPSS v 17.0). Second, repeated measures profile analyses were run to detect amount of shared variance and strength of

relationship between the macroinvertebrate community metrics and the independent variables: BU cases (BU+/BU-), pathogen (ER+/ER-), sampling season (June 2007, November 2007, February 2008, April 2008, July 2008), and between waterbodies (n= 6)(SPSS 17.0). Finally, Pearson correlation coefficient was used to identify potential relationships between individual macroinvertebrate taxa (family) and the independent variables BU cases (BU+/BU-) and pathogen (ER+/ER-)(SPSS 17.0). Correlation coefficients were performed through parametric tests using SPSS 17.0 with $p > 0.05$ and $p > 0.01$ as threshold for significance.

RESULTS

Macroinvertebrate composition and distribution among sites. A total of 25,104 invertebrates from 69 unique taxa were identified and used for analyses in this study (Table 3.2). The greatest abundance of benthic macroinvertebrates was recorded in February (n= 9456), despite the fact that the waterbody at Teimen village was not sampled during this month, followed by June (n= 5854), November (n= 5016), April (n= 2829) and July (n=2083). The greatest number of different taxa collected was recorded in June (n= 54); followed by November (n= 50), April (n= 49), February (n= 46) and July (n= 42). The most abundant taxa throughout the entire study were mayflies (Ephemeroptera: Baetidae, n= 4030, 16%); followed by midges (Diptera: Chironomidae, n= 3725, 14.8%) and cladocerans (n= 2621, 10.4%). The majority of the cladocerans were collected from one waterbody during February (n= 2522), but were completely absent in all April collections and relatively few

were collected during June (n= 54), November (n= 5) and July (n= 40). Taxa dominance shifted throughout the season. Baetid mayflies were the most abundant taxa collected February, April and July (n= 983, 734, 418, respectively); however, mosquitoes (Diptera: Culicidae) were the dominant taxa in June (n= 896) and midges (Chironomidae) were the dominant taxa in November (n= 1241). Several taxa were found only during one season. The phantom midge (Diptera: Chaoboridae, n= 19) was only collected June 2007, but it occurred in three of six study sites that season. The collembolan family Entomobryiidae was collected during every season; however, only one specimen from the collembolan family Sminthuridae was collected during this entire study and it was collected during June, as were Sphaerid clams (n= 8) and polymitarcid mayflies (n= 7). In November, three beetle taxa (Elmidae, Gyrinidae and Hydrobiidae) were collected that were not found during the other sampling events.

There also were - taxa that were recorded from only one waterbody. Three taxa (Hemiptera: Saldidae, Neuroptera: Sysiridae, and Coleoptera: Elmidae) were only found in the waterbody at Nsakima village; two taxa (Collembola: Sminthuridae and Veneroida: Sphaeriidae) were only found in the waterbody at Kotoku village; and one taxa was only found in the waterbodies at Danfa (Ephemeroptera: Oligoneuridae), Afieman (Ephemeroptera: Polymitarcidae) and Teimen (Diptera: Simuliidae). Belostomatidae were completely absent from one site (Kotoku, Ga West) and Naucoridae were absent from two sites (Otinibi, Ga East and Afieman, Ga West). Together, these data demonstrate the importance of sampling multiple sites over a period of time to

more completely understand the macroinvertebrate communities in these waterbodies and to make more accurate associations between these communities and the ecology of BU disease.

Results for multiple linear regression analysis based on BU cases. Multiple linear regression analysis was run to investigate relationships between macroinvertebrate communities within waterbodies identified as either BU+ or BU- (SPSS 17.0). There was not a significant relationship between BU case data based on the combined group of macroinvertebrate metrics (Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness); R = .321, $R2$ = .103, F (6, 113) = 2.160, p = .052 (two-tailed). The R-squared value demonstrates that 10.3% of the variance in the macroinvertebrate metrics can be explained the by the presence or absence of BU cases. Table 3.3 presents a model summary of the multiple regression analysis of macroinvertebrate metric relationships with presence and absence of BU cases, and Table 3.4 shows the descriptive statistics from the regression analysis.

Results for multiple linear regression analysis for pathogen (ER+/ER-). Multiple linear regression analysis was run to investigate relationships between macroinvertebrate communities within waterbodies identified as either ER+ or ER- (SPSS 17.0). There was a significant relationship between ER presence and absence based on the combined group of macroinvertebrate metrics (Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness); R = .384, $R2$ = .148,

F (6, 113) = 3.26, *p* = .005 (two-tailed). The R-squared value demonstrates that 14.8% of the variance in the macroinvertebrate metrics can be explained the by presence or absence ER within waterbodies. Table 3.5 is a model summary of the multiple regression analysis of macroinvertebrate metric relationships with presence and absence of ER in waterbodies. The contribution of each predictor variable, when the others are controlled for, was evaluated using the standardized Beta for each coefficient. None of the individual variables made a statistically unique contribution to the model. Together, these results indicate that the combined macroinvertebrate metrics are potential predictors of *M. ulcerans*, but that not one individual metric is a predictor of the pathogen in the environment. Table 3.6 shows the descriptive statistics from the regression analysis.

Summary results for profile analyses. Repeated measures profile analyses were run to detect amount of shared variance and strength of relationship between macroinvertebrate community metrics and the independent variables: BU cases (BU+/BU-), pathogen (ER+/ER-), sampling season (June 2007, November 2007, February 2008, April 2008, July 2008) and individual waterbodies (n= 6)(SPSS 17.0). Results indicated there were significant differences in the dependant variables (Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness) depending on sampling season (n= 5) and individual waterbody (n= 6), but that there were no significant differences in the six macroinvertebrate metrics

between neither BU cases (BU+/BU-) nor pathogen (ER+/ER-)(p= .542)(Table 3.7).

Missing data and univariate outliers. A test for univariate outliers was conducted and none were found to exist within the distribution. Univariate outliers were sought by converting observed scores to *z*-scores and then comparing case values to the critical value of +/-3.29, $p < .001$. Case *z*-scores that exceed this value are greater than three standard deviations from the normalized mean. Missing data were investigated by running frequency counts (SPSS 17.0). No cases were missing, thus, 114 responses from participants were received and 114 were entered into the multiple regression models (n = 114). Before analysis, basic parametric assumptions were assessed. That is, for the criterion and predictor variables, assumptions of normality, linearity, and homoscedasticity of variance were evaluated. Results showed the variables to be normally distributed and assumed to meet parametric assumptions.

To examine the assumption of homogeneity of variance Box's M-Test of Equality of Covariance Matrices was run (Seber 1984). This test was run to determine if the dependent variable distributions were equal across the levels of the independent variable (BU+/BU-). Results from the test found that the distributions were not equal across groups for cases, F (21, 51212.449) = 7.846, $p < .001$. These results suggest that the two distributions were not equally distributed and therefore the homogeneity of variance assumption is not met.

Profile analysis for BU case data. Repeated measures profile analyses were run to detect amount of shared variance and strength of relationship between macroinvertebrate community metrics within waterbodies identified as either BU+ or BU- (SPSS 17.0). Using Wilks' criterion, the profiles did not significantly deviate from parallelism, F (5, 114) = 1.001, p = .420, partial eta-squared = .042. For the between-groups test, there were no statistically significant differences found for the dependent variables when scores were averaged over all BU case data; F (1,118) = 2.082, p = .152, partial-eta squared = .017. The partial eta-squared statistic means that 1.7% of the reason why the combined macroinvertebrate community metrics varied was due to the effect of the independent variable (BU cases). The test of within-subjects effects reveals that there are no significant differences in macroinvertebrate community metrics between the independent variable's (BU cases); F (5, 114) = .000, p = 1.00. In other words, the dependent variable measures (Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness) do not vary depending on the reported BU case data.

Missing data and univariate outliers. A test for univariate outliers was conducted and none were found to exist within the distribution. Univariate outliers were sought by converting observed scores to z-scores and then comparing case values to the critical value of +/-3.29, $p < .001$. Case z-scores that exceed this value are greater than three standard deviations from the normalized mean. Missing data were investigated by running frequency counts in SPSS (version 17.0). No cases were missing, thus, 114 responses from participants were

received and 114 were entered into the multiple regression models (n = 114). Before analysis, basic parametric assumptions were assessed. That is, for the criterion and predictor variables, assumptions of normality, linearity, and homoscedasticity of variance were evaluated. Results showed the variables were normally distributed and assumed to meet parametric assumptions.

To examine the assumption of homogeneity of variance Box's M-Test of Equality of Covariance Matrices was run. This test was run to determine if the dependent variable distributions were equal across the levels of the independent variable (ER+/ER-). Results from the test found that the distributions were not equal across groups for pathogen, F (df 21, 37359.566) = 10.630, $p < .001$. These results suggest that the two distributions were not equally distributed and therefore the homogeneity of variance assumption is not met.

Profile analysis for pathogen (ER+/ER-). Repeated measures profile analyses were run to detect amount of shared variance and strength of relationship between macroinvertebrate community metrics within waterbodies identified as either ER+ or ER- (SPSS 17.0). Using Wilks' criterion, the profiles did not deviate significantly from parallelism, F (5, 114) = .140, p = .983, partial eta-squared = .006. For the between-groups test, there were no statistically significant differences found for the dependent variables when scores were averaged for ER+ and ER- waterbodies; F (1,118) = .140, p = .339, partial-eta squared = .008. The partial eta-squared statistic means that 0.8% of the reason why the combined macroinvertebrate community metrics varied was due to the effect of the independent variable (ER). The test of within-subjects effects

reveals that there are no significant differences between dependant variables and independent variables; F (5, 114) = .140, p < .983. In other words, the macroinvertebrate metrics (Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness) did not vary between waterbodies that were either ER+ or ER-.

Profile analysis of macroinvertebrate associations with Season. All cases were examined for accuracy and found to be correctly recorded. Further, there were no cases with missing values. A test for univariate outliers was conducted for each group and none were found to exist within the distributions; thus, 120 responses were received and 120 were entered into the Profile Analysis model; n = 120. There were a number of variables that were either skewed, kurtotic, or both (Table 3.8). Normality of the distributions is assumed when z-skew coefficients were less than the critical value of +/- 3.29 (Tabachnick and Fidel 2008). In addition, the variables differed in ranges of scores. There are some tests of profile analysis that evaluate difference of scores in the dependent variables, making the scaling of the variables important. For this reason, dependent variables were standardized into z-scores around the mean for this analysis.

To examine the assumption of homogeneity of variance Box's M-Test of Equality of Covariance Matrices was run (Seber 1984). This test was run to determine if the dependent variable distributions were equal across the levels of the independent variable. Results from the test found that the distributions were not equal across groups, F (df 84, 25266.805) = 4.7, p < .001. These results

suggest that the two distributions were not equally distributed and therefore the homogeneity of variance assumption is not met.

Profile analysis based on season. Repeated measures profile analyses were run to detect amount of shared variance and strength of relationship between macroinvertebrate community metrics and sampling season (n= 5)(SPSS 17.0)(Figure 3.1). Using Wilks' criterion, the profiles deviated significantly from parallelism, F (20, 369.095) = 3.08, p < .001, partial eta-squared = .120. For the between-groups test, no statistically significant differences were found among groups (sampling season) when scores were averaged over all seasons F (4, 115) = 2.14, p = .08, partial-eta squared = .069. The partial eta-squared statistic means that 6.9% of the reason why the combined macroinvertebrate community metrics varied was due to the independent variable (sampling swason); F (5, 111) = .000, p = 1.0. In other words, while the grouped macroinvertebrate metrics were useful as predictors of sampling season, the macroinvertebrate metrics individually were not (Table 3.8).

Missing data and univariate outliers. All cases were examined for accuracy and found to be correctly recorded. Further, there were no cases with missing values. A test for univariate outliers was conducted for each group and none were found to exist within the distributions; thus, 114 responses were received and 114 were entered into the Profile Analysis model; n = 114. There were a number of variables that were either skewed, kurtotic, or both (Table 3.8). Normality of the distributions is assumed when z-skew coefficients were less than the critical value of +/- 3.29 (Tabachnick & Fidel, 2008). In addition, the

variables differed in ranges of scores. There are some tests of profile analysis that evaluate difference of scores in the dependent variable, making the scaling of the variables important. For this reason, dependent variables were standardized into z-scores around the mean for this analysis.

To examine the assumption of homogeneity of variance Box's M-Test of Equality of Covariance Matrices was run (Seber 1984). This test was run to determine if the dependent variable distributions were equal across the levels of the independent variables (waterbodies, n= 5). Results from the test found that the distributions were not equal across groups, F (df 105, 20867.275) = 5.89, $p <$ .001. These results suggest that the two distributions were not equally distributed and therefore the homogeneity of variance assumption is not met.

Profile analysis based on waterbody. Repeated measures profile analyses were run to detect amount of shared variance and strength of relationship between macroinvertebrate community metrics and individual waterbodies (n= 6)(SPSS version 17.0)(Figure 3.2). Using Wilks' criterion, the profiles deviated significantly from parallelism, F (25, 410.134) = 2.60, $p <$ .001, partial eta-squared = .104. For the between-groups test, there were statistically significant differences found among groups (waterbody) when scores were averaged over all sites F (5, 114) = 4.963, $p <$ .001, partial-eta squared = .179. The partial eta-squared statistic means that 17.9% of the reason why the combined macroinvertebrate community metrics varied was due to the effect of waterbody. The test of within-subjects effects reveals that there are no significant differences in the individual macroinvertebrate metrics between each

of the waterbodies; F (5, 110) = .000, p = 1.00, partial-eta squared = .000. In other words, while the grouped macroinvertebrate metrics were useful as predictors of different waterbodies, the macroinvertebrate metrics individually were not.

Macroinvertebrate taxa correlations with BU cases. Significant positive and negative Pearson correlations ($P < 0.05$) occurred between macroinvertebrate taxa collected within waterbodies from villages with reported cases of Buruli ulcer (Table 3.9). Significant positive correlations were observed between Buruli ulcer cases and total abundance of Caenidae (r= .214, $P < 0.01$), Chironomidae (r= .256, $P < 0.01$), Coenagrionidae (r= .280, $P < 0.01$), Hydraenidae (r= .191, $P < 0.05$), Naucoridae (r= .202, $P < 0.05$), Noteridae (r= .386, $P < 0.01$), Physidae (r= .246, $P < 0.01$), Planorbidae (r= .215, $P < 0.05$), Pleidae (r= .368, $P < 0.01$) and Scirtidae (r= .232, $P < 0.01$). Significant negative correlations were observed between Buruli ulcer cases and total abundance of Atyidae (r= -.261, $P < 0.01$) and Gerridae (r= -.341, $P < 0.01$).

Macroinvertebrate taxa correlations with pathogen. Significant positive and negative Pearson correlations ($P < 0.05$) occurred between macroinvertebrate taxa and waterbodies that were ER+ and ER- (Table 3.9). Significant positive correlations were observed between pathogen presence, based on ER detection from the waterbody, and total abundance of Atyidae (r= .213, $P < 0.05$) and Gerridae. Significant negative correlations were observed between pathogen presence, based on ER detection from the waterbody, and total abundance of Anclyidae (r= -.272, $P < 0.01$), Chironomidae (r= -.221, $P <$

0.05), Dytiscidae (r= -.234, $P < 0.01$), Hydraenidae (r= -.245, $P < 0.01$), Hydrophilidae (r= -.241, $P < 0.01$), Naucoridae (r= -.259, $P < 0.01$), Noteridae (r= -.300, $P < 0.01$), Planorbidae (r= -.184, $P < 0.05$) and Pleidae (r= -.195, $P < 0.05$).

Seasonal distributions and composition of Hemiptera taxa.

Hemiptera taxa abundances were relatively low throughout the entire study (n= 1571) and represented only 6.2% of the total macroinvertebrates collected. The greatest abundances of Hemiptera were recorded during the February (n= 411) collections; however, all other macroinvertebrate taxa were also collected in greater proportions during this season and total Hemipterans only compromised 4.3% of the February collections. The months with the greatest concentrations (relative abundance) of Hemipterans compared to all specimens collected were April (12.3%) and July (10.7%), whereas the concentrations of Hemiptera in June (5.5%), November (5.4%) and February (4.3%) were relatively low. Eleven families of Hemiptera were recorded during this study (Table 3.2), eight of which (Belostomatidae, Gerridae, Hydrometridae, Mesoveliidae, Naucoridae, Nepidae, Notonectidae and Pleidae) were collected in every month throughout the study period. Of the remaining three families, Veliidae and Corixidae were collected every month with the exception of July, and Hebridae were only collected in June and April. When only looking at the Hemiptera taxa composition, the most abundant families were the Notonectidae (29.9%) and Pleidae (26.5%), followed by Mesoveliidae (13.3%) and Gerridae (12.6%). Belostomatidae and Naucoridae were less abundant at 7.2% and 3.7%, respectively.

There were marked differences in Hemiptera composition between waterbodies sampled. The waterbody in Teimen village had the highest proportion of water bugs over all seasons compared to all other taxa (25.8%), whereas the proportion of water bugs collected from waterbodies in Otinibi (3.2%) and Nsakima (3.5%) villages were much lower. Of the 6 waterbodies sampled during this study, only the waterbody in Danfa village produced all eleven Hemiptera taxa. Naucoridae were never collected at Afieman and Nsakima village, and Belostomatidae were never collected at Kotoku village.

DISCUSSION

Aquatic macroinvertebrates have been proposed by several authors to be possible reservoirs of *M. ulcerans* or vectors of the pathogen to humans (Merritt et al. 2010). Despite these potential connections with both the ecology of *M. ulcerans* and BU transmission, standardized ecological studies aimed to investigate macroinvertebrate community associations with the pathogen and disease are limited (Benbow et al. 2008, Merritt et al. 2010). To date, most field studies investigating aquatic invertebrates have primarily targeted specific taxa, and sampling strategies have been either qualitative or lack adequate replication. An initial step in understanding the role aquatic macroinvertebrates might play in the ecology of BU, whether direct or indirect, is identifying the distribution and composition of the entire macroinvertebrate community in relation to the disease and disease pathogen. As part of a standardized assessment of the temporal patterns of macroinvertebrate communities, I surveyed 6 waterbodies selected from villages that were known to have reported cases of BU (n= 3) and villages

with no previous record of BU (n= 3), in Ghana, West Africa to characterize and compare overall macroinvertebrate seasonal variation, community metrics, with the presence and absence of BU cases and *M. ulcerans* within these environments.

Results generated from this study identified no significant relationship between macroinvertebrate community measurements in relation to BU+ and BU- villages. In other words, the aquatic macroinvertebrate communities in waterbodies within villages reporting at least one case of BU, prior to the beginning of this study, were similar to those in waterbodies from villages reporting no cases of BU. This result is comparable to what was reported by Benbow et al. (2008), who conducted survey studies of aquatic macroinvertebrate communities in endemic and non-endemic areas of southern Ghana. In central Cameroon, Marion et al. (2010) report higher abundances of Hemiptera taxa (water bugs) from a BU endemic stretch of the Nyong River than what were found in a stretch of the same river associated with a village identified as BU non-endemic. Marion et al. (2010) and I both present data that indicate greater concentrations of macroinvertebrates during dry seasons; however, while I found higher abundance of total macroinvertebrates in waterbodies from endemic areas, water bug total abundances and percent composition were highest in the non-endemic areas. One possible explanation for the differences in taxa abundances and composition between these two studies was that macroinvertebrate communities were expected to differ in relation to water body and habitat availability. My field sites were all lentic (slow-flowing) habitats,

whereas the study site sampled by Marion et al. (2010) was a large river characterized by flowing water (lotic). Together, these variations suggest additional collection sites should be included in order for a more comprehensive evaluation of invertebrate communities in relation to BU endemicity.

My profile analysis of macroinvertebrate community metrics in association with the presence and absence of *M. ulcerans*, based on presumptive ER testing, also generated no statistically significant relationships across seasons. The multiple linear regression model indicated a significant relationship between combined macroinvertebrate metrics and *M. ulcerans*, but not one individual metric could be used as a predictor of the presence of *M. ulcerans* in the environment. The significant result of the combined community analyses suggests a complex biological system, and points toward the importance for future studies to include collections of the entire macroinvertebrate community to elucidate associations between biological communities and BU disease.

The only other study that compared macroinvertebrate community measurements with the presence of *M. ulcerans* in the environment was conducted in a companion study to this and results were reported in Chapter 2 of this dissertation. In that study, using multivariate analyses (NMDS), I found no significant relationships between the macroinvertebrate community and presence of environmental *M. ulcerans* in lentic habitats, as was the case in the current study. There were, however, significant differences in the macroinvertebrate community profile and bioassessment metrics in relation to *M. ulcerans* in lotic habitats. This further underlines the importance of considering habitat for future

development of proper experimental designs and when analyzing data regarding macroinvertebrate communities in relation to *M. ulcerans* and BU endemicity.

I identified significant relationships between macroinvertebrate community metrics and both season and individual waterbodies during the current study. In both cases, the profile analysis of grouped means was significant but no individual macroinvertebrate metrics could be identified as good indicators of either season or waterbody. Variation in macroinvertebrate distribution and taxa composition between and among waterbodies can be due to several factors; including, physical, chemical, and biological parameters (USEPA 1996, 2001; Merritt et al. 2008). In Malawi, central Africa, McLachlan (1975) investigated the role of aquatic macrophytes in the recovery of the benthic fauna after a dry season and found associations between macroinvertebrate taxa and specific plant communities. Prior to that, Petr (1968) reported differences in macroinvertebrate taxa composition and abundance in relation to the habitats provided by the floating *Pistia stratiotes* and submersed *Ceratophyllum demersum* in Volta Lake (Ghana, West Africa). Aquatic plants have also been shown in the laboratory to promote biofilm and *M. ulcerans* development (Marsolier et al. 2004b), and associations between macrophyte communities and the presence of environmental *M. ulcerans* were reported by McIntosh et al. (submitted 2010). General macrophyte community measurements (dominant taxa, percent surface coverage) were collected during my study; however, the results reported here were based entirely on macroinvertebrate community assemblages in relation to BU disease parameters. Additional analyses

incorporating the abiotic and biotic measurements recorded during this study are under way.

A focal interest of this study was to investigate temporal variations in macroinvertebrate community distributions and compositions in relation to *M. ulcerans* and BU disease. While strong relationships were not found between macroinvertebrate communities and pathogen or BU case data, there were significant relationships between macroinvertebrates and season. In this study, overall macroinvertebrate abundances were greatest in the February sampling event, which for southern Ghana represents a dry season. Marion et al. (2010) reported high densities of Hemiptera densities from Cameroon during collections made in January, and studies on the life histories of Naucoridae in Costa Rica showed a similar pattern (Stout 1981, 1982). The work by Stout on Naucoridae indentified water bug distribution patterns that varied due to season and environmental condition, but it was also concluded that seasonal patterns in total abundance did not exist due to the presence of all life stages of Naucoridae that were collected throughout the year (Stout 1981). Asynchronous life history patterns are often a characteristic of aquatic macroinvertebrates in tropical environments (Merchant and Yule 1996; Huryn and Wallace 2000).

I found variation in the frequency and composition of Hemiptera taxa throughout the sampling period, but in general Hemipterans only represented a small percent (6.2%) of the total macroinvertebrate community, and in February the percent composition of hemipterans dropped to 4.3% of the total community. When comparing the composition of the entire hemipteran community,

Belostomatidae and Naucoridae represented a small percentage of the total Hemiptera taxa composition at 7.2% and 3.7%, respectively. Benbow et al. (2008) found comparable abundance and composition patterns in Hemiptera taxa during their investigation of macroinvertebrate communities in Ghana, and concluded that these data do not rule out the possibility of biting Hemiptera or other invertebrates as vectors or possible reservoirs for *M. ulcerans*, but that caution should be used in describing their role in transmission based solely on abundance patterns.

Belostomatidae and Naucoridae have received the most attention from researchers in West Africa as potential vectors of *M. ulcerans*, and in my study the abundances of these two taxa were low and in some cases these water bugs were missing completely from entire waterbodies. Belostomatidae were completely absent from one site (Kotoku, Ga West) and Naucoridae were absent from two sites (Otinibi, Ga East and Afieman, Ga West), despite sampling each waterbody 5 times over the period of one year. Future investigators of macroinvertebrate associations with BU disease should consider the potential spatial and temporal variations in the entire community composition, as well as proper replication of study sites, in order to enhance the accuracy of statements that can be drawn from their studies.

Pearson correlation's identified several individual macroinvertebrate taxa that were correlated with either reported cases of BU, the presence and absence of environmental *M. ulcerans*, based on the presumptive ER test, or both. Of particular interest, considering their proposed associations with BU disease, are

the correlations found with Belostomatidae and Naucoridae. Belostomatid water bugs were found to be negatively correlated with BU case data, which differs from what has been found elsewhere (Marsoilier et al. 2004; Marion et al. 2010) and is not what would be expected if these insects are intimately involved with BU disease transmission, as outlined by Benbow et al. (2008). Naucorid water bugs on the other hand were positively correlated with BU cases data, but were also negatively correlated with the presence of *M. ulcerans*. This pattern of positively correlated with BU cases and at the same time negatively correlated with *M. ulcerans*, or negatively correlated with BU and positively correlated with *M. ulcerans* was found throughout the results from these analyses. There are multiple rationales that could explain these relationships; however, the Interpretation of these results should be made with caution.

First, the relatively low numbers of some of these taxa, particularly the Belostomatidae and Naucoridae which were missing completely from sites, might be contributing to decreased power and inability to detect a true effect (Kendall and Gibbons 1990). Second, although a number of taxa were identified as predictors of BU cases (n= 13) and *M. ulcerans* (n= 11), several more (n= 69) were used for this analysis and there is potential for unwarranted artifacts to emerge as a result (Kendall and Gibbons 1990). Finally, correlations between two variables do not automatically mean that they are directly associated with each other (correlation versus causation). What these results do is offer insight into the potential associations between specific macroinvertebrate taxa and BU disease ecology, which suggest a complex interaction between BU and biological

communities. Furthermore, these results provide a framework for further studies to be directed toward elucidating these relationships.

Epidemiological studies have identified patterns in the emergence of BU cases that can be attributed to seasonal variation (Daire et al. 1993, Portaels 1989, Meyers et al. 1996; Johnson et al. 2007), and empirical data suggest that seasonal patterns in rainfall and subsequent flooding may provide environmental conditions that are favorable for the establishment and proliferation of *M. ulcerans* (Hayman 1991b; Portaels 1999; Merritt et al. 2005, Williamson et al. 2008; McIntosh et al. submitted). For these reasons, and the proposed associations between aquatic macroinvertebrates and BU disease, an area of interest should be to develop a more complete understanding of the seasonal variations in macroinvertebrate communities within BU endemic and non-endemic areas. In this study, I identified variations in macroinvertebrate distributions and compositions in relation to both waterbody and season, and also identified individual taxa that show potential associations with both BU and *M. ulcerans*. Results from this study should be used as a framework to aid in the development of forthcoming studies with the goal of examining associations between macroinvertebrate communities, M. ulcerans, and BU.

It should be noted that the case data used in this study was obtained through passive surveillance practices, provided by the Ghana Ministry of Health, and may not reflect the true disease incidence. Likewise, many of the associations made between macroinvertebrates and *M. ulcerans* were based on the presumptive ER test, which might over-estimate the true distribution of *M.*

ulcerans in the environment. These data were also interpreted under the assumption that the waterbodies where the bacteria were not detected were in fact *M. ulcerans* negative sites. While these factors should not be overlooked, neither should the potential value that the information this study provides to scientists involved with BU research.

Table 3.1. Locations and general information for each of the six waterbodies used in the seasonal analysis of macroinvertebrate associations with BU and *M. ulcerans*. MW = modified wetland; MP = modified pond; BU- = no cases of BU had been reported from the village; and BU+ = at least 3 cases of BU had previously been reported from the village.

District	Village	Latitude	Longitude	Type	Dominant Macrophytes	BU+/BU-
Ga East	Otinibi	N 05.78889	W 000.14359	MW	Typha, Ludwigia,	BU-
Ga East	Danfa	N 05.78715	W 000.16475	MW	Brachiaria, Echinocloa,	BU-
Ga East	Teimen	N 05.75120	W 000.18143	MP	Paspalum, Cyperus	BU-
Ga West	Afieman	N 05.70809	W 000.28563	MP	Typha	BU+
Ga West	Kotoku	N 05.74115	W 000.35398	MP	Typha, Cyperus	BU+
Ga West	Nsakima	N 05.65478	W 000.32026	MW	Typha, Pistia	BU+

Table 3.2. Macroinvertebrate taxa and total specimens collected June 2007 to July 2008.

Taxon (Higher, Lowest)		June 2007	Nov. 2007	Feb. 2008	April 2008	July 2008	Total Spec.
Annelida							
Clitellata, Hirudinea		15	16	17	4	18	70
Clitellata, Oligochaeta		342	94	218	30	30	714
Arthropoda							
Arachnida, Araneae		94	136	86	73	59	448
Arachnida, Hydracarina		211	23	93	59	16	402
Crustacea, Branchiopoda	Cladocera	54	5	2522	0	40	2621
Crustacea, Decapoda	Atyidae	0	3	8	3	9	23
Crustacea, Maxillopoda	Copepoda	44	5	15	1	13	78
Crustacea, Ostracoda	Ostracoda	185	29	687	26	43	970
Insecta, Coleoptera	Curculionidae	4	8	16	0	4	32
	Dytiscidae	99	45	121	41	51	357
	Elmidae	0	1	0	0	0	1
	Gyrinidae	0	2	0	0	0	2
	Hydraenidae	4	3	495	0	3	505
	Hydrobiidae	0	1	0	0	0	1
	Hydrophilidae	98	93	957	45	41	1234

Table 3.2 (cont'd). Macroinvertebrate taxa and total specimens collected June 2007 to July 2008.

Insecta, Coleoptera	Lampyridae	2	0	0	4	0	6
	Noteridae	267	93	312	107	63	842
	Scirtidae	2	15	2	32	5	56
Insecta, Collembola	Entomobryiidae	28	22	24	28	12	114
Insecta, Diptera	Ceratopogonidae	192	105	88	3	14	402
	Chaoboridae	19	0	0.0	0.0	0	19
	Chironomidae	804	1241	982	386	312	3725
	Culicidae	896	500	147	66	67	1676
	Empididae	0	0	0.0	1.0	0	1
	Ephydridae	1	4	0.0	2.0	0	7
	Sciomyzidae	14	4	3.0	5.0	9	35
	Simuliidae	1	0	0	0	0	1
	Stratiomyiidae	5	5	24	11	6	51
	Syrphidae	0	1	0.0	5.0	0	6
	Tipulidae	2	2	2.0	1.0	0	7
Insecta, Ephemeroptera	Baetidae	687	1208	983	734	418	4030
	Caenidae	14	272	147	6	33	472
	Polymitarcyidae	7	0	0.0	0.0	0	7
Insecta, Hemiptera	Belostomatidae	26	32	20	21	14	113
	Corixidae	7	3	8	3	0	21
	Gerridae	55	48	30	53	35	221
	Hebridae	6	0	0	13	0	19
	Hydrometridae	9	3	1	3	1	17

Table 3.2 (cont'd). Macroinvertebrate taxa and total specimens collected June 2007 to July 2008.

	Mesoveliidae	23	39	60	51	38	211
	Naucoridae	4	28	13	7	6	58
	Nepidae	3	2	4	8	2	19
	Notonectidae	57	67	105	165	76	470
	Pleidae	153	39	149	26	50	417
	Saldidae	0	1	0	0	0	1
	Veliidae	1	8	23	1	0	33
Insecta, Odonata	Coenagrionidae	158	244	268	253	273	1196
	Corduliidae	524	11	4	60	66	665
	Libellulidae	269	160	247	20	6	702
	Protoneuridae	112	200	111	70	28	521
Mollusca							
Bivalvia, Veneroida	Sphaeriidae	0	8	0	0	0	8
Gastrpoda	Ancylidae	19	0	1	2	0	22
	Bithyniidae	1	0	0	0	9	10
	Lymnaeidae	0	24	58	2	15	99
	Physidae	26	19	30	102	72	249
	Pilidae	1	1	0	0	1	3
	Planorbidae	277	139	347	179	107	1049
	Thiaridae	10	3	10	40	2	65

Table 3.3. Model summary of the multiple regression analysis of macroinvertebrate metric relationships and between the presence and absence of BU cases, within the six seasonal study sites.

Regression Model Detail							
	R	R-Squared	Std. Error	*F*	Sig		
Omnibus Model	0.321	0.103	0.488	2.16	0.052		
			Unstandarized Coefficients		Standardized Coefficients	*t*	Sig.
			B	Std. Error	Beta		
(Constant)			0.500	0.045		11.223	0.000
ZTotal Specimens			-0.008	0.078	-0.017	-0.107	0.915
ZTotal Taxa			0.516	0.216	1.028	2.393	0.018
ZShannon Diversity			-0.280	0.187	-0.557	-1.496	0.137
ZSimpsons			-0.439	0.306	-0.874	-1.436	0.154
ZMargalef			-0.111	0.154	-0.221	-0.719	0.473
ZEvenness			0.594	0.284	1.183	2.092	0.039

Table 3.4. Descriptive statistics for regression analysis of macroinvertebrate metrics and the presence and absence of BU cases. BU- = no cases of BU reported prior to the beginning of this study and BU+ = at least 3 cases of BU reported prior to the beginning of this study.

Variable	Min	Max	Mean	Std. Dev	Skew	*z*-Skew	Kurtosis	*z*-Kurtosis
BU-								
Total Spec.	0	1089	132.517	176.968	3.504	11.352	15.782	25.936
Total Taxa	0	28	14.350	6.709	-0.265	-0.858	-0.234	-0.385
Shannon	0	2.68	1.957	0.587	-2.485	-8.050	6.246	10.264
Simpsons	0	1	0.785	0.220	-3.130	-10.141	9.057	14.885
Margalef	0	5.01	2.962	1.047	-1.261	-4.084	2.398	3.942
Evenness	0	1	0.745	0.219	-2.611	-8.458	6.928	11.386
BU+								
Total Spec.	7	3018	288.117	471.805	3.985	12.910	19.603	32.216
Total Taxa	4	31	17.117	6.633	0.036	0.115	-0.438	-0.721
Shannon	0.63	2.7	2.020	0.401	-1.057	-3.424	1.708	2.807
Simpsons	0.31	0.91	0.794	0.116	-2.118	-6.862	5.556	9.130
Margalef	1.04	4.79	3.177	0.790	-0.690	-2.235	0.571	0.938
Evenness	0.46	0.96	0.739	0.113	-0.475	-1.538	-0.143	-0.236

Note. Absent: $n = 60$, Std. Error Skew = .309, Std. Error Kurtosis = .608
Note. Present: $n = 60$, Std. Error Skew = .309, Std. Error Kurtosis = .608

Table 3.5. Model summary of the multiple regression analysis of macroinvertebrate metric relationships with presence and absence of ER, within the six seasonal study sites.

Regression Model Detail							
	R	R-Squared	Std. Error	*F*	Sig		
Omnibus Model	0.384	0.148	0.466	3.26	0.005		
			Unstandarized Coefficients		Standardized Coefficients	*t*	Sig.
			B	Std. Error	Beta		
(Constant)			0.600	0.043		14.101	0.000
ZTotal Specimens			-0.135	0.074	-0.274	-1.811	0.073
ZTotal Taxa			0.343	0.206	0.698	1.666	0.099
ZShannon			-0.268	0.179	-0.545	-1.500	0.136
ZSimpsons			-0.060	0.292	-0.122	-0.206	0.837
ZMargalef			-0.166	0.147	-0.338	-1.129	0.261
ZEvenness			0.418	0.271	0.849	1.540	0.126

Table 3.6. Descriptive statistics for regression analysis of macroinvertebrate metrics and the presence and absence of ER within each waterbody during the entire study period.

Variable	Min	Max	Mean	Std. Dev	Skew	z-Skew	Kurtosis	z-Kurtosis
ER Absent								
Total Spec.	0	3018	289.375	521.995	3.719	10.838	16.328	24.212
Total Taxa	0	30	16.500	7.389	-0.398	-1.159	0.395	0.585
Shannon	0	2.53	1.913	0.633	-2.348	-6.843	5.005	7.421
Simpsons	0	0.91	0.738	0.237	-2.601	-7.581	5.915	8.770
Margalef	0	4.39	3.064	1.081	-1.821	-5.307	3.333	4.942
Evenness	0	0.9	0.676	0.224	-2.295	-6.689	4.951	7.342
ER Present								
Total Spec.	4	1089	157.611	183.893	2.599	9.186	9.325	16.687
Total Taxa	4	31	15.222	6.356	0.084	0.298	-0.746	-1.334
Shannon	0.63	2.7	2.039	0.388	-1.042	-3.682	1.772	3.170
Simpsons	0.31	1	0.824	0.106	-2.724	-9.627	9.887	17.692
Margalef	1.04	5.01	3.073	0.823	-0.185	-0.653	0.121	0.217
Evenness	0.46	1	0.785	0.112	-0.787	-2.782	0.819	1.466

Note. Absent: $n = 48$, Std. Error Skew = .434, Std. Error Kurtosis = .674
Note. Present: $n = 72$, Std. Error Skew = .283, Std. Error Kurtosis = .559

Table 3.7. Summary table indicating that two of four hypotheses reached statistically significant differences for the repeated measures profile analyses. The dependent variables were total macroinvertebrate abundance, total taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness, and Pielou's Eveness. The significant differences were between the dependent variables and both season and waterbody.

Dependent Variables	Independent Variables	Analysis	Sig.
Macroinvertebrate Community Metrics (n= 6):	Season (n= 5)	Profile Analysis	$p < .001$
	Waterbody (n= 6)	Profile Analysis	$p < .001$
Total Abundance, Total Taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness and Pielou's Eveness	BU Cases	Profile Analysis	$p = .420$
	Pathogen	Profile Analysis	$p = .982$

Table 3.8. Descriptive statistics for the repeated measures profile analysis of macroinvertebrate metrics. Results presented here are for both season and waterbody.

Dependent Variable	Min	Max	Mean	Std. Dev	Skew	z-Skew	Kurtosis	z-Kurtosis
Total Spec.	.00	3018.00	210.317	363.312	4.921	22.278	31.787	72.518
Total Taxa	.00	31.00	15.733	6.787	-.115	-0.522	-.221	-0.503
Shannon-Weiner Diversity	.00	2.70	1.988	.502	-2.276	-10.303	6.639	15.147
Simpson's Heterogeneity	.00	1.00	.789	.175	-3.368	-15.249	12.334	28.138
Margalef's Richness	.00	5.01	3.070	.930	-1.179	-5.337	2.450	5.589
Pielou's Evenness	.00	1.00	.742	.174	-2.589	-11.722	9.041	20.626

Note. n = 120, Std. Error Skew = .221, Std. Error Kurtosis = .438

Table 3.9. Significant positive and negative correlations between taxa and BU cases and ER.

Macroinvertebrate Taxa (Family)	BU Cases +/-	ER +/-
Anclyidae	ns	r= -.272, *P* < 0.01
Atyidae	r= -.261, *P* < 0.01	r= .213, *P* < 0.05
Belostomatidae	r= -.280, *P* < 0.01	ns
Caenidae	r= .214, *P* < 0.01	ns
Chironomidae	r= .256, *P* < 0.01	r= -.221, *P* < 0.05
Coenagrionidae	r= .280, *P* < 0.01	ns
Dytiscidae	ns	r= -.234, *P* < 0.01
Gerridae	r= -.341, *P* < 0.01	r= .237, *P* < 0.01
Hydraenidae	r= .191, *P* < 0.05	r= -.245, *P* < 0.01
Hydrophilidae	ns	r= -.241, *P* < 0.01
Naucoridae	r= .202, *P* < 0.05	r= -.259, *P* < 0.01
Noteridae	r= .386, *P* < 0.01	r= -.300, *P* < 0.01
Physidae	r= .246, *P* < 0.01	ns
Planorbidae	r= .215, *P* < 0.05	r= -.184, *P* < 0.05
Pleidae	r= .368, *P* < 0.01	r= -.195, *P* < 0.05
Scirtidae	r= .232, *P* < 0.01	ns

ns: nonsignificant

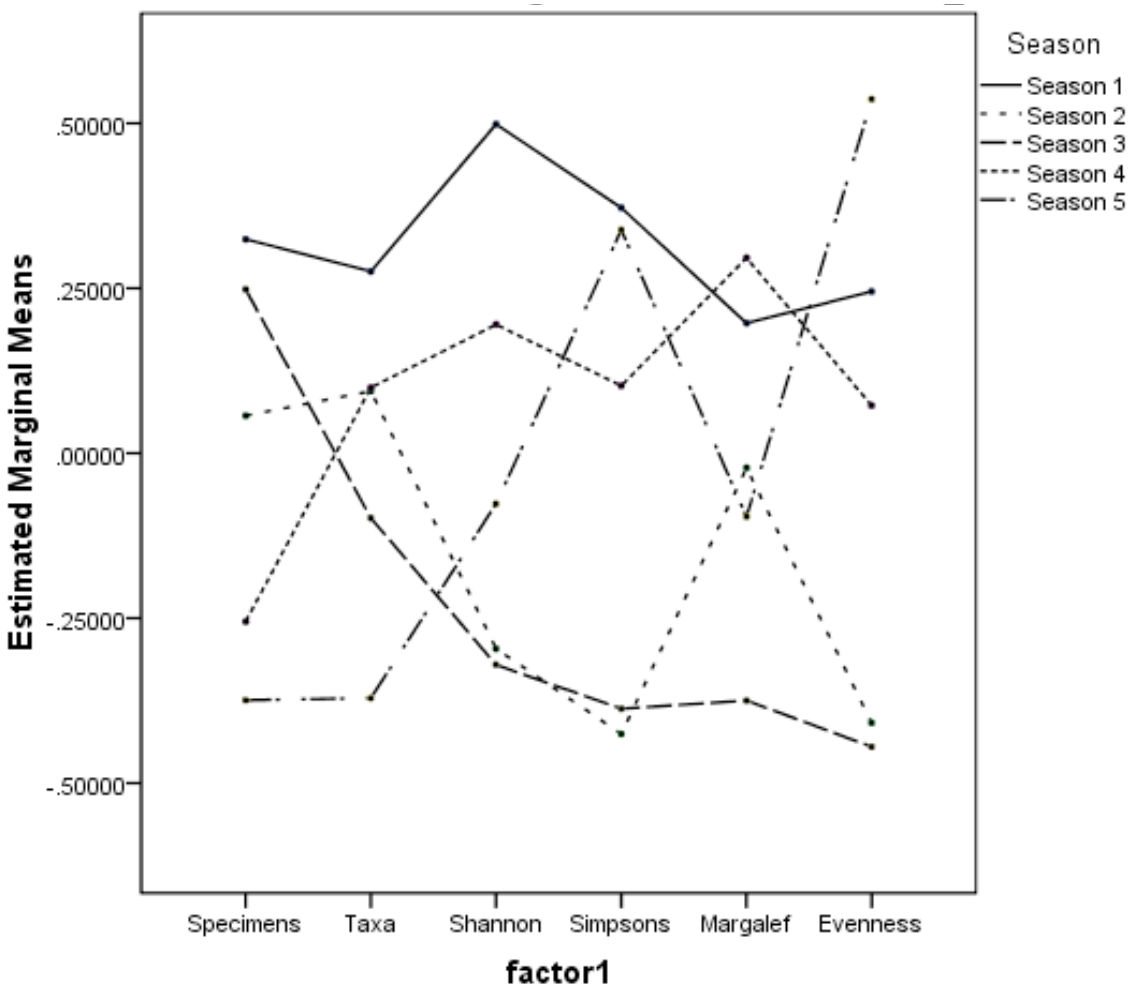


Figure 3.1. Estimated marginal means for six macroinvertebrate metrics across five sampling seasons. The macroinvertebrate metrics were total specimens, total taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness, and Pielou's Eveness. The sampling seasons are listed as Season 1 (June 2007), Season 2 (November 2007), Season 3 (February 2008), Season 4 (April 2008), and Season 5 (July 2008).

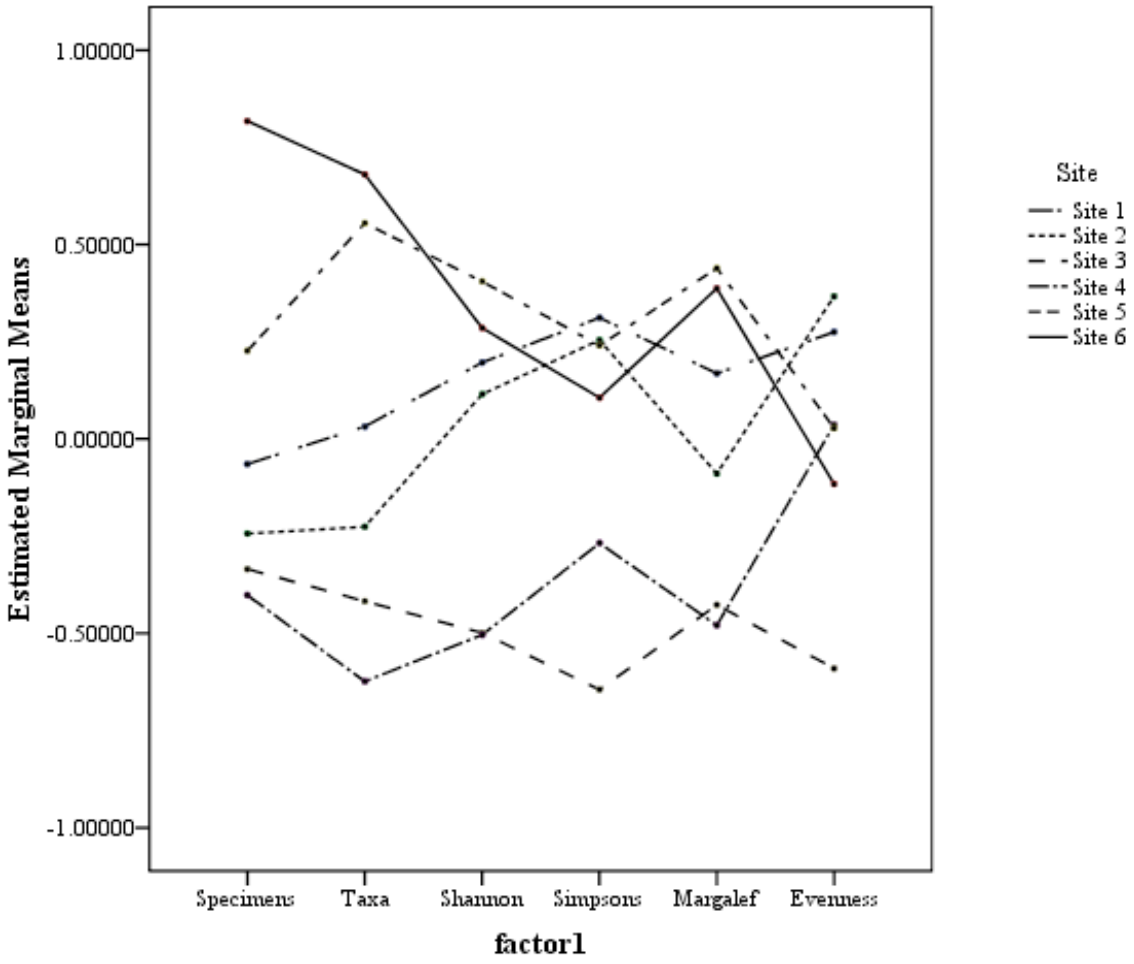


Figure 3.2. Estimated marginal means for six macroinvertebrate metrics across six waterbodies. The macroinvertebrate metrics were total specimens, total taxa, Shannon-Weiner Diversity, Simpson's Heterogeneity, Margalef's Richness, and Pielou's Eveness. The were from the following villages: Site 1=Otinibi, Site 2=Danfa, Site 3=Teimen, Site 4= Afieman, Site 5=Kotoku, Site 6=Nsakima.

LITERATURE CITED

Literature Cited

Ablordey A, J Swings, C Hubans, K Chemlal, C Locht, F Portaels, P Supply (2005) Multilocus variable-number tandem repeat typing of *Mycobacterium ulcerans*. *J Clin Microbiol* 43, 1546–1551.

Aiga H, Amano T, Cairncross S, Domako JA, Nanas OK, et al. (2004) Assessing water-related risk factors for Buruli ulcer: A case-control study in Ghana. Am J Trop Med Hyg 71: 387–392.

Barker DJP (1973) Epidemiology of Mycobacterium ulcerans infection. Trans R Soc Trop Med Hyg. 67:(1)43.

Barker DJP, Carswell JW (1973) Mycobacterium ulcerans infection among Tsetse control workers in Uganda. International Journal of Epidemiology 2: 161–165.

Batzer DP, SA Wissinger (1996) Ecology of insect communities in nontidal wetlands. Annu Rev Entomol. 41:75–100.

Benbow M, Williamson H, Kimbirauskus R, McIntosh M, Kolar R, et al. (2008) Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis 14: 1247–1254.

Darie H, T Le Guyadec, JE Touze (1993) "Aspects épidémiologiques et cliniques de l'ulcère de Buruli en Côte-d'Ivoire". *Bull Soc Pathol Exot Filiales* 86: 272–276.

Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, et al. (2004) Mycobacterium ulcerans disease (Buruli ulcer) in rural hospital, Southern Benin, 1997-2001. Emerg Infect Dis 10: 1391–1398.

Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, et al. (2006) Risk factors for Buruli ulcer, Benin. Emerg Infect Dis 12: 1325–1331.

Dobson M, Magana AM, Lancaster J, Mathooko JM. (2007) Aseasonality in the abundance and life history of an ecologically dominant freshwater crab in the Rift Valley, Kenya. Freshw Biol. 52:215–25.

Donohue I, Irvine K. (2004) Seasonal patterns of sediment loading and benthic invertebrate community dynamics in Lake Tanganyika, Africa. Freshw Biol. 49:320–31.

Duker AA, Carranza JM, Hale M (2004) Spatial dependency of Buruli ulcer prevalence on arsenic-enriched domains in Amansie West District, Ghana: implications for arsenic mediation in *Mycobacterium ulcerans* infection. Int J Health Geogr. 3:19.

Duker AA, Portaels F, Hale M (2006) Pathways of Mycobacterium ulcerans infection: A review. Environment International 32: 567–573.

Durand, JR, Leveque, C (1981) *Flore et Faune Aquatiques de l'Afrique Sahelo-Soudaniene,* 2 volumes. ORSTOM, Paris.

Fyfe JAM, Lavender CJ, Johnson P, Globan M, Sievers A, et al. (2007) Development and Application of Two Multiplex Real-Time PCR Assays for the Detection of Mycobacterium ulcerans in Clinical and Environmental Samples. Appl Environ Microbiol 73: 4733–4740.

George KM, D Chatterjee, G Gunawardana, D Welty, T Lee, et al. (1999) Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science 283: 854–857.

Hayman J (1991a) Postulated epidemiology of Mycobacterium ulcerans infection. International Journal of Epidemiology. 20(4): 1093-8.

Hayman J (1991b) Mycobacterium ulcerans infection. The Lancet 337: 124.

Huryn AD, Wallace JB (2000) Life history and production of stream insects. Annu Rev Entomol. 45:83–110.

Invertebrates of South Africa (1999-2007) Identification Keys, vols. 2-10. Rhodes University, Department of Zoology and Entomology, Johannesburg, South Africa.

Johnson PDR, Stinear TP, Hayman JA (1999) Mycobacterium ulcerans — a minireview. J Med Microbiol 48: 511–513.

Johnson PDR, Stinear TP, Small PLC, Pluschke G, Merritt RW, et al. (2005) Buruli ulcer (M. ulcerans Infection): new insights, new hope for disease control.PLoS Med 2(4): e108.

Johnson PDR, Hayman JA, Quek TY, Fyfe JAM, Jenkin GA, et al. (2007) Consensus recommendations for the diagnosis, treatment and control of Mycobacterium ulcerans infection (Bairnsdale or Buruli ulcer) in Victoria, Australia. Medical Journal of Australia 186: 64–68.

Johnson PDR, Lavender CJ (2009) Correlation between Buruli ulcer and vector-borne notifiable diseases, Victoria, Australia. Emerging Infectious Diseases 15: 614–615.

Kendall MG, Gibbons JD (1990) *Rank Correlation Methods* (5th edition). London: Arnold.

Kibadi K, Panda M, Tamfum JM, Fraga AG, Filho AL, et al. (2008) New foci of Buruli ulcer, Angola and Democratic Republic of Congo. Emerging Infectious Diseases 14: 1790–1792.
Lavender CJ, Stinear TP, Johnson PDR, Azuolas J, Benbow ME, et al. (2008) Evaluation of VNTR typing for the identification of Mycobacterium ulcerans in environmental samples from Victoria, Australia. FEMS Microbiology Letters 287: 250–255.

Lunn HF, Connor DH, Wilks NE, Barnley GR, Kamunvi F, et al. (1965) Buruli (Mycobacterial) ulceration in Uganda. East African Medical Journal 42: 275–288.

Marion E, Eyangoh S, Yeramian E, Doannio J, Landier J Aubry J et al. (2010) Seasonal and regional dynamics of M. ulcerans transmission in environmental context: deciphering the role of water bugs as hosts and vectors. PLoS Negl Trop Dis. 6:4(7):731.

Marsollier L, Robert R, Aubry J, Andre JS, Kouakou H, et al. (2002) Aquatic insects as a vector for Mycobacterium ulcerans. Applied and Environmental Microbiology 68: 4623–4628.

Marsollier L, Aubry J, Saint-Andre JP, Robert R, Legras P, et al. (2003) Ecology and transmission of Mycobacterium ulcerans. Pathologie Biologie 51: 490–495.

Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, et al. (2004a) Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl Environ Microbiol 70: 6296–6298.

Marsollier L, Stinear TP, Aubry J, Saint-Andre J-P, Robert R, et al. (2004b) Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Applied and Environmental Microbiology 70: 1097–1103.

Marsollier L, Aubry J, Coutanceau E, Andre JPS, Small PL, et al. (2005) Colonization of the salivary glands of Naucoris cimicoides by Mycobacterium ulcerans requires host plasmatocytes and a macrolide toxin, mycolactone. Cellular Microbiology 7: 935–943.

Marsollier L, Andre J, Frigui W, Reysset G, Milon G, et al. (2006) Early trafficking events of Mycobacterium ulcerans within Naucoris cimicoides. Cellular Microbiology.

Marston BJ, Diallo MO, Horsburgh CR Jr., Diomande I, Saki MZ, et al. (1995) Emergence of Buruli ulcer disease in the Daloa region of Cote D'ivoire. Am J Trop Med Hyg 52: 219–224.

McIntosh MD, H Williamson, ME Benbow, RK Kimbirauskas, C Quaye, D

Boakye, PLC Small, RW Merritt (2010) Associations between *Mycobacterium ulcerans* and Aquatic Plant Communities of West Africa: implications for Buruli ulcer disease. (submitted 2010)

McLachlan AJ (1975) The role of aquatic macrophytes in the recovery of the benthic fauna of a tropical lake after a dry phase. Limnology and Oceanography. 20(1) pp. 54-63.

Merchant R and CM Yule (1996) A method for estimating larval life spans of aseasonal aquatic insects from streams on Bougainville Island, Papua New Guinea. Freshwater Biology 35, 101-107.

Merritt RW, Benbow ME, Small PLC (2005) Unraveling an Emerging Disease Associated with Disturbed Aquatic Environments: The Case of Buruli Ulcer. Frontiers in Ecology and the Environment 3: 323–331.

Merritt, RW, Cummins, KW, MB Berg (2008) An introduction to the aquatic insects of North America. Fourth Edition. Kendall/Hunt Publishing Co., Dubuque, Iowa. 1158pp.

Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review. PLoS Negl Trop Dis 4(12): 1-15.

Meyers WM, Shelly TA, Conner DH, Meyers EK (1974) Human Mycobacterium ulcerans infections developing at sites of trauma. American Journal Trop Med Hyg 23: 919-923.

Meyers WM, Tignokpa WM, Priuli GB, Portaels F (1996) Mycobacterium ulcerans infection (Buruli ulcer): first reported patients in Togo. British Journal of Dermatology 134: 1116–1121.

Meyers WM, Tignokpa WM, Priuli GB, Portaels F (1996) Mycobacterium ulcerans infection (Buruli ulcer): first reported patients in Togo. British Journal of Dermatology 134: 1116–1121.

Mosi L, Williamson H, Wallace JR, Merritt RW, Small PLC (2008) Persistent association of Mycobacterium ulcerans with West African predaceous insects of the family Belostomatidae. Applied and Environmental Microbiology 74: 7036–7042.

Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, et al. (2004) Buruli ulcer disease in Cameroon rediscovered. Am J Trop Med Hyg 70: 520–526.

PASW Statistics v. 17.0 (SPSS: An IBM Company).

Petr T (1968) Population changes in aquatic invertebrates living on two water

plants in a tropical man-made lake. Hydrobiologia 32:449–85
Portaels, F (1989) Epidemiologie des ulcères à Mycobacterium ulcerans. *Ann Soc Belg Med Trop.* 69: 91–103.

Portaels, F (1995) Epidemiology of mycobacterial diseases. Clin. Dermatol. 13:207-222.

Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P, Meyers WM (1999) Insects in the transmission of Mycobacterium ulcerans infection. The Lancet 353: 986.

Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, et al. (2001) Mycobacterium ulcerans in wild animals. Rev sci tech Off int Epiz 20: 252–264.

Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, et al. (2008) First Cultivation and Characterization of Mycobacterium ulcerans from the Environment. PLoS Neglected Tropical Diseases 2: e178.

Raghunathan PL, Whitney EAS, Asamoa K, Stienstra Y, Taylor TH Jr., et al. (2005) Risk factors for Buruli Ulcer disease (Mycobacterium ulcerans Infection): results from a case-control study in Ghana. Clinical Infectious Diseases 40:1445–1453.

Revill WDL, Barker DJP (1972) Seasonal distribution of mycobacterial skin ulcers. Brit J prev soc Med 26: 23–27.

Seber GAF (1984) *Multivariate observations*. New York: John Wiley & Sons, Inc. (Section 9.2.6).

Silva MT, Portaels F, Pedrosa J (2007) Aquatic Insects and Mycobacterium ulcerans: an association relevant to Buruli ulcer control? PLoS Medicine 4: e63.

Sizaire V, F Nackers, E Comte, F Portaels (2006) *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. *Lancet Infect Dis* 6, 288–296.

Sopoh GE, Johnson RC, Chauty A, Dossou AD, Aguiar J, et al. (2007) Buruli ulcer surveillance, Benin, 2003-2005. Emerg Infect Dis 13: 1374–1376.

Stout JR (1981) How abiotic factors affect the distribution of two species of tropical predaceous aquatic bugs (Family: Naucoridae). Ecology. 62:1170–8.

Stout JR (1982) Effects of harsh environment on the life history patterns of two species of tropical aquatic hemiptera (Family: Naucoridae). Ecology 63: 75–83.

Tabachnick BG, LS Fidel (2008) Using Multivariate Statistics (5th Edition). Allyn and Bacon, Inc. Boston, MA USA.

Tobias N, Seemann T, Pidot S, Porter J, Marsollier L, et al. (2009) Mycolactone gene expression is controlled by strong SigA-like promoters with utility in studies of Mycobacterium ulcerans and buruli ulcer. PLoS Negl Trop Dis 3: e553.

U.S. Environmental Protection Agency (1996) The volunteer monitor's guide to quality assurance project plans. U.S. Environmental Protection Agency, Office of Wetlands, Oceans, and Watersheds, Washington, D.C. EPA 841-B-96-003.

U.S. Environmental Protection Agency (2001) Indicators for Monitoring Bilogical Integrity of Inland Freshwater Wetlands. U.S. Environmental Protection Agency, Office of Water, Wetlands Divison, Washington, D.C. EPA 843-R-01.

Wagner T, Benbow ME, Burns M, Johnson RC, Merritt R, et al. (2008a) A Landscape-based Model for Predicting Mycobacterium ulcerans Infection (Buruli Ulcer Disease) Presence in Benin, West Africa. EcoHealth 5: 69–79.

Wagner T, Benbow ME, Brenden T, Qi J, Johnson RC (2008b) Buruli ulcer disease prevalence in Benin, West Africa: associations with land use/cover and the identification of disease clusters. International Journal of Health Geographics 7: 25.

Wallace J, Gordon M, Hartsell L, Mosi L, Benbow M, et al. (2010) Interaction of Mycobacterium ulcerans with mosquito species: Implications for transmission and trophic relationships. Applied Environ Microbiol 76: 6215–6222.

Walsh D, Portaels F, Meyers W (2008) Buruli ulcer (Mycobacterium ulcerans infection). Transactions of the Royal Society of Tropical Medicine and Hygiene 102: 969–978.

Walsh D, Eyase F, Onyango D, Odindo A, Waitumbi JN et al. (2010) Short Report: Clinical and Molecular Evidence for a Case of Buruli Ulcer (Mycobacterium ulcerans Infection) in Kenya. Am. J. Trop. Med. Hyg., 81(6), pp. 1110–1113.

Wansbrough-Jones M, Phillips R (2006) Buruli ulcer: emerging from obscurity. Lancet 367: 1849–1858.

Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, et al. (2008) Distribution of Mycobacterium ulcerans in Buruli Ulcer Endemic and Non-Endemic Aquatic Sites in Ghana. PLoS Neglected Tropical Diseases 2: e205.

WHO, ed (2000) Buruli ulcer. Mycobacterium ulcerans infection. Geneva, Switzerland: WHO. 118 p.

WHO (2008) Buruli ulcer: progress report, 2004–2008. In: WHO, ed. Weekly epidemiological record. Geneva, Switzerland: World Health Organization 83: 145–156.

CHAPTER 4

DETECTION OF NATURAL PREY IN THE GUTS OF AFRICAN CREEPING WATER BUGS (HEMPTERA: NAUCORIDAE) USING SEQUENCED CLONES OF PCR-AMPLIFIED GUT CONTENTS

Introduction

Buruli ulcer (BU) is a neglected emerging disease of skin and soft tissue that leads to scarring and disability (Johnson et al. 2005, Merritt et al. 2005). It is caused by *Mycobacterium ulcerans*, an environmental pathogen that produces a destructive polyketide toxin (George et al. 1999). The disease has been reported in humans from at least 32 countries, with a large number of cases reported from West Africa (Duker et al. 2006; Walsh et al. 2008). While transmission of the disease to human beings remains unclear, BU outbreaks have been associated with freshwater habitats (Thangaraj et al. 1999), particularly in areas where the landscape is disturbed by natural events such as flooding, or through deforestation, dam construction, agricultural diversion, or mining (Thangaraj et al. 1999, Johnson et al. 2005, Merritt et al. 2005, Duker et al. 2006, Wansbrough-Jones and Philips 2006).

A critical step in understanding BU transmission is elucidating the diet of organisms that may potentially act as reservoirs and vectors of the *Mycobacterium* pathogen in nature. Non-human mammals and reptiles have been tested in the environment without positive findings for the pathogen (Radford 1974), and several arthropod disease vectors (i.e., bedbugs, black flies, mosquitoes) tested negative in early studies (Revill and Barker 1972, Portaels et al. 2001). However, only a few organisms in each taxonomic group were tested in these early studies, and insect

sampling methods were neither systematically employed nor standardized as discussed by Benbow et al. (2008). Portaels et al. (1999) were first to suggest that aquatic bugs (Hemiptera) might be reservoirs of *M. ulcerans* in nature, and recently they described the first isolation in pure culture of *M. ulcerans* from a water strider (Hemiptera: Gerridae, *Gerris* sp.) from Benin, West Africa (Portaels et al. 2008). A survey study (Portaels et al. 2001) based on detecting *M. ulcerans* DNA in aquatic insects (Hemiptera, Odonata, Coleoptera) in African BU-endemic swamps confirmed the earlier findings. More recent studies in Australia have suggested that mosquitoes may be involved in transmission (Johnson et al. 2007).

Using experiments, Marsollier et al. (2002, 2003) demonstrated that *M. ulcerans* could survive and multiply within the salivary glands of the aquatic bug *Naucoris cimicoides* (Hemiptera, Naucoridae), and that *N. cimicoides* could transmit the mycobacteria to mice (Marsollier et al. 2002). *Naucoris* spp. live in freshwater ponds, lakes, and slow-flowing sections of streams and rivers. Naucoridae are predacious in both the immature (nymph) and adult stages, although little is known of the ecology and prey preferences of Naucoridae in nature. This is particularly true in developing countries where Buruli ulcer is most prevalent (WHO 2008). Most aquatic hemipterans are believed to be generalist predators on other aquatic invertebrates (Merritt et al. 2008), although some, including naucorid species, have mouthparts designed to aid in feeding on prey larger then themselves (e.g., Cohen 1995) such as tadpoles (Polhemus and Polhemus 1988) and larval fish (Louarn and Cloarec 1997).

Most Hemiptera feed by injecting digestive enzymes into prey and ingesting the liquefied tissues through a tube-like proboscis (extra-oral digestion) (Cohen

1995). This feeding mode presents a challenge to the study of their diet, largely eliminating the use of standard morphological identification of chitinous body parts in the gut. As a result, studies of hemipteran diets have typically used immunoassays employing prey-specific monoclonal antibodies (Greenstone 1996), PCR tests (Sheppard and Harwood 2005), or both (e.g., Fournier et al. 2008). One limitation of most antibody- and DNA-based applications is that some knowledge of the potential prey is required. Antibodies target epitopes that are specific to proteins from target prey species, and most DNA methods employ species- or taxon-specific primers in PCR tests to determine the presence/absence of target prey species or taxa (e.g., Agustí et al. 1999, 2000, 2003, Read 2002, Cuthbertson et al. 2003, Jarman et al. 2004, de León et al. 2006). While these methods can be powerful and have been verified for their accuracy using laboratory feeding experiments (Chen et al. 2000, Foltan et al. 2005, Harper et al. 2005, 2006, Sheppard et al. 2005, Harwood et al. 2007, McMillan et al. 2007), a major limitation arises when there is little or no prior knowledge of the prey in their natural habitat.

Here I examined the diet of a common predator in freshwater ecosystems in West Africa (*Naucoris* sp.) in a first attempt to understand its role in a tropical pond food web and potential trophic connections in relation to the pathogen *M. ulcerans*. In the absence of any *a priori* knowledge of their prey, I attempted to PCR-amplify all DNA in the *Naucoris* sp. gut using universal primers, clone the PCR product, and sequence a subset of clones. I then matched the resulting gut-content sequences to sequences obtained from potential prey collected from the same habitat, and to publically available sequence databases.

Materials and Methods

Sample collection and preparation. *Naucoris* sp. water bugs and potential prey populations were sampled 9 August 2009 from one waterbody within the village of Saduase, Ga East District, Ghana, Africa. All macroinvertebrates were collected using a 500µm D-frame aquatic net. All *Naucoris* sp. were transferred immediately to individual vials, while all other macroinvertebrates were considered to be potential prey items and stored separately. All specimens were preserved in 95% EtOH in the field. In the laboratory, *Naucoris* sp. were sexed and guts were carefully removed under a dissecting microscope. To expose the guts, heads were dissected and incisions were made laterally along the abdomen to peel back the exoseleton. Guts were then removed with forceps and stored separately in fresh 95% EtOH. Prior to handling each specimen, all instruments were rinsed with distilled water, flame treated, and wiped with individual Kimwipes. All samples were stored at 4°C prior to DNA extraction.

DNA extraction and PCR. Genomic DNA was extracted from *Naucoris* sp. adults (n = 29) and nymphs (n = 14) as well as the potential prey sampled: Ephemeroptera (mayflies, n = 4), Odonata (damselflies, Zygoptera) (n = 8), Coleoptera (beetles, n = 4), Diptera (flies, Chironomidae, n = 3), and Arachnidae (spiders, n = 3) using DNeasy tissue kits (Qiagen GmbH, Hilden, Germany). Genomic DNA was extracted from *Naucoris* sp. guts (n = 60) using the QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) (King et al. 2008). Field samples were first centrifuged for 1 min at 2000 g. The ethanol was poured off and the dry weight of the pellet was determined. All remaining steps followed the manufacturer's protocol, except that only half the recommended volume of

buffers/InhibitEX was used. Primers LR-N-13389 (alias 16ar, Simon et al. 1994) and 16b2 (5' - TTTAATCCAACATCGAGG - 3') were used to amplify a ca. 440-bp fragment of mitochondrial *rrnL* (16S) for all samples using standard methods. The 5' (DNA barcode) region of *cox1* (COI) was amplified for four *Naucoris* sp. adults using primers LCO-1490 and HCO-2198 (Folmer et al. 2004) in order to potentially match individuals with existing databases. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions using the PCR primers. Samples were analyzed on either a CEQ 8000 (Beckman Coulter) or a 3500xL (Applied Biosystems) automated sequencer.

Molecular cloning. Cloning was used to differentiate among multiple possible PCR products obtained from *Naucoris* sp. guts. Both the *rrnL* and *cox1* PCR primers (above) target a broad range of organisms including crustaceans, insects, and vertebrates, thus could be useful for simultaneously amplifying multiple taxa that may be present in the gut. PCR products were run out on a 2 % agarose gel and purified using the MinElute gel extraction kit (Qiagen GmbH, Hilden, Germany). The clone libraries were created with the pGEM-T-Easy-kit (Promega GmbH, Mannheim, Germany) following the manufacturer's protocol. Insert size was examined using PCR of plasmids with the primers SP6 (5'-ATTTAGGTACACTATAG) and T7 (5'-AATACGACTCACTATAGG). Large inserts (n = 576) were cleaned with PEG and sequenced using primer SP6.

Data analysis. All sequences were assembled and edited using CodonCode Aligner v 3.5 (Codon Code Corporation, Dedham, USA). For *Naucoris* sp. and prey, forward and reverse sequences were assembled and edited for each specimen. *Naucoris* sp. *cox1* and *rrnL* sequences were first compared to the NCBI

nucleotide database using blastn queries (http://blast.ncbi.nlm.nih.gov). Clone sequences were assembled and contigs were edited in order to generate consensus sequences for each contig (contig sizes on Table 4.1). All full-length sequences obtained from clone libraries (*ca*. 450-478 bp *rrnL*, 658 bp *cox1*) were compared to the NCBI nucleotide database using blastn queries.

Phylogenetic analysis was conducted on all insect and arachnid *rrnL* sequences that were newly generated by direct sequencing or cloning. I also included 18 other Hemiptera *rrnL* sequences obtained from the list of blastn hits, most of which came from a recent mtDNA phylogeny (Hua et al. 2009). For comparison of potential prey sequences with the NCBI database, I downloaded all blastn hits with > 90 % identity to each query genotype. All sequences were aligned using clustalW (align.genome.jp) and a phylogenetic tree search was conducted on the matrix using a maximum likelihood approach in PhyML v 3.0 (Guindon and Gascuel 2003) under a GTR model of evolution (as determined by Modeltest v 3.7, Posada and Crandall 1998).

Results

PCR amplification of *cox1* and *rrnL* was equally successful for *Naucoris* sp. but *rrnL* was more consistently amplified for gut samples and potential prey. There were no *Naucoris* sp. *cox1* sequences available on the NCBI nucleotide database and the top hit of the blastn query was an unclassified Hemiptera (AAG5301 voucher ENT-OUBS-156, HM381306), whereas the database contained 8 *rrnL* sequences for Naucoridae collected from Madagascar, Europe, North and Central America, and the Philippines (Hebsgaard et al. 2004). Combining the newly generated *rrnL* sequences with blastn query results produced an aligned matrix of

62 taxa and 460 characters (sequence length 336 – 443 bp). In the maximum likelihood *rrnL* gene tree (ln L = -8123.81514; Fig. 4.1) our *Naucoris* sp. sequences were clearly nested within published data for the Hemiptera, the closest relative being *Ambrysus* sp. collected from North America (Fig. 4.1). *Ambrysus* sp. was the second-ranked blastn hit, with *Macrocoris* sp. from Madagascar (Hebsgaard et al. 2004) the top hit but phylogenetically more distant in our analysis of the same sequences (Fig. 4.1).

Three of our cloned *rrnL* sequences were identical to a field-caught Coleoptera species sampled at the study site as potential prey. The top database match (using blastn) to this sequence was *Spercheus* (Spercheidae) (Table 4.1) and the second-ranked match was *Hydrobius* sp. (Hydrophilidae)(not shown), but it is clear from our phylogenetic search that it is distantly related to both (Fig. 4.1). None of the other potential prey species that we collected from the sampling site and sequenced were recovered from gut sequence clones (Fig. 4.1). Nonetheless, a number of interesting non-insect species were recovered from guts and identified with blastn queries (Table 4.1). These included *Afrixalus* sp. (Anura: Hyperoliidae), a sub-Saharan genus of frog for which one *rrnL* sequence was recovered from the clone library. Sequences from the *cox1* clone library included *Embata* and *Floscularia* (Rotifera), and *Pythium* (Oomycete fungi). All other full-length clone sequences were identical to our Naucoridae sequences obtained using direct sequencing of PCR products (*rrnL* shown in Fig. 4.1).

Discussion

Buruli ulcer (*Mycobacterium ulcerans* infection) causes severe morbidity in human populations associated with degraded freshwater habitats, but neither the

reservoir nor the mode of transmission of *M. ulcerans* is known (Merritt et al. 2005). Here I investigated an abundant aquatic predator from a BU-endemic area in Ghana, *Naucoris* sp. water bugs (Hemiptera, Naucoridae). While Naucorids have been implicated in the transmission of *M. ulcerans* in laboratory studies, a limited knowledge of their place in aquatic food webs in nature makes it difficult assess the potential source and sinks of the pathogen. This is the first investigation of which I am aware to clone and sequence PCR products from universal primers to determine a Hemipteran diet without any prior knowledge of potential prey.

Our approach led to a broader perspective of the role of *Naucoris* sp. in the aquatic food web. Using a standard PCR-based method, prey-specific primers for the 5 taxa of field-caught prey would have designed (e.g., Agusti et al. 2003). From this, we would have probably generated positive tests for the Coleoptera. In contrast, the universal primers, sequenced clones, and database queries used here allowed us to identify DNA in the guts that came from taxa that were not field-collected. These included fungi, rotifers, and an anuran, although immature anurans were collected from the field site and thus known to be present. A limitation of the database queries is clearly the fact that the extent of the database plays an important role. Using the newly generated *Naucoris* sp. sequences, none of the blastn query results gave a close match. Even the barcode *cox1* sequence gave a fairly meaningless match (Hemiptera sp.) to the Barcode of Life database (www.barcodinglife.org).

Combining public databases and our own newly generated prey sequences was beneficial for confirming that the prey Coleoptera sequence cloned from the gut matched the field-caught Coleoptera species at the same habitat. Although

neither the database nor our new sequence could provide identification, at least I could conclude with confidence that *Naucoris* sp. prey on the Coleoptera resident to the sample site in Ghana, West Africa. These sequences were identical and, based on the phylogenetic gene tree, quite different from any species in GenBank. Interestingly, the blastn query and phylogenetic analysis gave different results. The second-ranked blastn result was phylogenetically closer to the query sequence than the top-ranked blastn query result. The more detailed phylogenetic analysis, using more of the available data and a GTR model of sequence evolution, probably revealed the closer relative. Although both query hits were relatively distant and neither is probably a good match, it does suggest that a phylogenetic approach is more accurate than a blast result in the absence of a complete database.

In conclusion, this approach provided the means to study an aquatic hemipteran diet without any prior knowledge of potential prey and despite the difficulties of extra-oral digestion. *Naucoris* sp. in this area of West Africa feed on a wide range of prey and body sizes, including rotifers, insects, and anurans. Further work on *M. ulcerans* transmission will be aided by this food web information. These results also suggest the approach could be successfully used to study the complex interactions within aquatic food webs, including even feeding on fungi. These results corroborate previous suggestions that DNA-based approaches using universal primers and cloning provide an important tool for studying the prey spectrum of predators with unknown diets.

Table 4.1. Results of comparisons to the NCBI nucleotide database using blastn queries of *rrnL* and *cox1*sequences that were PCR-amplified from Naucoridae guts and cloned (see methods). Taxa listed were, in each case, first on the hit table. Only full-length inserts (450 – 478 bp rrnL, 658 bp cox1) were considered. Most full-length inserts were identical to our sequences obtained from direct sequencing of sampled Naucoridae and resulted in a top blastn hit of *Macrocoris* sp. (*rrnL*) or Hemiptera sp. (*cox1*) (data not shown).

gene region	**Taxon**	**Accession**	***n***	**% identity**	**query length / alignment length**	**e-value**	**Source**
rrnL	*Spercheus emarginatus* (Coleoptera)	AM287063	3	85.59	450 / 444	4 E -135	Bernhard et al. (2006)
	Afrixalus sp.(Anura)	AF215431	1	98.47	478 / 457	0	Vences (2000)
cox1	*Embata parasitica* (Rotifera)	EF650597	3	88.87	658 / 602	0	Unpublished A
	Floscularia melicerta (Rotifera)	EU499896	2	75.99	658 / 633	1 E -124	Unpublished B
	Pythium acanthophoron (Oomycetes)	EU350529	4	80.18	658 / 661	0	Unpublished C

A- Herniou, E.A. and Fontaneto, D. (unpubl.)

B- Fontaneto, D., Chen, K. and Castillo, K. (unpubl.)

C- Jackson, C. A. R., de Cock, A. W. A. M., Vijayan, P., Robideau, G. P. and Levesque, C. A. (unpubl.)

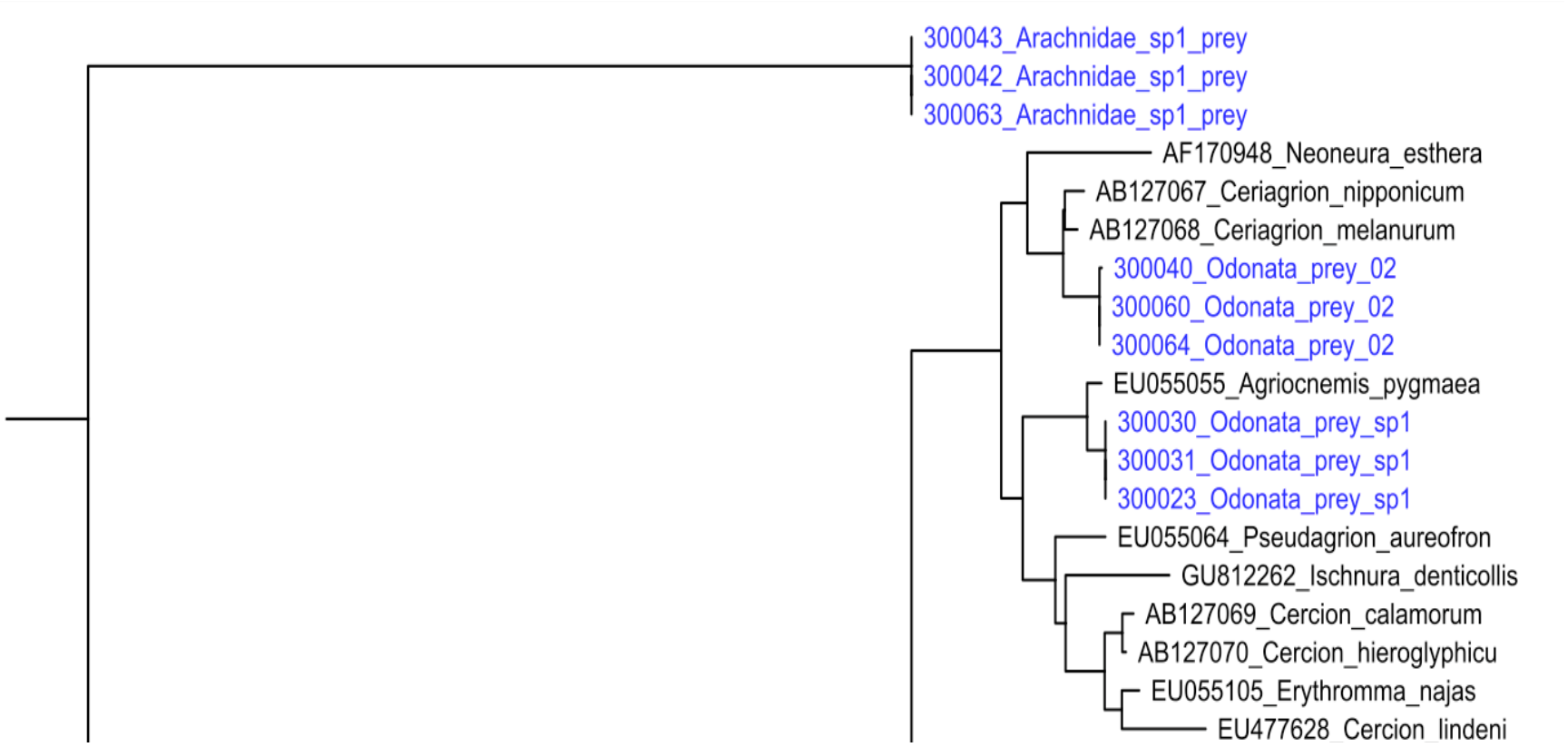


Figure 4.1. Maximum likelihood phylogenetic tree of *rrnL* using a GTR model of evolution, including newly sequenced *Naucoris* sp. and potential prey (blue terminals), cloned PCR products from *Naucoris* sp. guts and mouthparts (red terminals), and highly ranked sequences according to blastn queries (black, see text for criteria).

Figure 4.1 (cont'd).

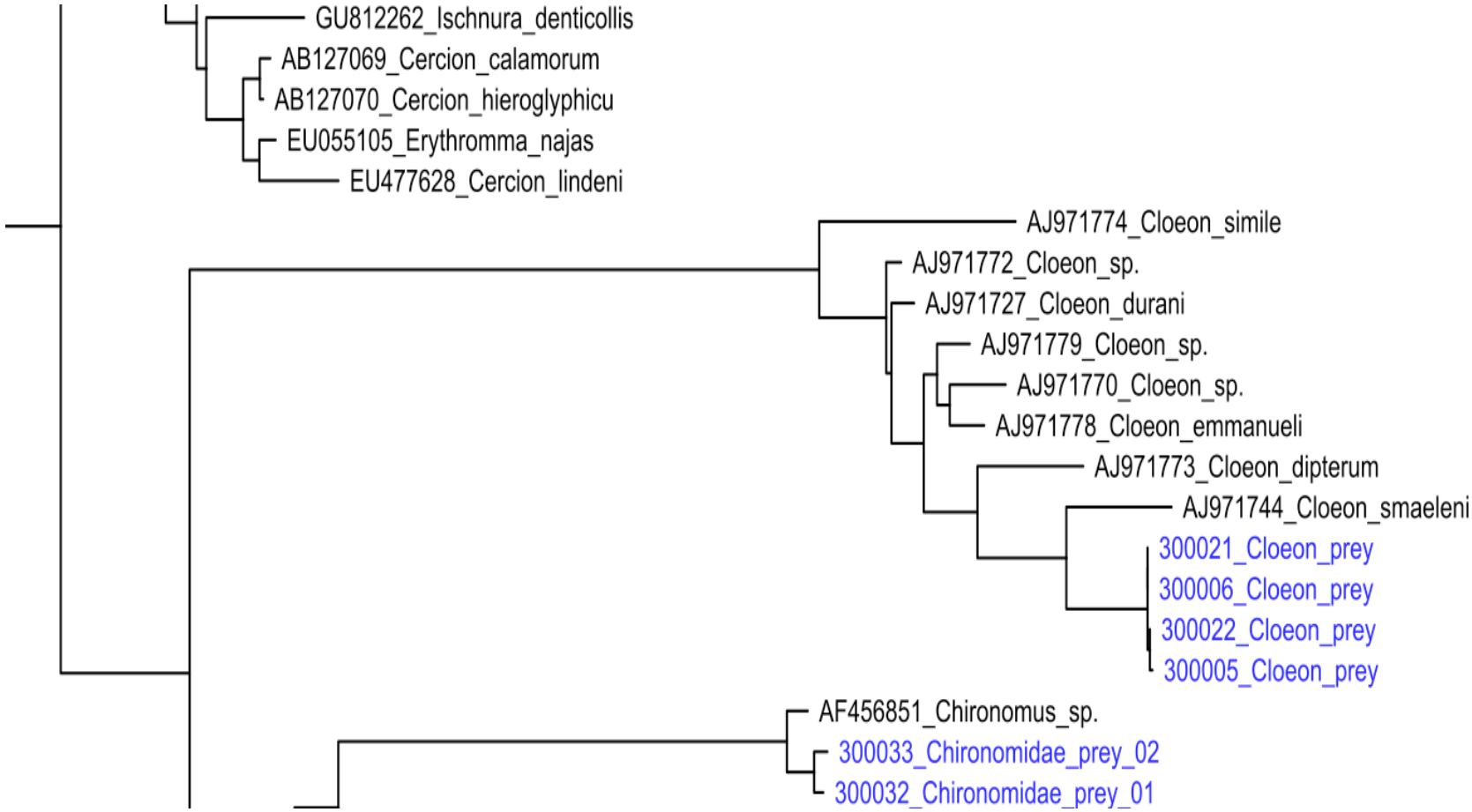
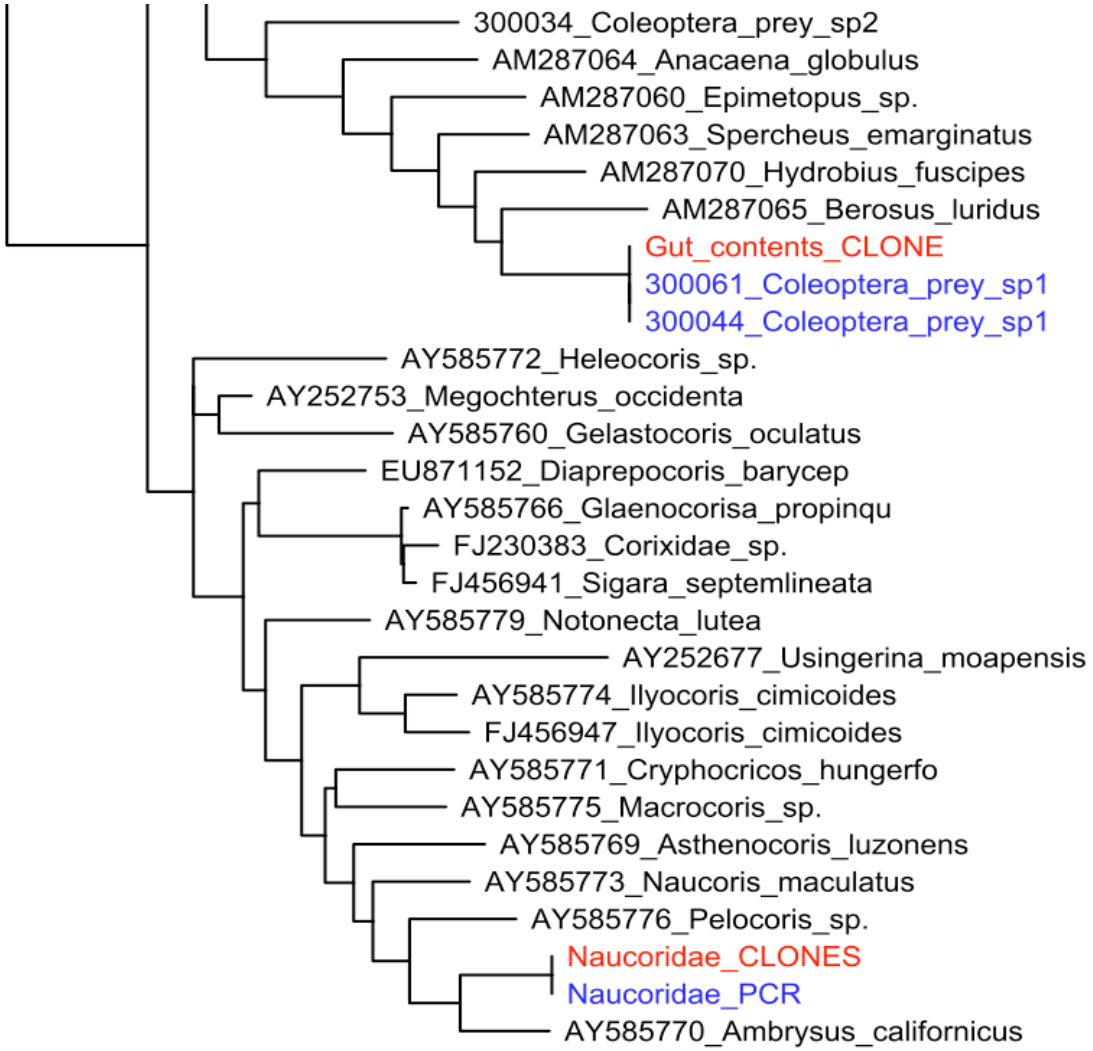


Figure 4.1 (cont'd).



LITERATURE CITED

Literature Cited

Agustí N, Unruh TR, Welter SC (2003) Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of Entomological Research*, 93, 179–185.

Agustí N, de Vicente MC, Gabarra R (1999) Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. *Molecular ecology*, 8, 1467-1474.

Agustí N, de Vicente MC, Gabarra R (2000) Developing scar markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology*, 9, 263–268.

Benbow M, Williamson H, Kimbirauskus R, McIntosh M, Kolar R, et al. (2008) Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis 14: 1247–1254.

Bernhard D, Schmidt C, Korte A, Fritzsch G, Beutel R G (2006) From terrestrial to aquatic habitats and back again – molecular insights into the evolution and phylogeny of Hydrophiloidea (Coleoptera) using multigene analyses. *Zoologica Scripta*, 35, 597-606.

Chen Y, Giles KL, Payton ME, Greenstone MH (2000) Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology*, 9, 1887–1898.

Cohen AC (1995) Extra-oral digestion in predaceous terrestrial Arthropoda. *Annual Review of Entomology*, 40, 85-103.

Cuthbertson AGS, Fleming CC, Murchie AK (2003) Detection of *Rhopalosiphum insertum* (apple-grass aphid) predation by the predatory mite *Anystis baccarum* using molecular gut analysis. *Agricultural and Forest Entomology*, 5, 219–225.

de León JH, Fournier V, Hagler JR, Daane KM (2006) Development of molecular diagnostic markers for sharpshooters *Homalodisca coagulata* and *Homalodisca liturata* for use in predator gut content examinations. *Entomologia Experimentalis et Applicata*, 119, 109–119.

Duker AA, Portaels F, Hale M (2006) Pathways of *Mycobacterium ulcerans* infection: A review. *Environment International*, 32, 567-573.

Foltan P, Sheppard S, Konvicka M, Symondson WOC (2005) The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. *Molecular Ecology*, 14, 4147–4158.

Fournier V, Hagler J, Daane K, de León J, Groves R (2008) Identifying the predator complex of *Homalodisca vitripennis* (Hemiptera: Cicadellidae): a comparative study of the efficacy of an ELISA and PCR gut content assay. *Oecologia*, 157, 629-40.

Greenstone MH (1996) Serological analysis of arthropod predation: past, present and future. In: *The Ecology of Agricultural Pests: Biochemical Approaches* (eds Symondson WOC, Liddell JE), pp. 265–300. Chapman & Hall, London.

George KM, Chatterjee D, Gunawardana G *et al*. (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. *Science*, 283, 854–7.

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52(5), 696-704.

Harper GL, King RA, Dodd CS *et al*. (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology*, 14, 819–827.

Harper GL, Sheppard SK, Harwood JD *et al*. (2006) Evaluation of temperature gradient gel electrophoresis for the analysis of prey DNA within the guts of invertebrate predators. *Bulletin of Entomological Research*, 96, 295–304.

Harwood JD, Desneux N, Yoo HJS *et al*. (2007) Tracking the role of alternative prey in soybean aphid predation by *Orius insidiosus*: a molecular approach. *Molecular Ecology*, 16, 4390–4400.

Hebsgaard MB, Andersen NM, Damgaard J (2004) Phylogeny of the true water bugs (Nepomorpha: Hemiptera-Heteroptera) based on 16S and 28S rDNA and morphology. *Systematic Entomology*, 29, 488-508.

Jarman SN, Deagle BE, Gales NJ (2004) Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. *Molecular Ecology*, 13, 1313–1322.

Johnson PD, Stinear TP, Small PL *et al*. (2005) Buruli ulcer (*Mycobacterium ulcerans*): new insights, new hope for disease control. *PLoS Med*, 2, e108.

Johnson PDR, Azuolas J, Lavender CJ *et al*. (2007) *Mycobacterium ulcerans* in mosquitoes captured during outbreak of Buruli ulcer, Southeastern Australia. *Emerging Infectious Diseases*, 13, 1653-1660.

King RA, Read DS, Traugott M, Symondson WOC (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular ecology*, 17, 947-963.

Louarn H, Cloarec A (1997) Insect predation on pike fry. *Journal of Fish Biology*, *50*(2), 366-370. doi: 10.1111/j.1095-8649.1997.tb01364.x.)

McMillan S, Kuusk AK, Cassel-Lundhagen A, Ekbom B (2007) The influence of time and temperature on molecular gut content analysis: *Adalia bipunctata* fed with *Rhopalosiphum padi*. *Insect Science*, 14, 353–358.

Marsollier L, Aubry J, Saint-Andre JP *et al*. (2003) Ecology and transmission of *Mycobacterium ulcerans*. *Pathologie Biologie*, 51, 490-495.

Marsollier L, Robert R, Aubry J *et al*. (2002) Aquatic insects as a vector for *Mycobacterium ulcerans*. *Applied and Environmental Microbiology*, 68, 4623-4628.

Merritt RW, Cummins KW, Berg MB (2008) *An introduction to the aquatic insects of North America*. 4th ed. Kendal/Hunt Pub. Co., Duduque, IA. 1158 p.

Merritt R, Benbow M, Small P (2005) Unraveling an emerging disease associated with disturbed aquatic environments: the case of Buruli ulcer. *Frontiers in Ecology and the Environment*, 3, 323-331.

Polhemus DA, Polhemus JT (1988) The Aphelocheirinae of Tropical Asia (Heteroptera: Naucoridae). *Raffles Bulletin Zoology*, 36, 167-300.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Portaels F, Chemlal K, Elsen P *et al*. (2001) *Mycobacterium ulcerans* in wild animals. *Revue Scientifique et Technique (International Office of Epizootics)*, 20, 252-264.

Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P, Meyers WM (1999) Insects in the transmission of *Mycobacterium ulcerans* infection. *The Lancet*, 353, 986.

Portaels F, Meyers WM, Ablordey A, *et al*. (2008) First cultivation and characterization of *Mycobacterium ulcerans* from the environment. *PLoS Neglected Tropical Diseases*, 2, e178.

Radford AJ (1974) *Mycobacterium ulcerans* infection in Papua New Guinea. *Papua and New Guinea Medical Journal*, 17, 145-149.

Read DS (2002) *Sequencing of aphid DNA and primer design for the detection of aphid remains in predators*. Undergraduate Dissertation, Cardiff University. Cardiff, UK.

Revill WDL, Barker DJP (1972) Seasonal distribution of mycobacterial skin ulcers. *British Journal of Preventive & Social Medicine*, 26, 23-27

Sheppard SK, Harwood JD (2005) Advances in molecular ecology: tracking trophic links through predator-prey food-webs. *Functional ecology*, 19, 751-762.

Sheppard SK, Bell J, Sunderland KD, Fenlon J, Skervin D, Symondoson WOC (2005) Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. *Molecular Ecology*, 14, 4461–4468.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook PC (1994) Evolution weighting and phylogenetic utility of mitochondrial gene sequences and compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87, 651- 700.

Swanstrom J, Chen K, Castillo K, Barraclough TG, Fontaneto D (in press) Testing for evidence of inefficient selection in bdelloid rotifers: do sample size and heterogeneity matter? *Hydrobiologia*

Thangaraj HS, Evans MRW, Wansbrough-Jones MH (1999) *Mycobacterium ulcerans*: Buruli ulcer. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 93, 337-340.

Vences M (2000) Phylogenetic studies of ranoid frogs (Amphibia:Anura), with a discussion of the origin and evolution of the vertebrate clades of Madagascar. *PhD Thesis Universitaet Bonn*.

Walsh D, Portaels F, Meyers W (2008) Buruli ulcer (*Mycobacterium ulcerans* infection). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102, 969-978.

Wansbrough-Jones M, Phillips R (2006) Buruli ulcer: emerging from obscurity. *Lancet*, 367, 1849-1858.

WHO (2008) Buruli ulcer: progress report, 2004–2008. In: WHO, ed. Weekly epidemiological record. Geneva, Switzerland: World Health Organization 83: 145–156.