
Aquatic Invertebrates as Unlikely Vectors of Buruli Ulcer Disease

M. Eric Benbow,^{*1} Heather Williamson,[†] Ryan Kimbirauskas,^{*} Mollie D. McIntosh,^{*} Rebecca Kolar,^{*} Charles Quaye,[‡] Felix Akpabey,[§] D. Boakye,[‡] Pam Small,[†] and Richard W. Merritt^{*}

Buruli ulcer is a necrotizing skin disease caused by *Mycobacterium ulcerans* and associated with exposure to aquatic habitats. To assess possible transmission of *M. ulcerans* by aquatic biting insects, we conducted a field examination of biting water bugs (Hemiptera: Naucoridae, Belostomatidae, Nepidae) in 15 disease-endemic and 12 non-disease-endemic areas of Ghana, Africa. From collections of 22,832 invertebrates, we compared composition, abundance, and associated *M. ulcerans* positivity among sites. Biting hemipterans were rare and represented a small percentage (usually <2%) of invertebrate communities. No significant differences were found in hemipteran abundance or pathogen positivity between disease-endemic and non-disease-endemic sites, and between abundance of biting hemipterans and *M. ulcerans* positivity. Therefore, although infection through insect bites is possible, little field evidence supports the assumption that biting hemipterans are primary vectors of *M. ulcerans*.

Mycobacterium ulcerans infection is an emerging skin disease often called Buruli ulcer (BU). Infection results in illness and lasting negative socioeconomic effects in rural areas of the tropics and subtropics (1). The pathologic changes, clinical signs and symptoms, and treatment have been reviewed elsewhere (2–5). In this article we evaluate field evidence for the potential of aquatic invertebrates to be vectors of *M. ulcerans*.

The exact mode of BU transmission remains unknown; however, past epidemiologic studies have associated BU with human activity near, or within, slow-flowing or standing water bodies that have been created or disturbed by

humans (2–4). Although several water-related risk factors have been recognized, none has been consistently reported, making it difficult to identify specific water-related risk activities (6–8). Most studies suggest that infection occurs through inoculation of *M. ulcerans* into skin lesions or insect bites (2,4,9–11). Portaels et al. (11) were the first to propose that aquatic insects might serve as vectors of *M. ulcerans*. This hypothesis maintains that *M. ulcerans* is found in biofilms of aquatic habitats and concentrated by grazing or filter-feeding invertebrates that are then consumed by predators known to bite humans (11). Initial evidence for this hypothesis used PCR detection of the insertion sequence IS2404 to document *M. ulcerans*' association with biting water bugs (Hemiptera), filtered concentrates of water, detritus, and aquatic plants (4,12–14). These studies were important for understanding the possible environmental reservoirs of *M. ulcerans*. However, IS2404 is now understood to be not specific for *M. ulcerans* because this insertion sequence has been found in a number of other aquatic mycobacterial species, including *M. marinum* (15–17). When more discriminatory methods based on detection of variable number tandem repeats were used, many IS2404-positive environmental samples were reported to lack *M. ulcerans* (18). In light of these recent findings, the relative frequency or abundance of *M. ulcerans* among aquatic invertebrates or other environmental reservoirs, remains tenuous, and thus, the role of aquatic insect vectors is uncertain.

A series of laboratory experiments provided initial evidence for biting hemipteran vectors of *M. ulcerans* (19–23). Marsollier et al. (9,24) demonstrated that a South American isolate of *M. ulcerans* could survive and multiply within the salivary glands of aquatic bugs indigenous to France (Naucoridae: *Naucoris cimicoides*). Furthermore, *N. cimicoides* could transmit *M. ulcerans* by feeding on inoculated prey

^{*}Michigan State University, East Lansing, Michigan, USA; [†]University of Tennessee, Knoxville, Tennessee, USA; [‡]University of Ghana, East Legon, Ghana; and [§]Water Resources Research Institute, Accra, Ghana

¹Current affiliation: University of Dayton, Dayton, Ohio, USA.

and then biting mice, which then exhibited BU (9). Most recently, exposure to hemipteran insect saliva was reported to infer protection against lesion development in laboratory mouse models (21). That study also reported correlations between aquatic insect salivary gland antibodies in humans categorized as exposed or patient, when the former group had exhibited BU. However, 3 limitations of that study have been noted (25): 1) the antibodies against salivary proteins might only be biomarkers of protection; 2) possible geographically related polymorphisms in the salivary proteins among hemipteran taxa could limit the generalizability of protection among distant communities; and 3) the overall relevance of biting aquatic insects infected with *M. ulcerans* in the natural environment is unknown.

A confounding factor in these experimental studies is that they used 1 South American isolate of *M. ulcerans*. Recent data support 2 major lineages of *M. ulcerans*: the ancestral strains that closely resemble *M. marinum* in chromosomal content, and the classic strains that have undergone substantial genome reduction (26). The latter strains account for all severe disease and include the African, Malaysian, and Australian isolates. The aforementioned laboratory studies have been elegantly performed, but the use of a French species of Naucoridae and a South American isolate of *M. ulcerans* makes it difficult to assess the importance of insect transmission in Africa. Thus, although provocative experimental data support a potential role for aquatic hemipterans as vectors of *M. ulcerans* in laboratory settings, no supporting evidence has been obtained from studies conducted in the natural setting. Results from field studies that identify the relative abundance and exposure potential of biting aquatic hemipterans can provide insight into the importance of biting insects in BU transmission.

This study had 3 objectives: 1) to describe the aquatic invertebrate samples collected during a large-scale, 2-year standardized field-sampling program of 27 bodies of water in Ghana, West Africa; 2) to investigate *M. ulcerans* positivity among the same aquatic invertebrates from those water bodies, directly linking aquatic invertebrate communities with pathogen positivity; and 3) to discuss the role of human-biting hemipterans as primary vectors of *M. ulcerans*. Data on the detection of *M. ulcerans* within aquatic samples based on the use of variable number tandem repeats analysis are presented in another article (18). In the current article, we associate presumptive *M. ulcerans* positivity rates with relative abundance and percentage composition of the same aquatic communities.

Methods

Study Sites

In June 2004 and August 2005, we sampled 27 water bodies associated with human communities in southern

Ghana (Figure 1). The water bodies were located within or very near (<100–200 m) each community of housing structures and were routinely used for daily domestic purposes and reflect habitats of routine human exposure. These water bodies were chosen after discussions with community members who directed us to the main water source for drinking water, recreation, domestic washing, irrigation, or bathing for that community. Six of these sites were sampled in both years, providing information on annual variation: Afuaman, Amasaman, Abbeypanya, Afienyaa, Odumse, and Weija. Human BU case data for the years 2003–2005 were provided by the Ghana Ministry of Health and used to classify communities into 2 site types: 15 BU–endemic (BU+) and 12 BU–nonendemic (BU–). A site was classified as a BU+ type if at least 1 case of BU had been reported during the 3-year period.

Aquatic Invertebrate Sampling and Processing

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, we randomly placed two 1-m² polyvinyl chloride quadrats and collected invertebrates by sweeping within the quadrat with a 500- μ m mesh dip net. The quadrats floated on top of the water and delineated 1 m² of area to be sampled by using an aquatic dip net designed to capture the aquatic life stages of invertebrates. 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. All

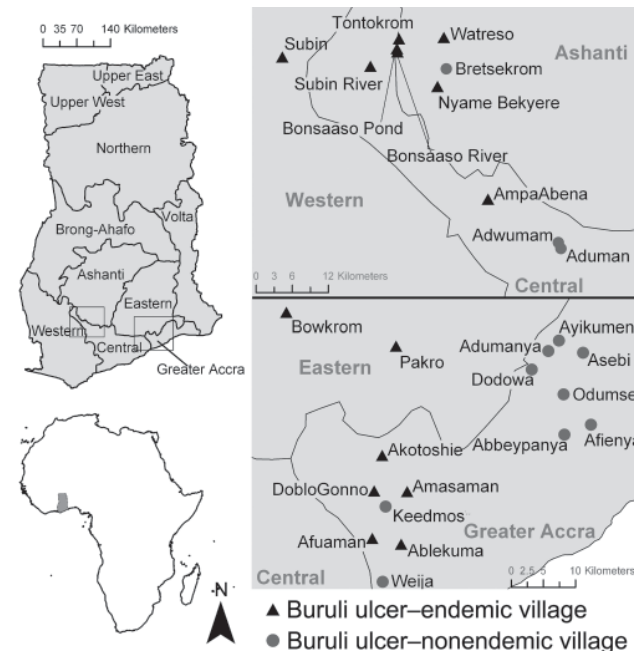


Figure 1. Regional site map of water bodies sampled in Ghana for aquatic invertebrates during 2004, 2005, or both. Small maps on left show location of Ghana in Africa and location of regions sampled within Ghana (boxes).

contents were washed through a 500- μ m sieve and preserved in 100% ethanol for laboratory identification and PCR. The 2 quadrats were combined into 1 composite sample.

***M. ulcerans* Detection in Invertebrate Samples**

Samples were analyzed in a 2-step procedure so that an initial screening reduced sample numbers. Small invertebrates were analyzed in pools of 3–15, whereas larger specimens were tested individually. DNA was extracted by using a protocol adapted from Lamour and Finley (27). Samples were ground and vortexed in 400 μ L of lysis solution (100 mmol/L Tris, pH 8.0), 50 mmol/L EDTA, 500 mmol/L NaCl, 1.33% sodium dodecyl sulfate, and 0.2 mg/mL RNase A) and 1 g of 1.0-mm glass beads (Sigma-Aldrich, St. Louis, MO, USA), then centrifuged. After 150 μ L of 5 mol/L potassium acetate was added, each sample was incubated overnight at -20° C. After a 30-min centrifugation, supernatants were transferred to new tubes containing 0.66 mol/L guanidine hydrochloride in a 63.3% ethanol solution. The samples were added to a spin filter (MO BIO Laboratories Inc., Carlsbad, CA, USA) in a 2-mL microcentrifuge tube (MO BIO Laboratories Inc.). The flow-through was discarded and the filter was rinsed first with 500 μ L of wash solution (10 mmol/L Tris, pH 8, 1 mmol/L EDTA, 50 mmol/L NaCl, 67% ethanol) and then with 500 μ L of 95% ethanol. The spin filters were dried by centrifugation and transferred to new 2-mL microcentrifuge tubes, immersed in 200 μ L elution solution (10 mmol/L Tris, pH 8), and incubated at room temperature for 15 min. The DNA was eluted and stored at -20° C.

Presumptive identification of *M. ulcerans* in invertebrates was based on detection of the enoyl reduction domain (ER) in *mlsA* that encodes the lactone core of the mycolactone toxin, the major virulence determinant of *M. ulcerans*. All samples were screened for the presence of the ER gene, which has been evaluated for *M. ulcerans* specificity in a companion study that used a multitiered PCR approach (18). Amplification of the ER gene was achieved using a 50- μ L reaction mixture containing 1 μ L each of forward and reverse primer (15,18), 10 μ L 5 \times Go Taq reaction buffer (Promega, Madison, WI, USA), 1 μ L 10 mmol/L PCR nucleotide mix (Promega), 31.7 μ L double-distilled water, 1.6 units Go Taq polymerase enzyme (Promega), and 5 μ L DNA template. Cycling conditions began with an initial denaturation at 94° C for 5 min, 35 cycles of 94° C for 1 min, 58° C for 45 seconds, 72° C for 1 min, and a final 10-min extension at 72° C. The amplified DNA was subjected to gel electrophoresis by using a 1.5% agarose gel, and band sizes were compared by using a 1-kb DNA ladder (Invitrogen, Carlsbad, CA, USA). PCR products of appropriate size were cloned into the pCR2.1 Topo vector (Invitrogen) and sequenced by using an ABI 3100 automated genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Data Analysis

Using all invertebrate data, we initially evaluated differences between site types (i.e., BU+ vs BU-) by comparing total abundance and percentage composition. Only those taxa that represented >3% of total invertebrates collected from all sites were used for subsequent statistical analyses because some taxa were so rare that any comparisons would limit meaningful conclusions. However, because we were interested in evaluating Hemiptera known to bite humans, the families Belostomatidae, Naucoridae, and Nepidae also were included, although each represented <2% of total collections.

To compare abundance differences between site types, *t* tests were used after data were log + 1 transformed to meet the assumptions of normality and equal variances. For percentage composition differences, data were arc-sine square root transformed, but they still did not demonstrate a normal distribution, so the nonparametric Wilcoxon/Kruskal-Wallis rank sum test was used. Because multiple tests were performed, it was necessary to calculate a Bonferroni adjusted α (and corresponding *p* value) of 0.006 to assist in interpreting statistically significant differences. However, to evaluate the biological meaning of these multiple tests, Cohen *d* effect size (and 95% confidence intervals) was calculated with Hedges adjustment (28). To compare overall ER positivity proportions between BU+ and BU- sites, a *t* test was used after data were arc-sine square root transformed. Lastly, we evaluated correlations between total biting hemipterans (and each individual family) and ER positivity using Spearman rank correlations with a Bonferroni adjusted $\alpha = 0.008$. This nonparametric test was used after attempts to transform the data for normality and homogeneity of variances failed.

Results

Invertebrate Abundance and Composition

Of 22,832 invertebrates collected, \approx 50% came from each group of BU+ and BU- site types (online Technical Appendix, available from www.cdc.gov/EID/content/14/8/1247-Techapp.pdf). A total of 85 taxa were represented among all sites: 80 taxa were collected from BU+ sites compared with 71 from BU- sites. The abundance of specific taxa was not consistent between site types, indicating that the invertebrate communities were highly variable. This variability was confirmed in statistical analyses comparing the most abundant taxa (>3%) with substantial effect size variation within and among taxa (online Technical Appendix). The invertebrates found in greatest abundance were 2 families of Diptera (i.e., Chironomidae and Culicidae), 1 family of Ephemeroptera (Baetidae), and several Crustacea. More than 300 individuals of some families of Hemiptera, Coleoptera, and

Odonata were encountered (online Technical Appendix). The biting Hemiptera were usually rare. For instance, 55 Naucoridae in total were collected, which was about 0.2% of all invertebrates sampled (online Technical Appendix).

Insects made up the greatest percentage of the invertebrates collected from BU+ sites but were nearly equivalent to the Crustacea in BU- sites. In BU- sites, Anura made up a relatively higher percentage, but most (1,231 of 1,303 individuals) were from a single site (Figure 2; online Technical Appendix). The Crustacea were most often represented by copepods, ostracods, and shrimp (Athyidae); fewer shrimp were collected from BU+ sites. Most shrimp were from BU- sites Adumanya (197) and Keedmos (120). Further, in BU- sites the large copepod abundance occurred primarily at Odumse, where 1,723 were collected from a total 1,884 (online Technical Appendix). Insects were reduced by 40% in BU- sites compared with BU+ sites (Figures 2, 3). When individual insect orders were compared, the Ephemeroptera (mayflies) and Diptera (true flies) made up the greatest percentages of insects in both BU+ and BU- site types (Figure 3; online Technical Appendix).

When the abundance and percent composition of dominant taxa were statistically compared between BU+ and BU- site types, there were no significant differences for any taxa (online Technical Appendix). However, the effect size varied greatly, reflecting a need to collect from more sites in future studies. On average, the Chironomidae (midges) made up the greatest percentage of the invertebrate communities, representing 9%–20% of the total, while the Baetidae (mayflies) ranged from 6% to 15% and the Culicidae (mosquitoes) from 2% to 5%. The biting Hemiptera made up a very small percentage of the dominant invertebrate communities, with Naucoridae \leq 0.5%, Belostomatidae \leq 2%, and Nepidae \leq 0.3% (online Technical Appendix).

Presumptive Identification of *M. ulcerans* from Invertebrates

Presumptive identification of *M. ulcerans* from a total of 1,032 invertebrate sample pools tested found no significant difference between BU+ and BU- site types (online Technical Appendix). Furthermore, there was no detectable pattern of invertebrate taxa ER positivity among sites, indicating that no single taxon was more often likely to be positive at a particular site. The number of ER positive taxa that were detected at any site ranged from 0 to 15 and 0 to 6 in BU+ and BU- sites, respectively (Figure 4). Clearly, not all BU+ or BU- sites had ER positive invertebrates. There were 6/15 BU+ sites without a single taxon positive compared with only 3/12 BU- sites (Figure 4).

Taxon-specific ER positivity was highly variable, and percentage positivity ranged from 0% to 100% among taxa

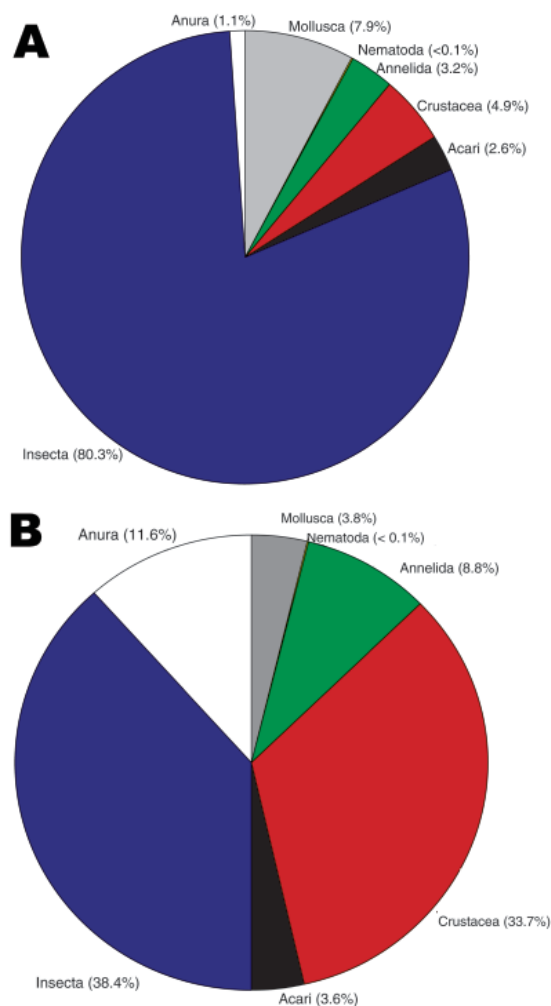


Figure 2. Higher level classification (e.g., class, phylum) taxa percentage composition between A) Buruli ulcer-endemic (n = 15) and B) Buruli ulcer-nonendemic (n = 12) site types, Ghana.

(online Technical Appendix). There were 26 taxa positive from BU+ site types compared with only 18 from BU- sites. Only 2 taxa were positive in BU- and not in BU+ sites, and for those taxa, $<$ 5 samples were tested from the BU+ type. When only those taxa with $>$ 5 samples tested were compared, no observable pattern in ER positivity was apparent among sites or taxa. The most abundant taxa did not always have the greatest ER positivity. For instance, positivity of Chironomidae (19.5% of all invertebrates) was only about 7%, even though positivity of Caenidae ($<$ 2% of all invertebrates) ranged from 6% to 17% (online Technical Appendix). For taxa with $>$ 5 samples tested from either BU+ or BU- sites, the ER positivity was \geq 20% for 5 taxa and from 10% to 20% for 12 taxa (online Technical Appendix). The biting Hemiptera had neither the highest nor consistently higher ER positivity compared with more abundant taxa (online Technical Appendix). Fifteen taxa with $>$ 5 samples

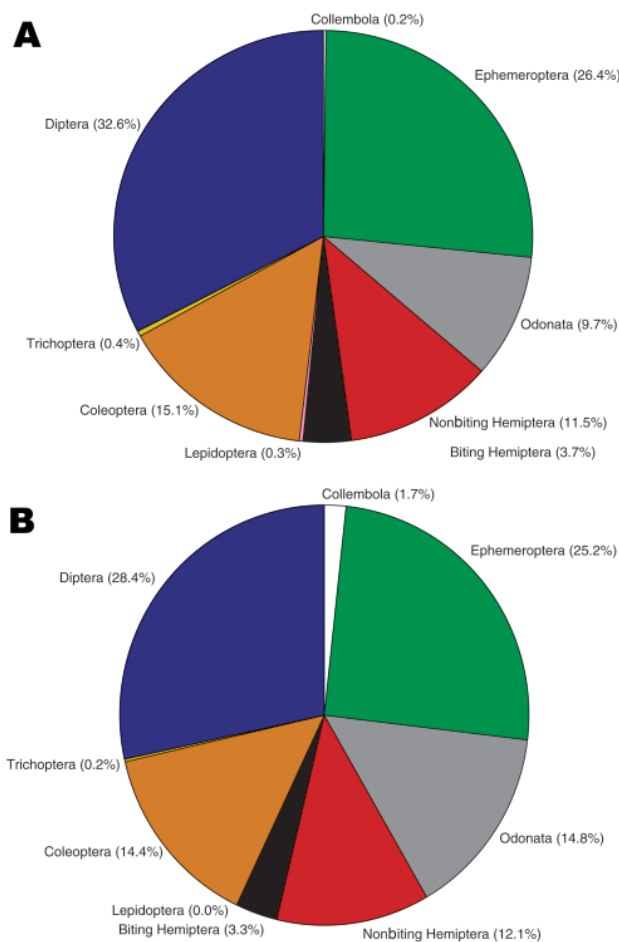


Figure 3. Insect order percentage composition between Buruli ulcer–endemic (n = 15) and Buruli ulcer–nonendemic site types (n = 12), Ghana.

tested had 0 positivity. These taxa represented all invertebrate functional feeding groups (e.g., predators, shredders, scrapers, collector-gatherers, and filterers).

Biting Hemiptera Correlations

No significant correlation was found between mean ER positivity and total biting Hemiptera ($r = 0.25$; $p = 0.218$) or any individual family: Belostomatidae ($r = 0.31$; $p = 0.118$), Naucoridae ($r = -0.03$; $p = 0.850$), and Nepidae ($r = 0.37$; $p = 0.060$). These results confirmed that biting Hemiptera were not significantly associated with the pathogen in the environment.

Discussion

The role of aquatic invertebrates in the transmission of BU has been proposed several times (3,4,29). However, to date, no large-scale field studies have assessed aquatic invertebrate communities from multiple loca-

tions or evaluated associated *M. ulcerans* positivity rates for specific invertebrate communities. Understanding the relative abundance and composition of the invertebrate taxa is a useful initial approach for assessing exposure risk of populations that use waterbodies for domestic needs. If biting water bugs are primary vectors of *M. ulcerans*, then the minimum (but not only) supporting evidence should confirm at least 1 of the following characteristics: 1) biting water bugs should be relatively more abundant at sites with BU cases compared with those without BU, indicating increased exposure potential to the vector in disease-endemic communities; 2) biting water bugs should have relatively higher *M. ulcerans* positivity rates within disease-endemic sites compared with disease-nonendemic sites; 3) *M. ulcerans* positivity rates should be higher in biting water bugs than in other invertebrates in the same sites, demonstrating increased potential pathogen exposure in the vector compared with background exposure; or 4) a correlation should exist between *M. ulcerans* positivity and vector abundance. This study addressed each of these characteristics and did not find strong confirming evidence that biting water bugs were any more important in the transmission of *M. ulcerans* than passive contact exposure to the environment. This finding is consistent with reports that few infected persons remember being bitten by water bugs (30). Although our results do not prove that infection could never occur from biting water bugs, they suggest that such an event would be rare.

In a companion study, Williamson et al. (18) reported *M. ulcerans* ER positivity from a broad spectrum of environmental samples, including animals, water filtrate, and biofilm on glass slides. They found that *M. ulcerans* DNA was detectable, not only at sites with or without a history of BU cases, but also in the environment, independent of invertebrates; positive results were detected for all sample types. Although *M. ulcerans* has been detected on the exoskeleton of experimentally infected Naucoridae (9), the possibility that invertebrates could serve as substrates for *M. ulcerans* in a natural environment has not been addressed, but it is certainly possible and may explain the wide range of taxa that were found positive in this study.

The invertebrate communities in this study demonstrated high intersite variation (online Technical Appendix), a finding similar to those of other studies of lentic invertebrate habitats (31,32). This variation suggests that additional collection sites should be included for a more comprehensive evaluation of invertebrate communities; an expanded study is under way. Hydrologic and physical/chemical attributes regulate the structure and abundance of invertebrate communities (31), while biotic factors such as macrophytes and fish can also influence communities (33). Few basic ecologic studies have been conducted on non-disease-related aquatic invertebrates in West Africa. The

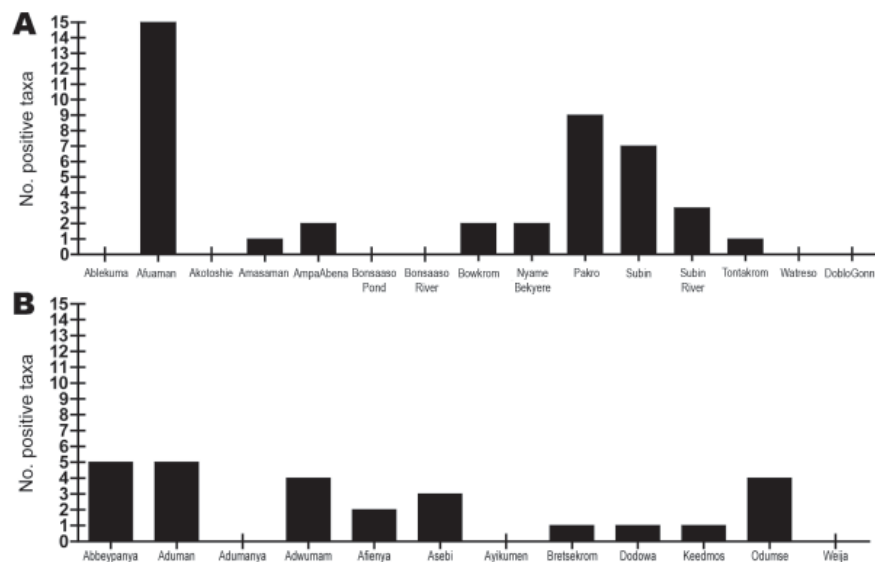


Figure 4. Number of enoyl-reduction-domain-positive taxa detected for each A) Buruli ulcer-endemic site ($n = 15$) and B) Buruli ulcer-nonendemic site ($n = 12$), Ghana.

most comprehensive articles on ecology have come from studies of small, fast-flowing streams or large lakes (34,35), which are different habitats than those in this study.

Season may also play a role in invertebrate abundance patterns; however, in many tropical and subtropical regions, most invertebrate taxa show minimal seasonally based abundance patterns (36–38). Most tropical species have multivoltine (multiple generations) and asynchronous (overlapping) life cycles throughout the year (39). For instance, all life stages of tropical naucorids have been reported through both wet and dry seasons over 2 years (38), and the same has been documented for other aquatic invertebrates in Kenya (36) and Lake Tanganyika (37). Therefore, although season might have had a small effect on the abundance variation of biting hemipterans and other invertebrates, this influence was unlikely to have limited our potential for detecting differences between BU+ and BU– sites.

If season affects biting Hemiptera populations, and these insects are important vectors, then human BU case data should reflect seasonal patterns, but this is not generally reported (4). In a recent study, no seasonal pattern was shown in monthly BU cases for 2003, 2004, and 2005 (40). In the current study, sampling each site throughout the year was not logistically feasible. In other ongoing studies, we have sampled an additional 55 sites, including 22 sites from 2004 to 2005 that have been sampled at least twice and 6 sites sampled 3 times over 3 years. The abundance of biting Hemiptera and other invertebrates from these additional sites are similar to what is reported here. Therefore, although season may have influenced our invertebrate community abundances, little evidence suggests that BU+ and BU– sites would be differentially affected, that Ghanaian invertebrate communities should respond differently to season compared with communities in other tropical and

subtropical regions, or that any seasonal pattern in BU cases is related to seasonal population changes of biting hemipterans.

Various researchers have proposed that biting water bugs could be vectors for *M. ulcerans*, and laboratory studies have provided evidence for this possibility. However, no complementary field studies had tested these laboratory results. Results from this field study do not support the hypothesis that biting aquatic insects are primary vectors of *M. ulcerans*. The results do not rule out the possibility of biting Hemiptera or other invertebrates as vectors or possible reservoirs for *M. ulcerans*, but rather, they suggest caution in describing their role in transmission. These field data on biting hemipteran abundance and *M. ulcerans* positivity suggest a need to reevaluate future research directions for understanding BU transmission.

Acknowledgements

We are grateful to E. Ampadu for providing the BU case data and Kingsley Asiedu for continued support of our research. We also thank Todd White and Lydia Mosi for field assistance and invertebrate collections and identifications, and Lindsay Campbell and Jenni van Ravensway for generating the site map.

This work was funded by the World Health Organization and the National Institutes of Health (NIH). The project described was supported by grant no. R01TW007550 from the Fogarty International Center through the NIH/National Science Foundation Ecology of Infectious Diseases Program and grant no. R03AI062719.

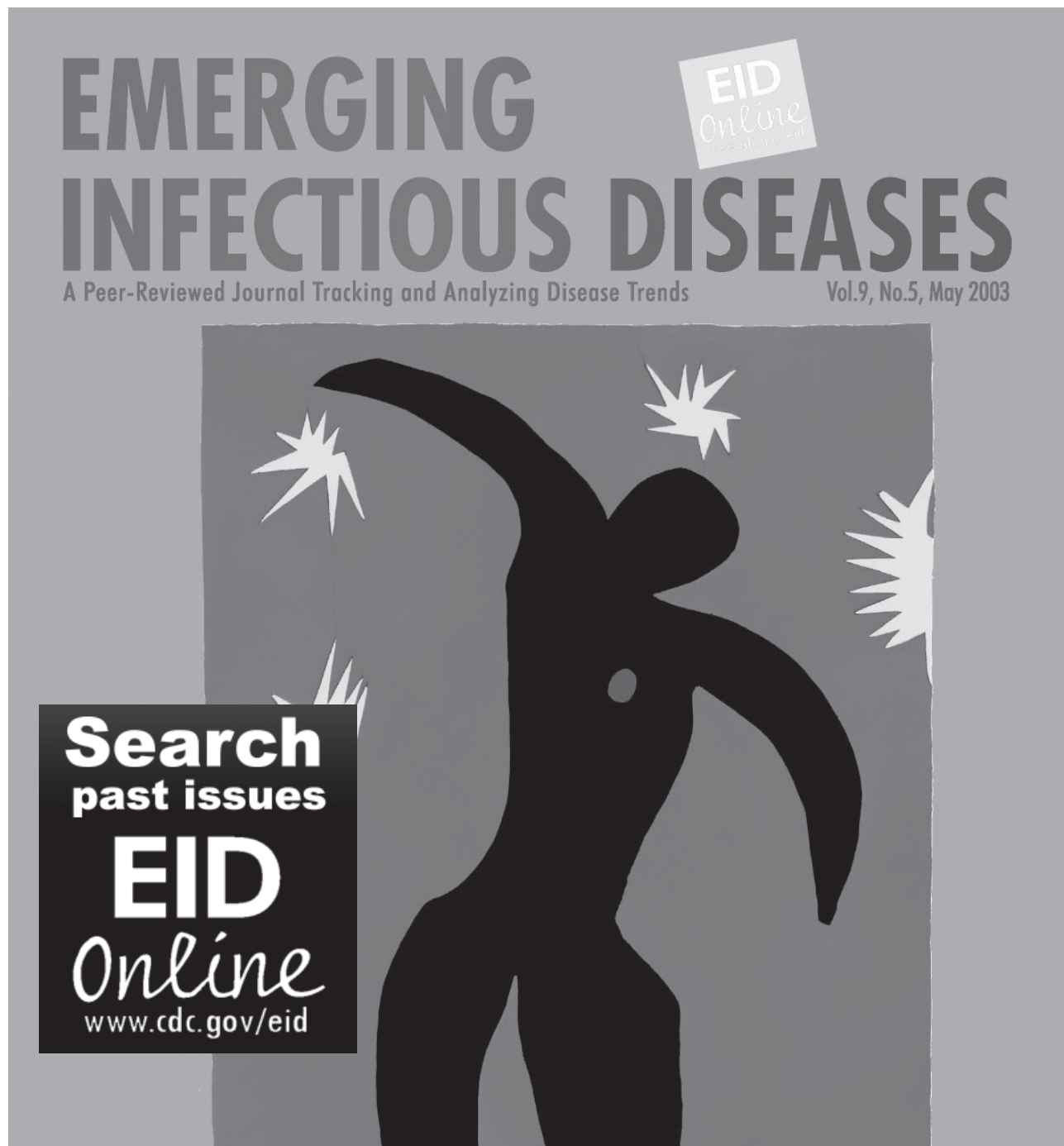
Dr Benbow is an assistant professor in the Department of Biology at the University of Dayton. His research interests include the role of human-mediated environmental changes in aquatic ecosystem structure and function, with emphasis on the ecology of *M. ulcerans* infection in West Africa.

References

- Asiedu K, Etuafu S. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. *Am J Trop Med Hyg.* 1998;59:1015–22.
- Duker AA, Portaels F, Hale M. Pathways of *Mycobacterium ulcerans* infection: a review. *Environ Int.* 2006;32:567–73. DOI: 10.1016/j.envint.2006.01.002
- Johnson PDR, Stinear TP, Small PLC, Pluschke G, Merritt RW, Portaels F, et al. Buruli ulcer (*M. ulcerans* infection): new insights, new hope for disease control. *PLoS Med.* 2005;2:e108. DOI: 10.1371/journal.pmed.0020108
- Merritt RW, Benbow ME, Small PLC. Unraveling an emerging disease associated with disturbed aquatic environments: the case of Buruli ulcer. *Front Ecol Environ.* 2005;3:323–31.
- Thangaraj HS, Phillips RO, Evans MRW, Wansbrough-Jones MH. Emerging aspects of Buruli ulcer. *Expert Rev Anti Infect Ther.* 2003;1:217–22. DOI: 10.1586/14787210.1.2.217
- Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, Meyers W, et al. Risk factors for Buruli ulcer, Benin. *Emerg Infect Dis.* 2006;12:1325–31.
- Raghunathan PL, Whitney EAS, Asamoah K, Stienstra Y, Taylor TH Jr, Amofah GK, et al. Risk factors for Buruli ulcer disease (*Mycobacterium ulcerans* infection): results from a case-control study in Ghana. *Clin Infect Dis.* 2005;40:1445–53. DOI: 10.1086/429623
- Aiga H, Amano T, Cairncross S, Domako JA, Nanas O-K, Coleman S. Assessing water-related risk factors for Buruli ulcer: a case-control study in Ghana. *Am J Trop Med Hyg.* 2004;71:387–92.
- Marsollier L, Robert R, Aubry J, Andre JS, Kouakou H, Legras P, et al. Aquatic insects as a vector for *Mycobacterium ulcerans*. *Appl Environ Microbiol.* 2002;68:4623–8. DOI: 10.1128/AEM.68.9.4623-4628.2002
- Portaels F, Chemlal K, Elsen P, Johnson PDR, Hayman JA, Hibble J, et al. *Mycobacterium ulcerans* in wild animals. *Rev Sci Tech.* 2001;20:252–64.
- Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P, Meyers WM. Insects in the transmission of *Mycobacterium ulcerans* infection. *Lancet.* 1999;353:986. DOI: 10.1016/S0140-6736(98)05177-0
- Ross BC, Johnson PD, Oppedisano F, Marino L, Sievers A, Stinear T, et al. Detection of *Mycobacterium ulcerans* in environmental samples during an outbreak of ulcerative disease. *Appl Environ Microbiol.* 1997;63:4135–8.
- Stinear T, Davies JK, Jenkin GA, Hayman JA, Oppedisano F, Johnson PDR. Identification of *Mycobacterium ulcerans* in the environment from regions in Southeast Australia in which it is endemic with sequence capture-PCR. *Appl Environ Microbiol.* 2000;66:3206–13. DOI: 10.1128/AEM.66.8.3206-3213.2000
- Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne P, Portaels F. One-tube cell lysis and DNA extraction procedure for PCR-based detection of *Mycobacterium ulcerans* in aquatic insects, molluscs and fish. *J Med Microbiol.* 2004;53:927–33. DOI: 10.1099/jmm.0.45593-0
- Mve-Obiang A, Lee RE, Umstot ES, Trott KA, Grammer TC, Parker JM, et al. A newly discovered mycobacterial pathogen isolated from laboratory colonies of *Xenopus species* with lethal infections produces a novel form of mycolactone, the *Mycobacterium ulcerans* macrolide toxin. *Infect Immun.* 2005;73:3307–12. DOI: 10.1128/IAI.73.6.3307-3312.2005
- Yip MJ, Porter JL, Fyfe JAM, Lavender CJ, Portaels F, Rhodes M, et al. Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. *J Bacteriol.* 2007;189:2021–9. DOI: 10.1128/JB.01442-06
- Ranger BS, Mahrous EA, Mosi L, Adusumilli S, Lee RE, Colomi A, et al. Globally distributed mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. *Infect Immun.* 2006;74:6037–45. DOI: 10.1128/IAI.00970-06
- Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, et al. Distribution of *Mycobacterium ulcerans* in Buruli ulcer endemic and non-endemic aquatic sites in Ghana. *PLoS Neg Trop Dis.* 2008;2:e205. DOI: 10.1371/journal.pntd.0000205
- Marsollier L, Stinear TP, Aubry J, Saint-Andre J-P, Robert R, Legras P, et al. Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. *Appl Environ Microbiol.* 2004;70:1097–103. DOI: 10.1128/AEM.70.2.1097-1103.2004
- Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, Legras P, et al. Aquatic snails, passive hosts of *Mycobacterium ulcerans*. *Appl Environ Microbiol.* 2004;70:6296–8. DOI: 10.1128/AEM.70.10.6296-6298.2004
- Marsollier L, Deniaux E, Brodin P, Marot A, Wondje CM, Saint A, et al. Protection against *Mycobacterium ulcerans* lesion development by exposure to aquatic insect saliva. *PLoS Med.* 2007;4:e64. DOI: 10.1371/journal.pmed.0040064
- Marsollier L, Aubry J, Saint-Andre J-P, Robert R, Legras P, Manceau A-L, et al. Ecology and transmission of *Mycobacterium ulcerans*. *Pathol Biol (Paris).* 2003;51:490–5. DOI: 10.1016/S0369-8114(03)00151-2
- Marsollier L, Andre JS, Frigui W, Reysset G, Milon G, Carbonnelle B, et al. Early trafficking events of *Mycobacterium ulcerans* within *Naucoris cimicoides*. *Cell Microbiol.* 2007;9:347–55. DOI: 10.1111/j.1462-5822.2006.00790.x
- Marsollier L, Legras P, Manceau A-L, Saint-André J-P, Aubry J, Robert R, et al. Role des punaises d'eau dans la transmission de *M. ulcerans*. *Bull de l'ALLF.* 2002;10:23–5.
- Silva MT, Portaels F, Pedrosa J. Aquatic insects and *Mycobacterium ulcerans*: an association relevant to Buruli ulcer control? *PLoS Med.* 2007;4:e63. DOI: 10.1371/journal.pmed.0040063
- Rondini S, Käser M, Stinear T, Tessier M, Mangold C, Dernick G, et al. Ongoing genome reduction in *Mycobacterium ulcerans*. *Emerg Infect Dis.* 2007;13:1008–15.
- Lamour K, Finley L. A strategy for recovering high quality genomic DNA from a large number of *Phytophthora* isolates. *Mycologia.* 2006;98:514–7. DOI: 10.3852/mycologia.98.3.514
- Nakagawa S. A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behav Ecol.* 2004;15:1044–5. DOI: 10.1016/behco/arh107
- Wansbrough-Jones M, Phillips R. Buruli ulcer: emerging from obscurity. *Lancet.* 2006;367:1849–58. DOI: 10.1016/S0140-6736(06)68807-7
- World Health Organization. Buruli ulcer. *Mycobacterium ulcerans* infection. Geneva: The Organization; 2000.
- Batzer DP, Wissinger SA. Ecology of insect communities in non-tidal wetlands. *Annu Rev Entomol.* 1996;41:75–100. DOI: 10.1146/annurev.en.41.010196.000451
- Tangen BA, Butler MG, Ell MJ. Weak correspondence between macroinvertebrate assemblages and land use in Prairie Pothole Region wetlands, USA. *Wetlands.* 2003;23:104–15. DOI: 10.1672/0277-5212(2003)023[0104:WCBMAA]2.0.CO;2
- de Szalay FA, Resh VH. Factors influencing macroinvertebrate colonization of seasonal wetlands: responses to emergent plant cover. *Freshw Biol.* 2000;45:295–308. DOI: 10.1111/j.1365-2427.2000.00623.x
- Resh VH, Leveque C, Statzner B. 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. DOI: 10.1146/annurev.ento.49.061802.123231
- Petr T. Dynamics of benthic invertebrates in a tropical man-made lake (Volta Lake 1964–1968). Standing crop and bathymetric distribution. *Arch Hydrobiol.* 1974;73:245–65.

36. Dobson M, Magana AM, Lancaster J, Mathooko JM. Aseasonality in the abundance and life history of an ecologically dominant freshwater crab in the Rift Valley, Kenya. *Freshw Biol.* 2007;52:215–25. DOI: 10.1111/j.1365-2427.2006.01648.x
37. Donohue I, Irvine K. Seasonal patterns of sediment loading and benthic invertebrate community dynamics in Lake Tanganyika, Africa. *Freshw Biol.* 2004;49:320–31. DOI: 10.1111/j.1365-2427.2004.01187.x
38. Stout RJ. How abiotic factors affect the distribution of two species of tropical predaceous aquatic bugs (Family: Naucoridae). *Ecology.* 1981;62:1170–8. DOI: 10.2307/1937281
39. Hury AD, Wallace JB. Life history and production of stream insects. *Annu Rev Entomol.* 2000;45:83–110. DOI: 10.1146/annurev.ento.45.1.83
40. Sopoh GE, Johnson RC, Chauty A, Dossou AD, Aguiar J, Salmon O, et al. Buruli ulcer surveillance, Benin, 2003–2005. *Emerg Infect Dis.* 2007;13:1374–6.

Address for correspondence: M. Eric Benbow, Department of Biology, University of Dayton, Dayton, OH 45469-2320, USA; email: benbow@notes.udayton.edu



Aquatic Invertebrates as Unlikely Vectors of Buruli Ulcer Disease

Technical Appendix

Table 1. Total number of individual organisms collected for each taxon and grouped as BU–endemic or BU–nonendemic, based on human BU case data from 2003–2005*

Higher classification	Lower classification	Family	No. BU endemic (n = 15)	No. BU nonendemic (n = 12)	Total
Bivalvia	Gastropoda	Sphaeriidae	1	7	8
		Ancylidae	22	37	59
		Bulininae	162	14	176
		Lymnaeidae	12	0	12
		Physidae	214	12	226
		Planorbidae	315	210	525
		Thiaridae	16	44	60
		Viviparidae	2	1	3
	Gastropoda		171	99	270
Nematoda			10	7	17
Oligochaeta			317	947	1,264
Hirudinae			55	34	89
Crustacea	Cladocera		164	605	769
	Mysidacea		0	2	2
	Conchostraca		6	0	6
	Copepoda		78	1,884	1,962
	Ostracoda		248	833	1,081
	Decapoda	Atyidae	74	450	524
Arachnida	Acari		299	409	708
Insecta	Collembola	Entomobryiidae	22	45	67
		Isotomidae	0	27	27
	Ephemeroptera	Baetidae	2,127	736	2,863
		Caenidae	312	305	617
		Heptageniidae	12	29	41
		Leptophlebiidae	11	4	15
		Oligoneuriidae	1	0	1
		Polymitarcyidae	1	10	11
		Calopterygidae	0	2	2
	Odonata	Coenagrionidae	385	283	668
		Corduliidae	94	73	167
		Gomphidae	1	1	2
		Libellulidae	131	107	238
		Unknown	66	30	96
		Protoneuridae	232	140	372
	Hemiptera	Belostomatidae	283	118	401
		Corixidae	47	217	264
		Gerridae	22	18	40
		Hebridae	10	0	10
Hydrometridae		4	4	8	
Mesoveliidae		113	47	160	
Naucoridae		52	3	55	
Nepidae		12	22	34	
Notonectidae		198	189	387	
Ochteridae		1	0	1	

		Pleidae	652	31	683
		Saldidae	2	1	3
		Veliidae	27	16	43
Lepidoptera		Pyrilidae	31	1	32
Coleoptera		Carabidae	2	1	3
		Coleoptera	4	0	4
		Curculionidae	1	10	11
		Dryopidae	6	3	9
		Dytiscidae	272	86	358
		Elmidae	43	15	58
		Gyrinidae	23	2	25
		Hydraenidae	339	93	432
		Hydrophilidae	421	243	664
		Hygrobiidae	13	0	13
		Lampyridae	12	2	14
		Noteridae	251	83	334
		Psephenidae	1	0	1
		Scirtidae	52	44	96
		Staphylinidae	5	4	9
Trichoptera		Ecnomidae	1	0	1
		Helicopsychidae	0	2	2
		Hydropsychidae	12	0	12
		Leptoceridae	18	6	24
		Polycentropodidae	3	0	3
Diptera		Ceratopogonidae	126	135	261
		Chaoboridae	18	18	36
		Chironomidae	1,935	858	2,793
		Culicidae	871	182	1,053
		Diptera	1	1	2
		Empididae	6	0	6
		Ephydriidae	9	3	12
		Muscidae	1	0	1
		Psychodidae	22	5	27
		Sciomyzidae	2	1	3
		Simuliidae	5	0	5
		Stratiomyiidae	15	9	24
		Syrphidae	3	1	4
		Tabanidae	13	1	14
		Thaumaleidae	0	2	2
		Tipulidae	15	6	21
Vertebrata	Anura		123	1,303	1,426
		Total	11,659	11,173	22,832

*BU, Buruli ulcer. Invertebrates were collected from sites during 2004, 2005, or both; all taxa that were collected are included.

Table 2. Mean (SD) invertebrate taxon abundance and percentage composition for taxa that made up >3% of the total collected, and calculated by sites grouped as BU endemic or BU nonendemic, based on human BU case data from 2003 through 2005*

Category	Taxon	BU endemic (N = 15)		BU nonendemic (N = 12)		p value	Cohen <i>d</i> (95% CI)
		Mean	SD	Mean	SD		
Abundance†	Total organisms	787	644	943	1,096	0.678	0.17 (-0.59-0.93)
	Ephemeroptera						
	Baetidae	142	257	61	82	0.241	0.41 (-0.36-1.18)
	Diptera						
	Culicidae	58	81	15	28	0.455	0.69 (-0.09-1.47)
	Chironomidae	129	106	72	108	0.113	0.52 (-0.25-1.29)
	Hemiptera						
	Belostomatidae	19	30	10	11	0.882	0.39 (-0.38-1.15)
	Naucoridae	3	6	0	1	0.050	0.68 (-0.10-1.46)
	Nepidae	1	1	2	4	0.768	0.33 (-0.43-1.10)
	Pleidae	43	71	3	5	0.066	0.77 (-0.02-1.56)
	Oligochaeta	23	30	73	188	0.787	0.36 (-0.41-1.13)
	Copepoda	5	11	157	494	0.587	0.42 (-0.35-1.19)
	Ostracoda	17	31	69	165	0.344	0.42 (-0.34-1.19)
	Cladocera	11	17	50	115	0.768	0.46 (-0.31-1.23)
	Acari	20	35	34	62	0.552	0.27 (-0.49-1.03)
	Anura	8	7	109	354	0.181	0.39 (-0.38-1.16)
% Composition‡	Ephemeroptera						
	Baetidae	15.1	16.1	6.1	4.6	0.053	0.71 (-0.07-1.50)
	Diptera						
	Culicidae	4.8	6.5	1.2	1.7	0.055	0.74 (-0.05-1.52)
	Chironomidae	19.5	19.2	8.8	7.8	0.065	0.71 (-0.08-1.49)
	Hemiptera						
	Belostomatidae	1.4	1.9	2.1	3.6	0.544	0.24 (-0.52-1.00)
	Naucoridae	0.5	1.2	0.1	0.1	0.151	0.54 (-0.23-1.31)
	Nepidae	0.2	0.4	0.3	0.7	0.524	0.25 (-0.51-1.01)
	Pleidae	3.7	5.4	0.2	0.3	0.022	0.91 (0.11-1.70)
	Oligochaeta	2.2	2.7	3.5	5.4	0.476	0.28 (-0.48-1.05)
	Copepoda	0.6	1.2	5.3	13.8	0.246	0.48 (-0.29-1.25)
	Ostracoda	2.5	6.3	2.6	4.8	0.928	0.03 (-0.73- 0.79)
	Cladocera	1.4	2.1	3.1	5.7	0.369	0.36 (-0.40-1.13)
	Acari	1.7	2.1	4.8	11.6	0.392	0.35 (-0.41-1.12)
	Anura	1.5	1.9	7.1	17.8	0.309	0.42 (-0.35-1.19)

*BU, Buruli ulcer. Invertebrates were collected from sites during 2004, 2005, or both.

†Wilcoxon/Kruskal-Wallis rank sum test used for abundance comparisons.

‡*t* tests were used for percentage composition comparisons after data transformation. The effect size is given as Cohen *d* (with the Hedge adjustment) along with 95% confidence interval [CI]. Bonferroni multiple comparison adjustments gave an $\alpha = 0.006$ for significant differences based on multiple comparisons.

Table 3. Community-specific ER PCR sample numbers and positivity results*

Community	No. ER negative	No. ER positive	No. samples tested (% ER positive)
Endemic			
Ablekuma	23	0	23 (0)
Afuaman	67	17	84 (20.2)
Akotoshie	27	0	27 (0)
Amasaman	58	1	59 (1.7)
AmpaAbena	28	2	30 (6.7)
Bonsaaso Pond	36	0	36 (0)
Bonsaaso River	21	0	21 (0)
Bowkrom	45	1	46 (2.2)
Nyame Bekyere	18	2	20 (10.0)
Pakro	17	9	26 (34.6)
Subin	60	6	66 (9.1)
Subin River	43	3	46 (6.5)
Tontokrom	36	1	37 (2.7)
Watreso	37	0	37 (0)
DobloGonno	21	0	21 (0)
BU–endemic total	537	42	579 (6.3)
BU nonendemic			
Abbeypanya	49	6	55 (10.9)
Aduman	11	6	17 (35.3)
Adumanya	38	0	38 (0)
Adwumam	41	5	46 (10.9)
Afiencya	76	2	78 (2.6)
Asebi	22	3	25 (12.0)
Ayikumen	20	0	20 (0)
Bretsekrom	47	1	48 (2.1)
Dodowa	17	1	18 (5.6)
Keedmos	20	1	21 (4.8)
Odumse	47	4	51 (7.8)
Weija	36	0	36 (0)
BU–nonendemic total	424	29	453 (7.7)

*ER, enoyl-reduction-domain; BU, buruli ulcer. Communities are grouped as BU endemic or BU nonendemic based on BU cases during 2003–2005. Positive percentages are given for each group as well as for the overall total. *t* test showed no significant difference between BU–endemic and BU–nonendemic site (*t* = 0.378, *df* = 25, *p* = 0.709).

Table 4. Taxon-specific ER PCR sample numbers and positivity results*

Higher classification	Lower classification	Family	BU endemic (N = 15)		BU nonendemic (N = 12)		All sites	
			No. ER positive	No. samples tested (% ER positive)	No. ER positive	No. samples tested (% ER positive)	No. ER positive	No. samples tested (% ER positive)
Bivalvia		Bivalvia				2 (0)		2 (0)
		Sphaeriidae			1	1 (100.0)	1	1 (100.0)
Gastropoda		Ancylidae		1 (0)		3 (0)		4 (0)
		Lymnaeidae		2 (0)				2 (0)
		Physidae	1	7 (14.3)		2 (0)	1	9 (11.1)
		Pilidae		5 (0)				5 (0)
		Planorbidae	1	18 (5.6)	1	11 (9.1)	2	29 (6.9)
		Bulininae	1	8 (12.5)	0	2 (0)	1	10 (10.0)
		Thiaridae		4 (0)		4 (0)		8 (0)
		Viviparidae		2 (0)		1 (0)		3 (0)
		Unknown		1 (0)		8 (0)		9 (0)
Nematoda				2 (0)		1 (0)		3 (0)
Oligochaeta			1	13 (7.7)	1	12 (8.3)	2	25 (8.0)
Hirudinea			2	15 (13.3)	1	10 (10.0)	3	25 (12.0)
Crustacea	Cladocera			5 (0)		8 (0)		13 (0)
	Mysidacea					1 (0)		1 (0)
	Conchostraca			1 (0)		0		1 (0)
	Copepoda			5 (0)		8 (0)		13 (0)
	Ostracoda			4 (0)	2	10 (20.0)	2	14 (14.3)
	Decapoda	Atyidae		4 (0)		12 (0)		16 (0)
Arachnida	Acari		3	15 (20.0)		13 (0)	3	28 (10.7)
Insecta	Collembola	Entomobryidae		3 (0)		3 (0)		6 (0)
		Isotomidae		1 (0)				1 (0)
	Ephemeroptera	Baetidae	2	21 (9.5)		23 (0)	2	44 (4.5)
		Caenidae	2	12 (16.7)	1	16 (6.3)	3	28 (10.7)
		Heptageniidae		3 (0)		1 (0)		4 (0)
		Leptophlebiidae		3 (0)				3 (0)
		Polymitarcyidae				4 (0)		4 (0)
		Unknown		1 (0)				1 (0)
	Odonata	Aeshnidae		1 (0)		2 (0)		3 (0)
		Anisoptera		7 (0)		10 (0)		17 (0)
		Calopterygidae				1 (0)		1 (0)
		Coenagrionidae		12 (0)		7 (0)		19 (0)
		Corduliidae		3 (0)		2 (0)		5 (0)
		Gomphidae				1 (0)		1 (0)
		Libellulidae	1	16 (6.3)	3	10 (30.0)	4	26 (15.4)
		Protoneuridae	3	11 (27.3)	1	13 (7.7)	4	24 (16.7)
		Zygoptera		5 (0)		8 (0)		13 (0)
	Hemiptera	Belostomatidae	2	21 (9.5)	2	16 (12.5)	4	37 (10.8)
		Corixidae		4 (0)		11 (0)		15 (0)
		Gerridae		8 (0)		9 (0)		17 (0)
		Hebridae		2 (0)				2 (0)
		Hydrometridae		5 (0)		6 (0)		11 (0)
		Mesoveliidae		17 (0)		17 (0)		34 (0)

		Naucoridae	2	12 (16.7)	1	6 (16.7)	3	18 (16.7)
		Nepidae	1	9 (11.1)	3	12 (25.0)	4	21 (19.0)
		Notonectidae	1	18 (5.6)	2	19 (10.5)	3	37 (8.1)
		Pleidae		15 (0)		9 (0)		24 (0)
		Saldidae		1 (0)		1 (0)		2 (0)
		Veliidae		8 (0)		6 (0)		14 (0)
Lepidoptera		Pyrilidae	2	3 (66.7)			2	3 (66.7)
Coleoptera		Curculionidae				1 (0)		1 (0)
		Dytiscidae	1	27 (3.7)	2	17 (11.8)	3	44 (6.8)
		Elmidae		3 (0)	2	2 (100.0)	2	5 (40.0)
		Gyrinidae		2 (0)				2 (0)
		Hydraenidae	1	9 (11.1)	1	5 (20.0)	2	14 (14.3)
		Hydrophilidae	4	36 (11.1)	1	22 (4.5)	5	58 (8.6)
		Hygrobiidae		1 (0)				1 (0)
		Lampyridae		3 (0)		2 (0)		5 (0)
		Noteridae	1	23 (4.3)		15 (0)	1	38 (2.6)
		Psephenidae		1 (0)				1 (0)
		Scirtidae	1	6 (16.7)		4 (0)	1	10 (10.0)
		Unknown				7 (0)		7 (0)
Trichoptera		Helicopsychidae				1 (0)		1 (0)
		Hydropsychidae		2 (0)				2 (0)
		Leptoceridae		4 (0)		2 (0)		6 (0)
		Polycentropedidae		1 (0)				1 (0)
Diptera		Ceratopogonidae		15 (0)		13 (0)		28 (0)
		Chaoboridae		4 (0)		3 (0)		7 (0)
		Chironomidae	2	30 (6.7)	2	27 (7.4)	4	57 (7.0)
		Culicidae	2	19 (10.5)		11 (0)	2	30 (6.7)
		Dolichopodidae				1 (0)		1 (0)
		Ephydriidae		1 (0)				1 (0)
		Psychodidae	1	1 (100.0)			1	1 (100.0)
		Sciomyzidae	1	1 (100.0)		2 (0)	1	3 (33.3)
		Stratiomyidae		5 (0)		2 (0)		7 (0)
		Syrphidae	1	1 (100.0)		1 (0)	1	2 (50.0)
		Tabanidae		3 (0)				3 (0)
		Tipulidae		4 (0)		2 (0)		6 (0)
		Unknown				2 (0)		2 (0)
Vertebrata	Anura		2	21 (9.5)	2	10 (20.0)	4	31 (12.9)
		Total	42	557 (7.5)	29	474 (6.1)	71	1,031 (6.9)
		Mean	1.6	8.0 (8.8)	1.5	7.2 (6.5)	2.4	12.7 (7.8)
		SD	0.8	8.0 (21.7)	0.8	6.3 (18.2)	1.2	13.8 (18.6)

*ER, enoyl-reduction-domain; BU, Buruli ulcer. Results are grouped as endemic or nonendemic on the basis of BU cases during 2003–2005. Positivity percentages are given for each group as well as for the overall total. The table also includes the total number of endemic or nonendemic sites where there was at least one ER positive for the taxon.