Building evidence for conservation globally

Journal of Threatened Taxa

Open Access

10.11609/jott.2024.16.11.26063-26186
www.threatenedtaxa.org

26 November 2024 (Online & Print)
16(11): 26063–26186
ISSN 0974-7907 (Online)
ISSN 0974-7893 (Print)

ISSN 0974-7907 (Online); ISSN 0974-7893 (Print)

Publisher
Wildlife Information Liaison Development Society
www.wild.zooreach.org

Host
Zoo Outreach Organization
www.zooreach.org

Srivari Illam, No. 61, Karthik Nagar, 10th Street, Saravanampatty, Coimbatore, Tamil Nadu 641006, India
Registered Office: 3A2 Varadarajulu Nagar, FCI Road, Ganapathy, Coimbatore, Tamil Nadu 641006, India
Ph: +91 9385339863 | www.threatenedtaxa.org
Email: sanjay@threatenedtaxa.org

For Focus, Scope, Aims, and Policies, visit https://threatenedtaxa.org/index.php/JoTT/aims_scope
For Article Submission Guidelines, visit https://threatenedtaxa.org/index.php/JoTT/about/submissions
For Policies against Scientific Misconduct, visit https://threatenedtaxa.org/index.php/JoTT/policies_various

continued on the back inside cover

Cover: Mixed media with fine liners, colour pencils, and watercolour background of an Indian funnel web spider. © Elakshi Mahika Molur.

Journal of Threatened Taxa | www.threatenedtaxa.org | 26 November 2024 | 16(11): 26089–26103
ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)
https://doi.org/10.11609/jott.8954.16.11.26089-26103
#8954 | Received 08 February 2024 | Final received 11 October 2024 | Finally accepted 28 November 2024
OPEN ACCESS
ARTICLE

Watershed survey of streams in western Bhutan with macroinvertebrates, water chemistry, bacteria and DNA barcodes

Juliann M. Battle [1], Bernard W. Sweeney [2], Bryan Currinder [3], Anthony Aufdenkampe [4], Beth A. Fisher [5] & Naimul Islam [6]

[1,2] Stroud Water Research Center, 970 Spencer Road, Avondale, PA 19311, USA.
[3] Department of Environmental Science and Policy, 3134 Wickson Hall, University of California, Davis, CA 95616, USA.
[4] LimnoTech, 7300 Hudson Blvd, Suite 295, Oakdale, MN 55128, USA.
[5] Minnesota State University, 142 Ford Hall, Mankato, MN 56001, USA.
[6] The City of Philadelphia, 1101 Market St, Philadelphia, PA 19107, USA.
[1] jbattle@stroudcenter.org (corresponding author), [2] sweeney@stroudcenter.org, [3] bcurrinder@ucdavis.edu,
[4] aaufdenkampe@limno.com, [5] beth.fisher@mnsu.edu, [6] naimul.islam@phila.gov

Abstract: Bhutan in the eastern Himalaya contains some of the last pristine watersheds in the world, yet there has been limited monitoring of streams and rivers. Eighteen streams in three watersheds were surveyed for chemistry, bacteria, and macroinvertebrates in post-monsoon (2015) and monsoon (2016) seasons. Many water quality variables, including temperature, pH, specific conductivity, nitrite, nitrate, *E. coli,* and total coliform bacteria differed between seasons and between areas upstream and downstream of anthropogenic disturbance. In both seasons, total coliform bacteria and *E. coli* were significantly higher downstream of anthropogenic disturbance, with many urban sites having high coliform levels (>2000 cfu/100 ml) indicative of sewage inflow. A total of 50 insect families and six non-insect taxa were identified. During the post-monsoon, eight of 13 metrics (e.g., total richness, Ephemeroptera, Plecoptera, and Trichoptera (EPT) richness, % EPT, % non-insects, HKHbios, BMWP1983, ASPT1983, and ASPT2021) based on kick samples (qualitative) indicated impairment, while in the monsoon season composite Surbers (quantitative) had two metrics (e.g., total richness and Shannon) that differed between sites up and downstream of disturbance. DNA barcoding for cytochrome *c* oxidase subunit I (*COI*) in 63 morphological species of mayfly, stonefly, and caddisfly indicated 18 additional species, 17 mayflies and one stonefly. Forty-two barcode species were new additions to the Barcode of Life Data database. Results suggest macroinvertebrates are a viable method for evaluating human impacts on Bhutan streams. Bhutan faces future challenges of sanitation management, climate change, and shared river systems, and monitoring will need to be expanded. The monsoon season may be an ideal time to measure water chemistry and bacteria due to increased runoff, but macroinvertebrate sampling should occur in the post-monsoon season to obtain the best sampling conditions and larger individuals. Increasing the knowledge of species in the region, potentially with the help of DNA barcodes, will document the diversity of the region and help amplify the capacity for macroinvertebrates with future biomonitoring.

Keywords: Biomonitoring, coliform, COI gene, diversity, eastern Himalaya, EPT, hotspot, seasons, water quality.

Editor: J.A. Johnson, Wildlife Institute of India, Dehradun, India.

Date of publication: 26 November 2024 (online & print)

Citation: Battle, J.M., B.W. Sweeney, B. Currinder, A. Aufdenkampe, B.A. Fisher & N. Islam (2024). Watershed survey of streams in western Bhutan with macroinvertebrates, water chemistry, bacteria and DNA barcodes. *Journal of Threatened Taxa* 16(11): 26089–26103. https://doi.org/10.11609/jott.8954.16.11.26089-26103

Funding: The 2015 work was supported by gifts from Peter Kjellerup, Mandy Cabot, W.B. Dixon Stroud Jr., Lisa Stroud, and the Stroud Endowment for Environmental Research. The 2016 work was funded by the Greg and Susan Walker Endowment, a Trustees' Council of Penn Women Grant, and a Graduate & Professional Student Assembly Grant from the University of Pennsylvania. Barcode work was funded with help from a donation from MilliporeSigma, as facilitated by Tim Currinder. The preparation of this publication was supported by the Stroud Endowment for Environmental Research. This is Stroud Publication 2023001.

Competing interests: The authors declare no competing interests.

Author details, Author contributions, & Declarations: See end of this article.

Acknowledgements: The Waterkeeper Alliance was instrumental in organizing this project. Nedup Tshering (Clean Bhutan) and Pem Dekhi (Waterkeeper Alliance Bhutan) provided valuable assistance in Bhutan. We are grateful for the input from Dr. Sarah Willig, Dr. Yvette Bordeaux, Heather Kostick, Sally Cardy, Amanda Johnston, and William Morrissey from the University of Pennsylvania. We thank Peter Kjellerup, Mandy Cabot, and Dixon & Lisa Stroud for their assistance with the 2015 field collection. Special thanks to Dr. John Jackson from the Stroud Center for his support and access to various resources within the Stroud Center entomological section throughout the project. Stroud Center employees Kelly McIntyre and Mike Broomall were responsible for the EPT species identification, additional help from Stroud was provided by Katie McFadden Billé, Sally Peirson, and Matt Wilson for sample processing, and Charlie Dow, Tim Smith, and Ellie Kissis for data analyses. The 2016 data was from the Capstone Projects of B. Currinder and N. Islam from the University of Pennsylvania.

INTRODUCTION

Located in the eastern Himalaya, Bhutan is mountainous, 70% forested, and considered a world biodiversity "hotspot" (Wangdi et al. 2013). Human populations historically occurred largely in rural areas throughout the low to mid-elevations of the western part of the country (Worldometers 2022). Bhutan's urban population has grown by 40% from 2005 to 2017, and it is projected to comprise half of the country's population by 2037 (NSBB 2019). As of 2020, the capital Thimphu accommodated around 28% of the total urban population and ~13% of the total population (Worldometers 2022). This increased urbanization in the western part of the country has put significant stress on Bhutan's abundant, but fragile, forests and water resources (Wangdi et al. 2013). Thus, despite the country's relatively small population (791,817 people in 38,177 km^2; Worldometers 2022) and large forested areas, significant challenges currently exist in maintaining adequate water quality, particularly for humans and aquatic wildlife living downstream of population centers (WBMP 2016).

Although urban development tends to take up less area in the watershed than agriculture, it often has a larger impact on stream conditions (WBMP 2016). A recent survey of Bhutan wastewater management reported that only eight out of 35 towns (~7% of Bhutan's population) have a public sewage system, with the majority (80%) of the remaining urban population depending on on-site sanitation systems, with many being both inadequately designed and maintained (Dorji et al. 2019). Climate change poses additional threats to water quality in Bhutan through its impact on hydrology (WBMP 2016), causing localized droughts and frequent flooding (Tariq et al. 2021). Water scarcity, construction of roads, and hydropower dams are all factors that will impact water quality in Bhutan's streams and rivers (WBMP 2016; Thapa et al. 2020; Tariq et al. 2021). Because of its shared river systems (i.e., those flowing through multiple countries; sensu Price et al. 2014), the capacity to manage water quality becomes more complicated because of political challenges.

Agricultural land use and its impacts on water quality in Bhutan, as in other watersheds worldwide, varies with factors like intensity, region, livestock, and crop type, with virtually all water quality loss being due to modified flows, degraded channel habitat, altered temperature regimes, and high inputs of nutrients, pesticides, and sediments (Allan 2004). In Bhutan, the primary mode of livelihood in rural areas has historically consisted of traditional rain-fed and irrigated row crop agriculture, but recent times have seen a transition to more intensive agriculture using inorganic fertilizers (Dorji et al. 2011). Forestry (commercial and traditional firewood collection) and industry (mining, cement industry, fishery) are also impacting water quality in Bhutan (WBMP 2016; Tariq et al. 2021). Several studies in the Bhutan region examining water quality have pointed to the discharge of untreated sewage directly into streams as the major source of pollution in urban areas, while nutrient levels have been indicative of agricultural disturbance (Korte et al. 2010; Giri & Singh 2013; Dorji et al. 2021).

The use of aquatic macroinvertebrates (i.e., insects, crustaceans, molluscs, and worms) has been shown to be a powerful tool for monitoring freshwater around the world because of their high diversity, high abundance, and spectrum of pollution tolerances (Allan 2004). In Bhutan, macroinvertebrates have been shown to be of use for monitoring agricultural and urban impacts but most studies have focused on one stream and did not use a watershed approach (Moog et al. 2008; Ofenböck et al. 2010; Wangyal et al. 2011; Giri & Singh 2012; Dorji 2014a; Dorji et al. 2014, 2021; Gurung & Dorji 2014; Wangchuk & Dorji 2018).

This study was designed to further investigate the water quality of stream and river systems in western Bhutan for many sites throughout three watersheds. The study focused on how water quality responded to the presence and activities of human development within the districts of Thimphu and Paro in the Wangchhu basin and the districts of Punakha and Wangduephodrang in the Punatsangchhu basin. Family-level identifications were used to describe the macroinvertebrate assemblage of sites while species-level data on three major aquatic insect Orders (Ephemeroptera, Plecoptera, and Trichoptera; also known as EPT) were barcoded using the mitochondrial cytochrome *c* oxidase 1 (*COI*) gene. EPT has been shown worldwide to be the most sensitive (i.e., intolerant) of pollution and therefore most indicative of stream and water health (Resh & Jackson 1993). Species-level knowledge of macroinvertebrates needs to be expanded in Bhutan and the south Asian region to better connect taxa to water quality parameters. Recent advances in the use of deoxyribonucleic acid (DNA) barcoding to identify aquatic macroinvertebrate species have enhanced their use for biomonitoring (Sweeney et al. 2011; Jackson et al. 2014; Li et al. 2022).

METHODS

In this study, 18 streams and rivers in three watersheds were selected to measure water chemistry, bacteria, and macroinvertebrates (Table 1). Water quality was measured at 16 sites from 6–13 November 2015 (post-monsoon season) and 12 of the same sites were sampled from 15–20 August 2016 (monsoon season) with an additional two sites added (Figure 1; Table 1). Stream sites represented a gradient of anthropogenic disturbance (e.g., an undisturbed, forested upstream area was contrasted with a downstream area impacted by agriculture or urbanization) and were labeled as being either upstream or downstream of major human disturbance (Table 1).

Temperature, conductivity, pH, and dissolved oxygen were measured with an Orion 5-Star portable meter and turbidity was measured with a Campbell Scientific OBS3+ turbidity sensor. Water samples were analyzed for ammonia, nitrite, nitrate, and phosphorus using API-brand freshwater test kits and quantified using an open- source colorimeter by IO-Rodeo (http://iorodeo.com/pages/colorimeter-project accessed August 2016). Total coliform bacteria and *Escherichia coli* bacteria were measured within 24 h of collection using the 3M™ Petrifilm™ *E.coli*/Coliform Count Plate kit and expressed as colony-forming units (cfu)/100ml. Wilcoxon rank-sum test (t approximation, 2-sided test) was used to examine differences in water quality variables between sites classified upstream or downstream of disturbance for each year, and both years of data were combined to examine if differences existed upstream or downstream within the Paro and Thimphu watersheds. As BT03 was a drinking well, only chemistry and bacteria were sampled (Table 1).

In 2015, macroinvertebrates were qualitatively sampled using a 500-µm D-frame net in riffle and run areas. The stream bottom was disturbed by kicking the substrate and collecting downstream, in addition, rocks, leaf packs, and woody material were examined. In the field, collected material was placed in a tray, and specimens were picked by hand before preserving in 95% ethanol, which was changed within 24 h of collection.

In 2016, macroinvertebrates were quantitatively collected with a Surber sampler (0.093 m^2; 250-µm mesh net). For each site, 16 individual Surber samples were taken in riffle areas (and some run areas if riffle habitat was scarce) and the contents (macroinvertebrates and organic debris) were split evenly between two large buckets containing stream water. The content of each of the two buckets was then transferred to a field sample splitter and the sample was split evenly into four subsamples (0.1858 m^2; Arscott et al. 2006). Two subsamples were preserved in 70% isopropyl alcohol resulting in four samples per site. In the laboratory, the entire 2015 sample was identified but, in 2016 three of the four preserved samples were further subsampled and processed under a microscope until a minimum of 200 macroinvertebrate specimens were obtained (>600 individuals per site). For three sites (BT06, BT11, BT13), only 1–2 preserved samples were processed because of limited time. Macroinvertebrate insects were identified to family level and some non-insects (e.g., oligochaetes, planarians, nematodes, bivalves, snails, and mites) were identified to order level or higher.

In order to ensure that taxon richness metrics were not biased by the number of individuals examined, samples were standardized (i.e., rarefaction) using the SAS statistical package (version 9.4, SAS Institute Inc., Cary, North Carolina). The 2015 qualitative samples were standardized to 100 individuals (except sites BT06, BT12, and BT14, which had <100 individuals), and the 2016 quantitative samples were standardized to 200 individuals/sample with both datasets being resampled to 1,000 random draws. Macroinvertebrate samples were used to calculate richness and percentage metrics, as well as the Shannon and Simpson diversity indices (Resh & Jackson 1993). Using samples in their entirety to best mimic the original index methods, the Hindu Kush-Himalaya Index (HKHbios; Ofenböck et al. 2010), the Biological Monitoring Working Party (BMWP), and the Average Score per Taxon (ASPT) were calculated. BMWP and ASPT were based on Armitage et al. (1983) method (ASPT 1983; BMWP 1983) and a Bhutan version following Dorji et al. (2021), BMWP (2021), and ASPT (2021). Within each year, a Wilcoxon rank-sum test (normal distribution, one-sided) was used to examine differences in macroinvertebrate metrics between sites classified upstream or downstream of disturbance.

Non-metric multidimensional scaling (NMS) was used to examine how macroinvertebrate taxa assemblages differed among years and in relation to various types of disturbance (i.e., upstream or downstream) using PC-ORD (version 6.22, MjM Software, Gleneden Beach, Oregon). This analysis was done using Sorenson distance, the step length was set at 0.20, and Monte Carlo was used to determine the optimal number of axes. NMS was performed using presence/absence data of 42 common taxa (i.e., taxa found in at least 2 samples) and was run with 41 iterations, an r^2 set at 0.28, a final stress of 12.0, and a final instability was <0.00001.

In an effort to better document the EPT diversity,

Table 1. Description of the Bhutan sampling locations in 2015 and 2016. Sites in similar watersheds are listed in pairs or groups indicating ones that were upstream (US) or downstream (DS) of disturbance. Stream type (tributary or mainstem), size, and land use are general descriptors. Years of water chemistry, bacteria, and macroinvertebrates were sampled are provided.

Location		US or DS	Stream type	Size (discharge m^3/s Nov 2015)	Land use	Chem & bacteria yrs	Macroinvert yrs	Elevation (m)	Latitude	Longitude
Paro River watershed										
BT03	groundwater well accessed at Tiger's Nest Tea House	US	other		Forest	2015, 2016		2976	27.4884	89.3586
BT04	Stream below Tiger's Nest	US	trib	small	Forest	2015, 2016	2016	2982	27.4859	89.3621
BT02	Holy Water stream near Chilai La pass	US	trib	small (0.03)	Forest	2015, 2016	2015, 2016	3235	27.3709	89.3620
BT07	Woo Chhu at Woo Chhu village	DS	trib	small (0.30)	Suburban/ agriculture	2015, 2016	2015, 2016	2412	27.3912	89.4244
BT05	Stream 1 by Ramzi	US	trib	small (0.13)	Forest	2015, 2016	2015, 2016	2866	27.5415	89.3295
BT06	Stream 2 by Ramzi	DS	trib	small	Forest/ agriculture	2016	2015, 2016	2692	27.5226	89.3283
BT01	Paro Chhu at Udumwara Resort	US	main	medium	Suburban/ agriculture	2015, 2016	2015	2355	27.4651	89.3558
BT08	Paro Chhu at Shaba	DS	main	large	Suburban/ agriculture	2015, 2016	2015, 2016	2432	27.3548	89.4643
Thimphu River watershed										
BT13	Thimphu Chhu at Chagri Dorjeden Monastery	US	main	medium	Forest	2015, 2016	2015, 2016	2599	27.5961	89.6304
BT09	Thimphu Chhu at Dodena	US	main	medium	Forest/ suburban	2015, 2016	2015, 2016	2523	27.5792	89.6348
BT15	Thimphu Chhu at Chanjiji Football Ground	DS	main	large	Urban	2016	2015, 2016	2293	27.4565	89.6491
BT12	Thimphu Chhu at Lungtenphug	DS	main	large	Urban	2015	2015	2296	27.4502	89.6547
BT14	Ola Rong Chhu at Semtokha	DS	trib	medium (1.94)	Urban	2015, 2016	2015	2283	27.4434	89.6603
BT11	Thimphu Chhu at Zimda	DS	main	large	Urban	2015, 2016	2015, 2016	2283	27.4302	89.6426
Paro & Thimphu watersheds										
BT10	Wangchhu at Tamchu	DS	main	large	Urban	2015, 2016	2015	2021	27.2503	89.5252
Punatsangchhu watershed										
BT16	Mochhu River upstream of Punakha Dzong	US	main	medium	Forested	2015	2015	1481	27.7117	89.7652
BT17	Punatsangchu below Khuruthang	DS	main	large	Urban/ agriculture	2015	2015	1209	27.5452	89.8699
BT18	Punatsangchu at Wangdue Phodrang	DS	main	large	Urban/ agriculture	2015	2015	1203	27.4863	89.8959

DNA was sequenced (COI gene) for a subset of EPT specimens to evaluate if species could be separated by morphology alone, or whether there were cryptic species present. The process of selecting EPT specimens for barcoding involved inspecting all individuals and choosing specimens that could be identified to genus level, and further dividing them into groups based on morphology. Common mayfly specimens were selected from forested and urban streams with the goal of barcoding four individual larvae from both stream types (undisturbed vs. disturbed) and a variety of sites and drainages where possible. Caddisflies and stoneflies were also separated based on morphology and 3–6 individuals were barcoded where possible albeit not from both stream types. The majority of the barcoded specimens were from 2015 because 2016 specimens were mostly small and immature, and therefore difficult or impossible to identify to a low level. Leg tissue from each specimen was sent to the Canadian Centre for DNA Barcoding at the University of Guelph, where genomic mitochondrial DNA was extracted and the 658-base pair (bp) barcoding region of the COI gene was amplified and sequenced (Sweeney et al. 2011). Sequences and detailed information about all specimens including photographs are stored on the GenBank and Barcode of Life Data systems (BOLD) website (https://boldsystems.

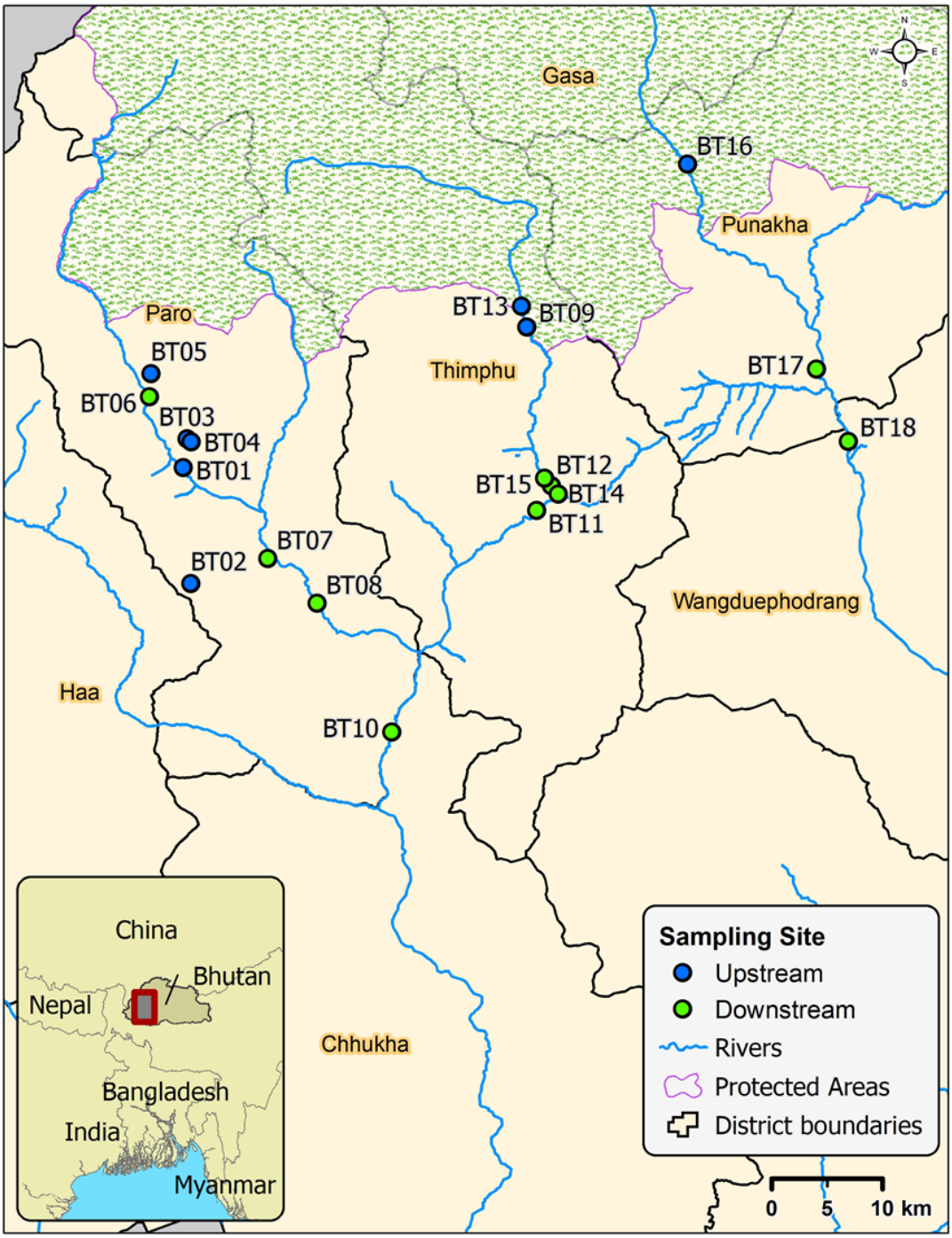

Figure 1. Map of Bhutan indicating sites sampled in 2015 and 2016. Blue circles indicate sites upstream of human disturbance and green circles are downstream.

org/). Of the 458 individuals submitted for barcoding, COI sequences ≥200 bp were determined for 281 specimens (61% of the total, 25 individuals with 200–350 bp; nine individuals with 351–450 bp; 247 individuals with 451–658 bp). The number of barcoded species and variance determined by BOLD Barcode Index Number (BIN) was based on their criteria for compliant barcode sequences (data accessed 8/2023). The study included 230 barcode-compliant individuals and 51 non-compliant (mainly because of short sequences). Sequences were aligned with a BOLD aligner and neighbor-joining trees (pairwise deletion and Kimura-2-parameter distance) were used to identify genetically distinct barcode species, which were confirmed using BINs where possible.

RESULTS

Water chemistry and bacteria variables for all study sites are summarized in Table 2. Results of the Wilcoxon rank-sum test showed that many of the 2015 water quality variables (specifically, pH, dissolved oxygen, specific conductivity, turbidity, ammonia, nitrite, nitrate, and phosphate) did not differ significantly ($p>0.05$) between sites upstream vs. downstream of disturbance. Temperature, *E. coli*, and total coliform were all significantly lower upstream of disturbance relative to downstream sites (Table 2). In 2016, pH, specific conductivity, and nitrite were all significantly higher upstream compared to sites downstream of disturbance, whereas *E. coli*, total coliform, and nitrate were significantly lower upstream than downstream. It is notable that differences in coliform bacteria, both total and *E. coli*, between upstream and downstream sites differed in both 2015 and 2016 by an average of thousands of cfu/100 ml. In contrast, for 2016 the differences in water quality variables were relatively small between upstream and downstream sites [e.g., pH (±0.2), nitrate and nitrite (±0.2 ppm)]. In the Paro and Thimphu watersheds, when the 2015 and 2016 data were combined, the coliform (total and *E. coli*) had the same patterns as the individual years with higher levels downstream than upstream. In addition, ammonia levels in the Thimphu watershed were significantly higher downstream (0.19 ppm) than upstream (0.04 ppm), while specific conductivity was higher upstream (181 µS/cm) than downstream (126 µS/cm).

A total of 50 insect families and six non-insect taxa were identified in 2015 and 2016; specifically, 36 taxa in 2015 and 49 taxa in 2016. The mayfly Baetidae was the only taxa collected from all 26 samples, while the mayflies Ephemerellidae and Heptageniidae, the caddisfly Hydropsychidae, and the true flies Chironomidae, Simuliidae, and Tipulidae were also common (>80% of the 26 samples). There were 22 rare taxa (i.e., 13 taxa were only recorded from one sample, and nine taxa were only recorded from two samples). Based on counts, Baetidae, Ephemerellidae, Heptageniidae, and Hydropsychidae were the most abundant in 2015 and Baetidae, Chironomidae, and Simuliidae were most abundant in 2016. For the 2015 data, the Wilcoxon test showed that for the 13 metrics examined, total richness, EPT richness, % EPT, % non-insects, HKHbios, BMWP 1983, ASPT 1983, and ASPT 2021 were significantly ($p\leq0.05$) different between upstream and downstream sites (Table 3). In 2016, total richness and Shannon diversity were higher in upstream sites than downstream ones, while EPT richness and % EPT were only slightly ($p\leq0.09$) different between the upstream and downstream sites.

The NMS revealed sites clustered by year and disturbance with years separating sites along axis 1 (32%) and disturbance separating sites along axis 2 (39%; Figure 2). Differences between years are likely related to the contrast in sampling seasons (post-monsoon vs. monsoon) and methods (qualitative dip net vs. quantitative Surber). For differences between years (axis 1), Stenopsychidae was the key taxa for 2015 whereas Acari, Empididae, Lepidostomatidae, Psychodidae, and individuals of mayflies, true flies, and caddisflies too small to identify beyond the family were the key taxa for 2016. Macroinvertebrate diversity was higher in 2016 (when samples were processed in the laboratory with a microscope) than in 2015 (when samples were processed in the field by eye). Microscope processing allows the counting of both small individuals (e.g., Acari, Ceratopogonidae, oligochaetes) as well as individuals that were too small to identify beyond order (e.g., Ephemeroptera, Trichoptera, Diptera). There were also more individuals examined in 2016 (>600 specimens per site) vs. 2015 (>100 specimens per site), increasing the likelihood of greater diversity. The NMS also showed sites upstream of disturbance were characterized by Perlodidae, Nemouridae, Rhyacophilidae, and Athericidae, whereas sites downstream of disturbance had fewer taxa and were more likely to have oligochaetes.

Although there were more morphological EPT taxa (76) than barcoded taxa (63), the actual barcode total may be underrepresented, because only 60% of the 458 individuals were successfully sequenced (Table 4). The 40% failure rate for barcoding may have resulted from the challenge of obtaining high-grade ethanol (95%, non-denatured) in Bhutan, making it difficult to properly preserve the DNA in samples. When only sequenced taxa were examined, there were 17 more taxa revealed with barcode than morphology alone, specifically 16 mayflies (in the families Baetidae, Ephemerellidae, Heptageniidae, Leptophlebiidae) and one stonefly (*Nemoura*). Barcoding indicated no additional caddisfly species. The average intraspecific variance across all groups was relatively low (average 0.36%; range = 0.0–1.18 %), in contrast to the interspecific variance (average 10.1%; range = 1.0–17.8 %). There were 19 barcoded species with <3 individuals so intraspecific variance could not be determined for those species. There were four taxa (*Acentrella* sp. C, *Drunella* sp. A, *Hydropsyche* sp. D, *Paragnetina*) that appeared to be morphologically distinct but grouped with another barcode species suggesting multiple morphotypes. Based on the BOLD database, there were 13 unique

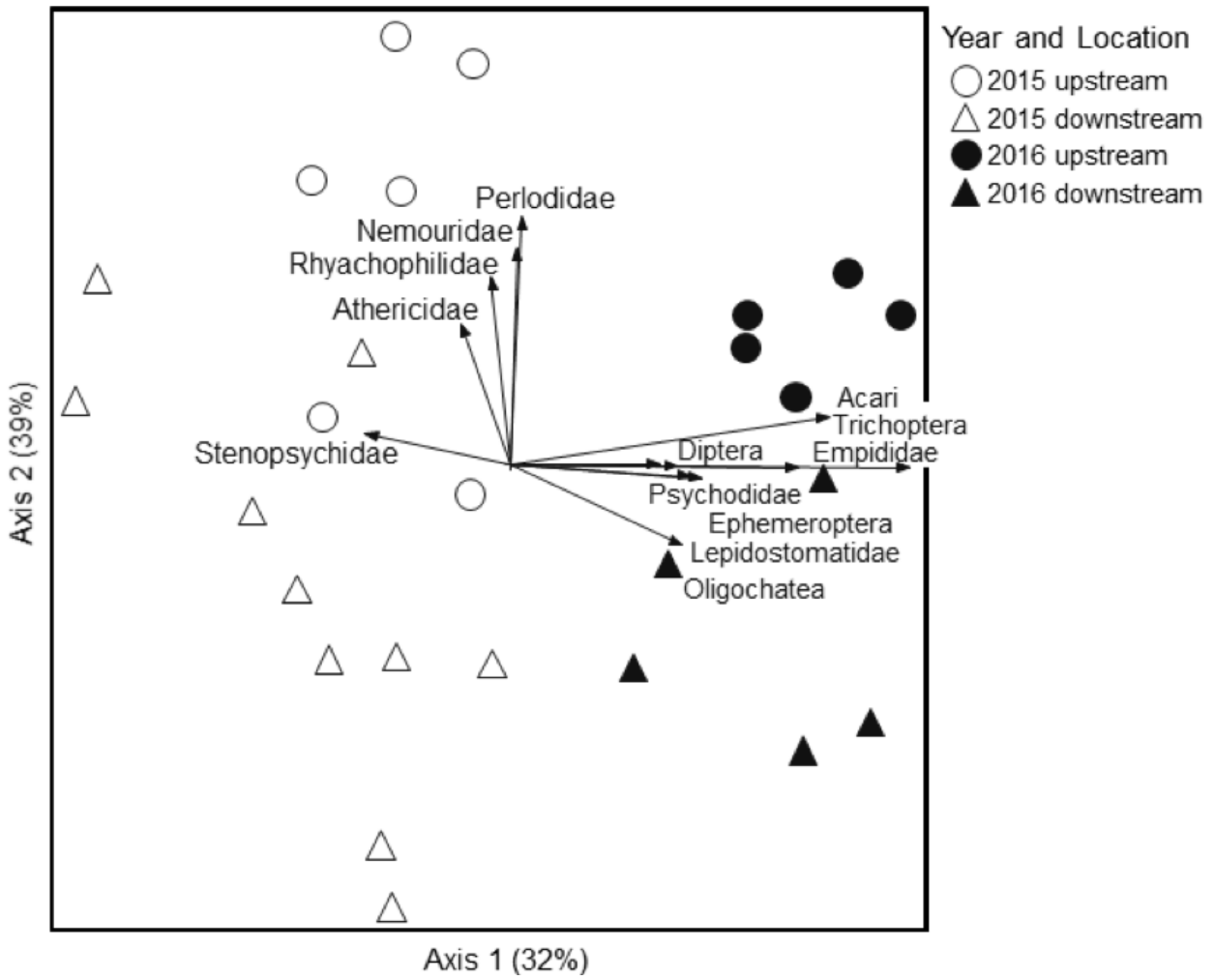

Figure 2. Non-metric multidimensional scaling of macroinvertebrates taxa groups (presence/absence) collected in Bhutan streams qualitatively in 2015 and quantitatively in 2016. Sites were classified as being upstream or downstream of disturbance.

species that were considered non-compliant, nine of them because sequences were too short (<500 bp) and they were not assigned to a BIN, and four are awaiting compliance with metadata requirements (Table 4). There were 42 barcode taxa (24 mayflies, six stoneflies, and 12 caddisflies) that were new sequences (e.g., new BINs) to the BOLD database (Table 4). One mayfly and seven caddisfly species had already been barcoded in other studies and had a species name available in BOLD (Table 4).

When mayfly sequences were compared between multiple sites (e.g., upstream vs. downstream and among drainage basins), they revealed differences that morphology failed to uncover. Overall, 201 of the 342 mayfly specimens (59%) were successfully barcoded, resulting in a total of 42 species versus 27 species based on morphology. There were several morphological taxa that looked similar but barcoding revealed that they did not occur at the same site (i.e., no spatial overlap; Figure 3). For example, barcodes indicated the presence of two species of *Epeorus* sp. C (29 individuals barcoded from eight streams) but one species was found in all the drainages (in small to medium streams) while the other species was only found at the large river sites of the Punatsangchhu drainage. This pattern of two (or more) species being morphologically similar but not overlapping geographically also occurred for *Cincticostella* sp. B (i.e., Paro and Thimphu sites vs. Punatsangchhu; 13 specimens), *Notacanthurus* sp. B (Paro vs. Punatsangchhu; 13 specimens), and *Epeorus* sp. B (i.e., upstream sites in the Paro vs. Thimphu; seven specimens). One caveat is that all of these spatial differences among species may be influenced by small sample sizes.

Barcoding revealed that one taxon, *Baetis* sp. A, was made up of five barcode species (34 specimens

Table 2. Water quality variables from November 2015 and August 2016 at Bhutan streams and rivers. Range of variables given for sites considered to be upstream (US) or downstream (DS) of a disturbance. Wilcoxon rank-sum test results (p≤0.01; * 0.05≤p>0.01; ns not significant) within years and watersheds.**

Variables		2015			2016			Paro			Thimphu		
	US (n = 8)	DS (n = 8)	t-test	US (n = 7)	DS (n = 7)	t-test	US (n = 9)	DS (n = 6)	t-test	US (n = 4)	DS (n = 8)	t-test
pH	7.47–8.39	7.55–8.56	ns	8.06–8.51	7.40–8.18	*	7.47–8.51	7.40–8.29	ns	8.06–8.39	7.41–8.56	ns
Temp (°C)	4.4–9.4	7.9–12.5	*	8.3–13.7	12.8–16.1	ns	4.4–13.7	9.2–14.9	ns	5.8–10.1	7.9–16.1	ns
Dissolved Oxygen (mg/L)	6.5–10.1	8.6–13.1	ns	7.7–8.6	7.3–8.5	ns	7.7–9.6	7.3–9.2	ns	6.5–9.0	7.3–9.8	ns
Spec. Conductivity (µS/cm)	21–198	90–156	ns	114–187	40–138	*	21–179	110–141	ns	166–198	40–156	*
Turbidity (NTU)	1–18	2–70	ns	1–13	2–20	ns	1–18	2–70	ns	1–13	2–20	ns
Total Coliform (cfu/100 ml)	0–44	21–443	**	0–267	367–16,000	**	0–267	22–2,400	*	0–100	107–16,000	*
E. coli (cfu/100ml)	0–1	<1–58	**	0	0–3933	**	0–1	1–433	*	0	14–4533	*
Ammonia (ppm)	0–0.28	0.06–0.30	ns	0.02–0.22	0.06–0.28	ns	0.07–0.28	0.07–0.28	ns	0.02–0.09	0.06–0.30	*
Nitrite (ppm)	0.03–0.41	0.03–0.10	ns	0.02–0.09	0.04–0.25	*	0.02–0.09	0.03–0.11	ns	0.04–0.41	0.05–0.25	ns
Nitrate (ppm)	0.95–2.31	1.45–1.96	ns	0.94–1.50	1.22–1.81	*	0.97–2.31	1.17–1.69	ns	0.94–1.69	1.22–1.96	ns
Phosphate (µM)	0.33–0.46	0.30–0.76	ns	0.25–0.41	0.32–0.81	ns	0.30–0.46	0.33–0.57	ns	0.25–0.49	0.30–0.81	ns

sequenced from 12 sites), with two common species being found in small to medium streams of the Paro and Thimphu watersheds, while a third common species preferred large sites in the Punatsangchhu and the confluence of the Paro and Thimphu Rivers. In addition, for some taxa only identified to a specific genus via morphology, barcoding was able to reveal multiple species, i.e., *Fallceon* (2 species), *Iron* (3 species), and *Paraleptophebia* (3 species), while in other cases morphology and barcoding were aligned (e.g.., *Acentrella* species A, B and C, *Baetis* sp. D, *Drunella* sp. A).

DISCUSSION

Based on health concerns and other management reasons, the World Health Organization (WHO 2017) has provided guidelines for drinking water that can be used as a baseline for stream conditions. They set levels not to be exceeded for nitrite (3 mg/L), nitrate (50 mg/L), and *E. coli* (zero cfu/100 ml) based on health concerns, a pH range (6.50–8.50) for sewage treatment operation, and an ammonia level (1.5 mg/L) for odor (WHO 2017). Using WHO criteria, many water quality parameters in this study were largely within the acceptable range (Table 2): specifically, nitrite, nitrate, and ammonia levels were below these limits and only one site had a pH slightly above the limit (8.56). *E. coli* was the only variable that was above the recommended limit (WHO 2017) and it was exceeded for the majority of the downstream sites (94%), while all the upstream sites registered no *E. coli* or extremely low levels (1 cfu/100 ml). This is good news for those living upstream, given that much of Bhutan's rural population draws untreated water for consumption directly from stream or river systems (Giri et al. 2010; Rahut et al. 2015) and *E coli* is a measure of fecal coliform and thus an indicator of fecal contamination. This is not good news for those living downstream of disturbance, because high fecal coliform indicates an increased risk of pathogen-borne illnesses (USEPA 2012; WHO 2017). In the United States, the Environmental Protection Agency (USEPA 2012) guideline for streams is that *E. coli* should not exceed a geometric mean of 126 cfu/100 ml to be considered safe for swimming. Unfortunately, 20% of the Bhutan samples exceeded this level, and for four of the 2016 sites (BT11, BT10, BT14, BT15) in the urban area of Thimphu watershed, levels were anywhere from 23 to 35 times higher (2900–4533 cfu/100 ml). This indicates that untreated sewage was entering the river at or near those sites. The results suggest fecal coliform or *E. coli* could be a powerful, yet easy and inexpensive tool

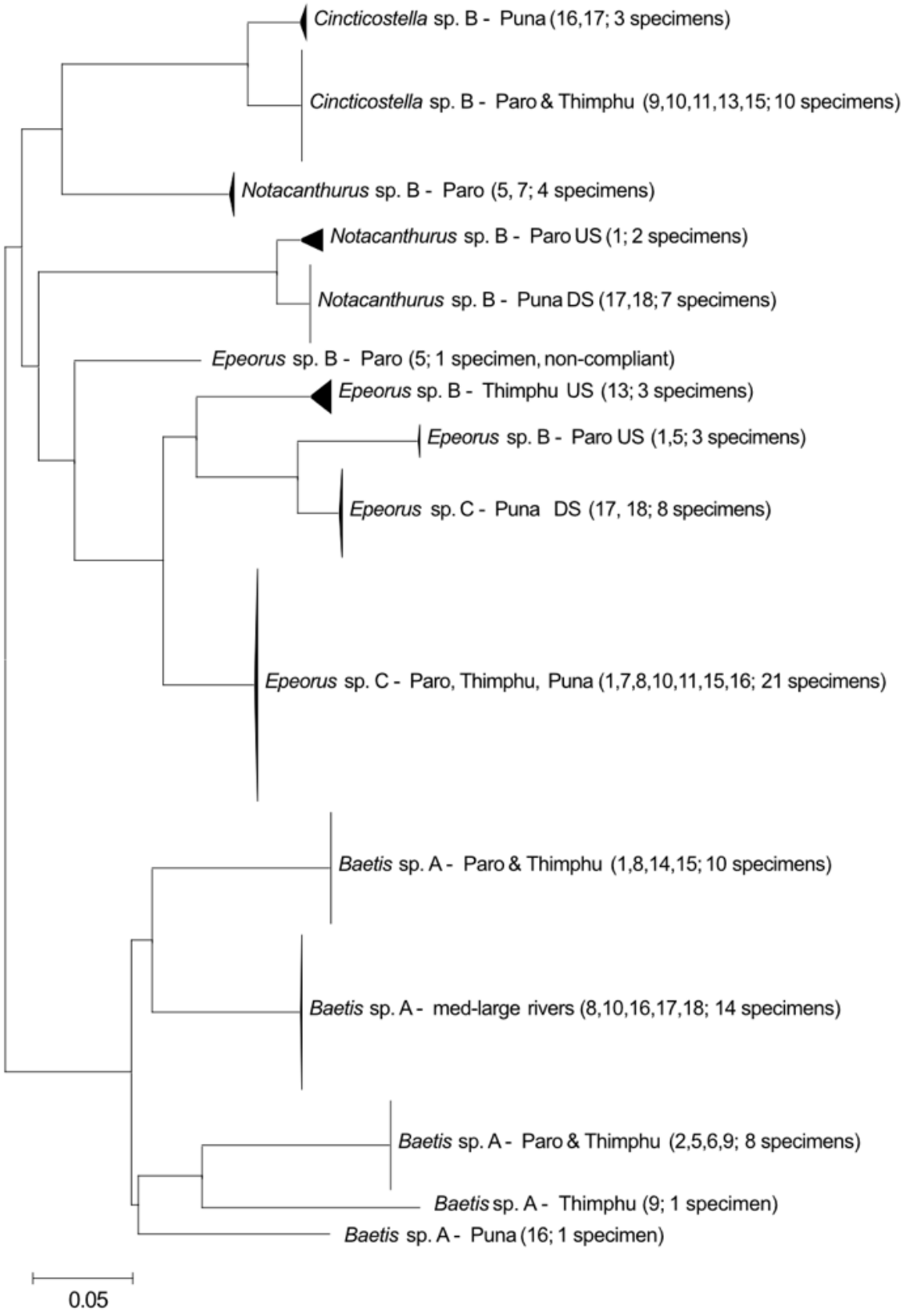

Figure 3. Neighbor-joining tree of five morphological mayfly species and their designations based on barcode. Taxa name is followed by the watershed it was found in [Paro, Thimphu, and Puna (-tsangchhu)], the specific sites (see Table 1), and the number of specimens barcoded. For each taxon, the vertical distance of the line indicates the number of individuals, and the horizontal distance is the maximum genetic diversity within the branch.

Table 3. Macroinvertebrate metrics for 2015 qualitative (dip nets) and 2016 quantitative (Surbers) sampling in Bhutan. Range of metrics provided for sites considered to be upstream (US) or downstream (DS) of disturbance. Wilcoxon rank-sum test results (* p≤0.001; ** 0.01≤p>0.001; * 0.05≤p>0.01; ● 0.09≤p>0.05; ns not significant) indicate if metrics differed based on disturbance.**

Metrics		2015			2016	
	US (n = 6)	DS (n = 10)	t-test	US (n = 5)	DS (n = 5)	t-test
Richness	12–14	9–16	*	14–18	12–15	*
EPT Richness	8–11	5–8	***	6–8	4–7	●
Diptera richness	2–4	2–3	ns	3–6	3–5	ns
% EPT	79–95	35–95	*	38–46	21–80	●
% Chironomidae	0–10	0–20	ns	13–47	5–19	ns
% Non-insects	0–4	0–25	*	5–10	4–28	ns
Shannon Diversity	1.88–2.14	1.42–2.38	ns	1.81–2.40	1.58–2.06	*
Simpson Diversity	0.79–0.86	0.64–0.88	ns	0.70–0.88	0.70–0.81	ns
HKHbios	7.6–8.7	6.2–7.6	***	5.9–7.8	6.7–7.8	ns
BMWP 1983	68–111	48–76	**	47–71	42–71	ns
BMWP 2021	46–86	41–77	ns	46–69	47–79	ns
ASPT 1983	6.5–7.6	6.0–7.3	**	5.4–7.4	5.2–6.8	ns
ASPT 2021	6.1–7.2	5.5–7.4	**	4.5–6.7	5.0–6.2	ns

to regularly monitor the safety of Bhutan streams for various public activities.

A previous study of four headwater streams in Bhutan reported that most environmental variables (i.e., temperature, conductivity, stream width, depth, velocity) did not differ between monsoon and pre-monsoon seasons (Dorji 2014b). Similarly, a 2008–2009 study of the river Wang Chhu near Thimphu city sampled in pre-monsoon, monsoon, and post-monsoon indicated similar patterns in response to urban pollution in all three seasons and that nitrate, total coliform, and biochemical oxygen demand [BOD] were the best parameters for monitoring urban impacts (Giri & Singh 2013). The results suggest that water chemistry in the monsoon season was better able to discern impacts than in the post-monsoon season. A study examining agricultural practices in a Bhutan stream in the Samtse district also indicated that the monsoon season was the optimal time to measure the highest levels of nitrate, BOD, and total dissolved solids (Giri et al. 2010).

It is important to note for this study that not all the same sites were measured in both years (e.g., Punatsangchhu sites were only sampled in 2015). Nevertheless, there were more water quality variables that differed between upstream and downstream sites in the monsoon season than the post-monsoon (3 vs. 6). This might be related to the fact that higher discharge in the monsoon season may result in more pollution entering the stream than for the pre- or post-monsoon seasons (Giri et al. 2010). Typically, 70% of the annual precipitation is concentrated during the monsoon season that occurs from June to September and a major portion of the water volume in the basins is attributed to rain-fed recharge (WBMP 2016).

In Bhutan, septic tanks are commonly reported to overflow into the environment due to poor design and maintenance and this problem is exacerbated by heavy monsoon rains because soak-pits and waste stabilization ponds can become full and overflow (Taylor-Dormond et al. 2018; Dorji et al. 2019). Also, because agriculture across this country occurs in steep topography, erosion is extensive in Bhutan and is exacerbated by heavy rain showers during the pre-monsoon season falling on bare soils prior to crop emergence (Dorji et al. 2011; WBMP 2016). Although the dominant soil type, gneissic, is resistant to erosion, the loss of fertile soils during storms results in increased nutrients and sediments washing into streams and rivers (Baillie et al. 2004; Dorji et al. 2011). Rapid runoff into Bhutan waters during flood events is further exacerbated by forest fires and overgrazing (Tariq et al. 2021). The above factors suggest that most of the water quality differences measured in this study (Table 2) between upstream and downstream sites were indicative of pollution (i.e., higher temperatures, coliform, and *E. coli* downstream than upstream in 2015 and higher coliform, *E. coli,* nitrite, and nitrate downstream than upstream in 2016). In contrast, the higher pH and conductivity levels in the upstream sites

Table 4. Morphological name followed by a letter is the designation of unique species. Variance is show as maximum within a barcode species (% intra) and distance to nearest neighbor (% inter). Number of barcode species and variance determined by BOLD BINs based on their criteria for compliant barcode (data accessed 8/2023). Intraspecific variance was listed as not available (na) if there was only one individual in the BIN. If there were multiple barcode species for a single morphological name then the range for % variance is shown. Instances where barcode species was based on noncompliant specimens are denoted with "b" followed by a number of basepairs (bp) in sequence; all of these were 1 individual with the exception of Skwala and Mystacides (2 individuals). Asterisks indicate a new sequence to the BOLD library.

Morphological name	No. individual barcoded	No. sequences (>200 bp)	No. barcode species	% Variance Intra	% Variance Inter	[a] Not unique sequence [b] Not barcode compliant [c] Name on BOLD BIN
EPHEMEROPTERA - 27 of 31 morphological taxa sequenced						
Total	**342**	**201**	**42**			
Baetidae						
Acentrella sp. A	17	14	1	1.96	15.2	
Acentrella sp. B	29	19	1	0.51	17.0	
Acentrella sp. C*	4	1	1	na	13.5	
Acentrella sp. D	1	1	0			[a] *Acentrella* sp. C
Baetis sp. A*	47	34	5	0–2.51	5.8–15.6	
Baetis sp. B*	2	1	1	na	15.5	
Baetis sp. C*	7	1	1	na	14.6	
Baetis sp. D	13	6	1	2.16	15.4	
*Fallceon***	14	9	2	Na–0	16.5–16.9	
Caenidae						
Caenis sp. A	4	0	—			
Caenis sp. B*	2	2	1	na	12.7	
Ephemerellidae	27	12	2	0–0.73	16.3–16.5	[c] *Spinorea gilliesi*
Cincticostella sp. A	3	0	—			
Cincticostella sp. B	27	13	2	0.36–0.92	4.8	
Cincticostella sp. C	6	0	—			
Drunella sp. A*	10	9	1	0.18	11.1	
Drunella sp. B	1	1	0			[a] *Drunella sp. A*
Drunella sp. C	2	1	1			[b] 268 bp
Teloganopsis	4	0	—			
Ephemeridae						
*Ephemera**	1	1	1	na	4.7	
Heptageniidae						
Afronurus	1	1	1	0.17	13.4	
*Cinygmula**	6	4	1	0.7	9.2	
Epeorus sp. A	11	1	1			[b] 329 bp
Epeorus sp. B*	10	7	3	1.61–1.77	3.7–11.8	[b] 217 bp, [c] *E. aculeatus*
Epeorus sp. C*	38	29	2	0–1.46	7.8–11.5	
*Iron****	4	4	3	na–0.96	12.2–14.3	
Notacanthurus sp. A*	6	5	1	1.0	17.0	
Notacanthurus sp. B***	19	13	3	na–0	2.5–14.0	
*Rhithrogena***	7	4	2	na–0.16	3.9	
Leptophlebiidae						
*Paraleptophlebia***	9	5	3	0.17–0.89	9.6	[b] 484 bp
Neoephemeridae*	10	3	1	0.96	9.6	
PLECOPTERA - 10 of 11 morphological taxa sequenced						
Total	**29**	**19**	**10**			
Capniidae	1	1	1			[b] 260 bp
Leuctridae						
Paraleuctra	1	1	1			[b] 441 bp
Nemouridae						
*Amphinemura**	4	2	1	0.2	10.7	
*Nemoura***	3	2	2	na	6.6	

Morphological name	No. individual barcoded	No. sequences (>200 bp)	No. barcode species	% Variance Intra	% Variance Inter	[a] Not unique sequence [b] Not barcode compliant [c] Name on BOLD BIN
Peltoperlidae						
*Cryptoperla**	3	3	1	0.37	12.7	
Perlidae						
Calineuria	3	0	—			
Kiotina sp. A*	2	2	1	0	15.7	
Kiotina sp. B *	2	2	1	na	15.6	
Paragnetina	5	3	1	2.5	14.6	
Tetropina	1	1	0			[a] *Paragnetina*
Perlodidae						
Skwala	4	2	1			[b] 202 & 459 bp
TRICHOPTERA - 26 of 34 morphological taxa sequenced						
Total	**87**	**61**	**25**			
Brachycentridae						
*Brachycentrus**	3	3	1	0	6.0	
*Micrasema**	1	1	1	na	10.5	
Glossosomatidae						
*Agapetus**	6	6	1	1.37	13.0	
Glossosoma	3	3	1	1.12	9.1	[c] *Glossosoma dentatum*
Hydropsychidae						
Arctopsyche	3	3	1	0.48	5.8	[c] *Arctopsyche lobata*
Hydropsyche sp. A	5	0	—			
Hydropsyche sp. B*	2	2	1	0.17	8.5	
Hydropsyche sp. C	4	1	0			[a] *Hydropsyche* sp. D
Hydropsyche sp. D*	3	3	1	0.33	2.7	
Hydropsyche sp. E	3	2	1	0.17	5.8	
Hydropsyche sp. F*	3	2	1	0.17	11.7	
Hydropsyche sp. G*	3	3	1	0.64	2.7	
*Lepidostoma**	3	3	1	1.36	10.1	
*Mystacides**	2	2	1	na	3.1	[b] 586 & 594
Limnephilidae	2	2	1	0.18	10.8	[c] *Phylostenax himalus*
*Chimarra**	3	3	1	0.34	2.7	
*Neurocyta**	1	1	1	na	3.6	[b] 637 bp
Psychomyiidae	1	0	—			
Rhyacophilidae						
Himalopsyche sp. A	3	3	1	1.19	2.5	[c] *Himalopsyche digitata*
Himalopsyche sp. B	2	1	1	1.81	8.5	
Himalopsyche sp. C	3	1	1	0.17	11.0	[c] *Himalopsyche horai*
Himalopsyche sp. D	1	0	—			
Rhyacophila sp. A*	3	1	1	na	2.3	
Rhyacophila sp. B	3	3	1	0.38	7.5	
Rhyacophila sp. C	1	0	—			
Rhyacophila sp. D	1	0	—			
Rhyacophila sp. E	1	1	1			[b] 317 bp
Rhyacophila sp. F	2	0	—			
Rhyacophila sp. G	2	2	1	0.17	1.0	[c] *Himalopsyche tibetana*
Rhyacophila sp. H	4	0	—			
Rhyacophila sp. I	1	0	—			
Rhyacophila sp. J	2	2	1			[b] 202 & 257 bp
Stenopsychidae						
Stenopsyche sp. A	4	4	1	0.64	6.2	
Stenopsyche sp. B	3	3	1	0.32	10.6	

versus the downstream sites in 2016 are likely due to a geological influence since these variables typically increase with pollution but were found to decrease downstream of disturbance areas.

For the post-monsoon season, the 2015 kick samples sorted in the field resulted in larger, more mature macroinvertebrate specimens, and many metrics indicated significant differences between upstream and downstream sites. The best metrics were related to sensitive groups known to become less abundant in response to disturbance (i.e., EPT richness and % EPT; Table 3). Other important metrics capable of measuring disturbance in 2015 were taxon richness (on average having two more taxa upstream than downstream, often families belonging to EPT) and % non-insects (averaging 1% upstream vs. 6% downstream). In addition, the metric BMWP1983 indicated the upstream sites had better environmental conditions than the downstream sites in 2015. It is noteworthy that although the BMWP1983 was initially designed for European streams, it worked better than the version (BMWP 2021) modified specifically for Bhutan (Dorji et al. 2021). To this end, BMWP1983 characterized some insect families (i.e., Ephemerellidae and Heptageniidae) as sensitive to disturbance even though they were found in nearly all the sites (including degraded sites) suggesting those families contain taxa somewhat pollution-tolerant, while other families (e.g., Perlidae and Perlodidae) seemed to be better indicators of "good" water quality or sites that lack major human disturbance. Also, both the 1983 and 2021 versions of the metric ASPT, which is the BMWP modified to account for richness, were sensitive to disturbance in 2015. The HKHbios was designed to monitor streams in the region (Bangladesh, Bhutan, Nepal, India, and Pakistan) and worked well in indicating impact in 2015, although it rated all the sites as "good", even the disturbed ones, but the sampling method in this study was modified, which may have inflated the scores (Ofenböck et al. 2010).

In 2016, the fact that taxon richness and Shannon were the only metrics associated with the Surber sampling to indicate a disturbance is likely related to multiple factors (Table 3). The monsoon season is a difficult time to sample, presenting a safety issue, and high-water levels may have scoured some streams more than others. Also challenging is achieving equal sampling effort at sites across a gradient of small streams to large rivers, especially since high flow limited sampling in some cases to only the stream edges. Regional studies of monsoon effects on macroinvertebrates are not all in agreement (Brewin et al. 2000; Ofenböck et al. 2010; Dorji 2014b; Wangchuk & Dorji 2018; Thapa et al. 2020). Most studies in tropical Asian streams suggest a tendency for an overall decline in macroinvertebrates abundance and richness during the monsoon versus drier seasons (see Dudgeon 1999; Brewin et al. 2000). In Bhutan, one study reported macroinvertebrate abundance in headwater streams also decreased after flash floods but found no difference in macroinvertebrate diversity between pre- and post-monsoon seasons (Dorji 2014b). In contrast, a study of springs in nearby Nepal found EPT richness was higher in the post-monsoon versus the pre-monsoon season (Thapa et al. 2020). In a relatively large survey, Ofenböck et al. (2010) studied 198 streams in the Hindu Kush-Himalayan region and found that both pre- and post-monsoon macroinvertebrate data were able to differentiate non-impacted and impacted sites. To evaluate disturbance, they recommended sampling in the pre-monsoon season to avoid the many complications (noted above) associated with flooding effects in the post-monsoon period (Ofenböck et al. 2010).

The NMS indicated distinct differences in the 2015 and 2016 macroinvertebrate assemblages, which may be attributed to both time of year and sampling methods (Figure 2). More importantly, both sampling years, independent of the method, resulted in the separation of upstream and downstream sites. Given that for 2016, only two of the 12 metrics showed a significant difference between upstream and downstream sites (Table 3), perhaps metrics more specific to the Bhutan macroinvertebrate assemblages like % Baetidae or % Plecoptera (or possibly % Nemouridae and % Perlodidae), might be more sensitive measures of disturbance but this would require a larger dataset to put it to the test.

The level of disturbance was not well-defined in this study. Not all sites designated downstream of disturbance had the same level of degradation. Hopefully, going forward, land use types may be quantified to better understand the relationship between disturbance in the watershed and its impact on macroinvertebrate assemblages (Giri & Singh 2013). Many macroinvertebrate studies in Bhutan are still using the higher family level identification, and although this level of identification is useful in instances of high degradation (Giri & Singh 2012; Dorji 2014a, Dorji et al. 2014; Gurung & Dorji 2014; Wangchuk & Dorji 2018), it has been shown in other studies not to be as sensitive as genus or species level identification in discerning small levels of disturbance (Arscott et al. 2006). Although progress has begun in creating species-level checklists for Bhutan (Wangdi et al. 2018; Dorji et al. 2021; Gyeltshen & Prasad 2022), research on the taxonomy

of most of the aquatic macroinvertebrate groups is very limited and lacks baseline data. Bhutan seems to have a high diversity of macroinvertebrates belonging to 18 orders and 89 families (Dorji & Gurung 2017), with current species counts of 38 stoneflies, 172 caddisflies, 33 dipterans, 41 beetles, five mites, 12 hemipterans, 114 dragonflies and damselflies, and one megalopteran (Wangdi et al. 2018). As of 2017, at least 566 new species of flora and fauna have been recorded for Bhutan, including 77 aquatic species (Takaoka & Somboon 2008; Gyeltshen et al. 2018).

The biggest challenge in species-level identification for aquatic macroinvertebrates is that taxonomic keys still need to be expanded or developed for many groups. This study shows that DNA barcoding may help in this regard. DNA barcoding expanded the EPT list by 17 species and highlighted the presence of cryptic taxa (e.g., four species for *Baetis sp. A*; Table 4, Figure 3). Moreover, it suggested that morphologically similar species of mayflies often segregate according to either drainage or disturbance. Other studies have shown DNA barcoding improves macroinvertebrate monitoring (Jackson et al. 2014; Li et al. 2022) and have shown that morphologically similar mayfly species were spatially separated within the same river based on pollution (Sweeney et al. 2011). The barcoding results (42 "new" DNA sequences) represent only a start for EPT and highlight the need for further additions to the DNA reference library for the region.

The largest water quality challenges Bhutan faces going forward are sanitation management, climate change, and shared river systems (WBMP 2016). Urban areas of Bhutan will have to provide adequate sanitation infrastructure and sufficient regulatory pollution control measures to be enforced to protect water quality (Karn & Harada 2001; Dorji et al. 2019). For example, macroinvertebrate monitoring, in conjunction with chemical and bacteria parameters, could help evaluate the effectiveness of the new 2021 biological processing plant in Thimphu city that replaced their outdated sewage facility (Lhaden 2021). Bioassessment with macroinvertebrates could also help in managing changes in hydrology due to climate change and guide policy in managing river systems shared with neighboring countries. Given its inexpensive and straightforward nature, biomonitoring of streams with macroinvertebrates seems to be an accessible tool for both public officials and community/citizen science. The study shows water chemistry and bacteria were best sampled in the monsoon season to have the greatest measure of human disturbance, while macroinvertebrates were most effective in detecting impacts when sampled in the post-monsoon season. The DNA findings (e.g., 18 more EPT species using barcode versus morphology and 42 new sequences added to the BOLD database) suggest the diversity of stream macroinvertebrates in this region is presently underestimated and the continued expansion of species identifications (either morphologically or through DNA barcoding) will greatly aid in the future assessments of Bhutan waterways.

REFERENCES

Allan, J.D. (2004). Landscapes and riverscapes: the influence of land use on stream ecosystems. *Annual Review of Ecology, Evolution, and Systematics* 35: 257–284.

Armitage, P.D., D. Moss, J.F. Wright & M.T. Furse (1983). The performance of a new biological water quality score system based on macroinvertebrates over a wide range of unpolluted running-water sites. *Water Research* 17(3): 333–347.

Arscott, D.B., J.K. Jackson & E.B. Kratzer (2006). Role of rarity and taxonomic resolution in a regional and spatial analysis of stream macroinvertebrates. *Journal of the North American Benthological Society* 25(4): 977–997. https://doi.org/10.1899/0887-3593(2006)025[0977:RORATR]2.0.CO;2

Baillie, I.C., K. Tshering, T. Dorji, H.B. Tamang, T. Dorji, C. Norbu, A.A. Hutcheon & R. Bäumler (2004). Regolith and soils in Bhutan, Eastern Himalayas. *European Journal of Soil Science* 55: 9–27.

Brewin, P.A., S.T. Buckton & S.J. Ormerod (2000). The seasonal dynamics and persistence of stream macroinvertebrates in Nepal: do monsoon floods represent disturbance? *Freshwater Biology* 44: 581–594. http://doi.10.1046/j.1365-2427.2000.00608.x

Currinder, B. (2017). *Land Use and Water Quality in Bangladesh and Bhutan*. Capstone Project in the Department of Environmental Studies, University of Pennsylvania, 57 pp.

Dorji, T. (2014a). Macroinvertebrate diversity and water quality of Messina stream: an agricultural stream impacted by semi-urbanization. *NeBIO* 5(5): 13–18.

Dorji, T. (2014b). Macroinvertebrate diversity in response to environmental variables in headwater streams: a Project Report. Royal University of Bhutan, Thimphu, 21 pp.

Dorji, T., K. Thinley & S. Jamtsho (2014). Macro-invertebrate diversity in Threlpang and Kawajangsa freshwater streams in Bhutan. *NeBIO* 5(1): 1–5.

Dorji, T., P. Gyeltshen, C. Norbu & I.C. Baillie (2011). *Land Degradation in Bhutan – An Overview.* National Soil Services Centre, Ministry of Agriculture, Royal Government of Bhutan, 8 pp.

Dorji, T., S. Tshering, N. Wangchuk & I.P. Acharja (2021). Biomonitoring of health of Chubachu stream using macroinvertebrate diversity. *Bhutan Journal of Research & Development* 73–90. https://doi.org/10.17102/bjrd.rub.10.2.005

Dorji, U. & D.B. Gurung (2017). *Aquatic Biodiversity of Bhutan. An Introduction to the Biodiversity of Bhutan: in the context of climate change and economic development*. Kuensel Corporation Limited, Thimphu.

Dorji, U., U.M. Tenzin, P. Dorji, U. Wangchuk, G. Tshering, C. Dorji, H. Shon, K.B. Nyarko & S. Phuntsho (2019). Wastewater management in urban Bhutan: assessing the current practices and challenges. *Process Safety and the Environmental Protection* 132: 82–93. https://doi.org/10.1016/j.psep.2019.09.023

Dudgeon, D. (1999). *Tropical Asian Streams: Zoobenthos, Ecology and Conservation.* Hong Kong University Press, Hong Kong, 844 pp.

Giri, N., O.P. Singh & G.K. Chhopel (2010). Physio-chemical characteristics of surface water in and around the agriculture field of Samtse district. *Journal Renewable Natural Resources Bhutan*

6(1): 128–145.

Giri, N. & O.P. Singh (2012). Bio-monitoring of water quality using benthic macro-invertebrates of river Singye Chhu in Bhutan. *Journal of Basic and Applied Biology* 6(3&4): 44–49.

Giri, N. & O.P. Singh (2013). Urban growth and water quality in Thimphu, Bhutan. *Journal of Urban and Environmental Engineering* 7(1): 82–95.

Gurung, P.B. & T. Dorji (2014). Macroinvertebrate diversity and relationship with environmental variables in the headwater streams of Toebirongchhu sub-watershed, Bhutan. *NeBIO* 5(3): 4–10.

Gyeltshen, C. & K. Prasad (2022). Biodiversity checklists for Bhutan. Biodiversity Data Journal, 10: e83798. https://doi.org/10.3897/BDJ.10.e83798

Gyeltshen, C., K. Tobgay, N. Gyeltshen, T. Dorji & S. Dema (2018). New species discoveries and records in Bhutan Himalaya, pp 59-82. In: M. Hartmann, M.V.L. Barclay & J. Weipert (eds.). *Biodiversität und Naturausstattung im Himalaya VI.* Erfurt, Germany.

Islam, N. (2017). Biomonitoring of the freshwater macroinvertebrate community in northeast Bangladesh and western Bhutan: An initiative to strengthen local scientific capacity. Capstone Project in the Department of Earth and Environment, University of Pennsylvania, 57 pp.

Jackson, J.K., J.M. Battle, B.P. White, E.M. Pilgrim, E.D. Stein, P.E. Miller & B.W. Sweeney (2014). Cryptic biodiversity in streams: a comparison of macroinvertebrate communities based on morphological and DNA barcode identifications. *Freshwater Science* 33(1): 312–324. https://doi.org/10.1086/675225

Karn, S.K. & H. Harada (2001). Surface water pollution in three urban territories of Nepal, India, and Bangladesh. *Environmental Management* 28(4): 483–496. https://doi.org/10.1007/s002670010238

Korte, T., A.B.M. Baki, T. Ofenböck, O. Moog, S. Sharma & D. Hering (2010). Assessing river ecological quality using benthic macroinvertebrates in the Hindu Kush-Himalayan region. *Hydrobiologia* 651: 59–76. https://10.1007/s10750-010-0290-z

Lhaden, Y. (2021). New plant struggles to treat sewage, pp. 287–298. Downloaded 2 November 2022. https://kuenselonline.com/new-plant-struggles-to-treat-sewage/

Li, F., S. Wang, Y. Zhang, N. Zhang, Y. Cai & Z. Yang (2022). DNA metabarcoding reveals human impacts on macroinvertebrate communities in polluted headwater streams: evidence from the Liao River in northeast China. *Environmental Pollution* 300(1): 118929. https://doi.org/10.1016/j.envpol.2022.118929

Moog, O., D. Hering, S. Sharma, I. Stubauer & T. Korte (eds.) (2008). *ASSESS-HKH: Proceedings of the Scientific Conference* "Rivers in the Hindu Kush-Himalaya. Ecology & Environmental Assessment", 202 pp.

NSBB (2019). Population Projections Bhutan 2017–2047. National Statistics Bureau of Bhutan. Thimphu, Bhutan, 87 pp.

Ofenböck T., O. Moog, S. Sharma & T. Korte (2010). Development of the HKHbios: a new biotic score to assess the river quality in the Hindu Kush-Himalaya. *Hydrobiologia* 651: 39–58. https://doi.org/10.1007/s10750-010-0289-5

Price, G., R. Alam, S. Hasan, F. Humayun, M.H. Kabir, C.S. Karki, S. Mittra, T. Saad, M. Saleem, S. Saran, P.R. Shakya, C. Snow & S. Tuladhar (2014). Attitudes to Water in South Asia. Chatham House Report, London. The Royal Institute of International Affairs, 100 pp.

Rahut, D.B., B. Behera & A. Ali (2015). Household access to water and choice of treatment methods: empirical evidence from Bhutan. *Water Resources & Rural Development* 5: 1–16.

Resh, V.H. & J.K. Jackson (1993). Rapid assessment approaches to biomonitoring using benthic macroinvertebrates, pp. 195–233. In: D.M. Rosenberg & V.H. Resh (eds.). *Freshwater Biomonitoring and Benthic Macroinvertebrates*. Chapman & Hall, New York, 488 pp.

Sweeney, B.W., J.M. Battle, J.K. Jackson & T. Dapkey (2011). Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? *Journal of the North American Benthological Society* 30: 195–216. https://doi.org/10.1899/10-016.1

Takaoka, H. & P. Somboon (2008). Eleven new species and one new record of black flies (Diptera: Simuliidae) from Bhutan. *Medical Entomology & Zoology* 59(3): 213–262.

Tariq, M.A.U.R., K. Wangchuk & N. Muttil (2021). A critical review of water resources and their management in Bhutan. *Hydrology* 8: 31. https://doi.org/10.3390/hydrology8010031

Taylor-Dormond, M., V. Salze-Lozac'h, N. Subramaniam & J. Jovellanos (2018). Bhutan: urban infrastructure development project — validation report. Project number 38049-013. Independent Evaluation ADB, 15 pp.

Thapa, B., R.R. Pant, S. Thakuri & G. Pond (2020). Assessment of spring water quality in Jhimruk River Watershed, Lesser Himalaya, Nepal. *Environmental Earth Sciences* 79: 504. https://doi.org/10.1007/s12665-020-09252-4

USEPA (2012). United States Environmental Protection Agency 2012 recreational water quality criteria. Office of Water EPA-820-F-12-058.

WBMP (2016). Wangchhu Basin Management Plan: Adapting to climate change through IWRM. Technical Assistance No.: ADB TA 8623 BHU

Wangchuk, J. & K. Dorji (2018). Stream macro-invertebrate diversity of the Phobjikha Valley, Bhutan. *Journal of Threatened Taxa* 10(1): 11126–11146. https://doi.org/10.11609/jott.3138.10.1.11126-11146

Wangdi, T., P. Lhendup & N. Wangdi (2013). An Analysis of Forestry Policy, Acts and Rules of Bhutan to Mainstream Climate Change Adaptation. Regional Climate Change Adaptation Knowledge Platform for Asia. Partner Report Series No. 13. Stockholm Environment Institute, Bangkok, 19 pp.

Wangdi, N., D. Yoezer & J. Wangchuk (2018). Bhutan Water Facts 2018. Ugyen Wangchuck Institute for Conservation and Environmental Research (UWICER), Department of Forests and Park Services, Ministry of Agriculture and Forests, Royal Government of Bhutan, Bumthang and WWF Bhutan Programme Office, Thimphu, Bhutan, 15 pp.

Wangyal, J.T., J. Dorji, U. Tshering, K. Dorjee, K. Jigme & K. Dawa (2011). Diversity of macroinvertebrates in Toebrongchu stream — a tributary of Punatsangchu. *SAARC Forestry* 1: 73–90.

WHO (2017). World Health Organization Guidelines for drinking-water quality: fourth edition incorporating the first addendum. Geneva.

Worldometers (2022). Worldometer Bhutan. https://www.worldometers.info/world-population/bhutan-population/. Downloaded on 20 November 2022.

Declarations: Data availability any data presented in this paper will be available from the corresponding author upon request. Barcode data is available on GenBank and Barcode of Life Data systems.

Author contributions: Conceptualization: BS, AA & BC; developing methodology: BS, AA & BC. Conducting the research: BS, BC, AA, BF, & NI. Formal analysis: JB; visualization: BS, AA, BC, & JB. Project administration: BS, AA, BC & NI. First-draft elaboration: JB, BC, & BS. Writing: JB and BS; review and editing: BC & BF. Funding acquisition: BS, AA & BC. All authors read and approved the final manuscript.

Author details: J. Battle is a staff scientist at Stroud Water Research Center in the entomology group. She has been doing research in rivers, streams, and wetlands for >30 years. B. Sweeney is distinguished research scientist and executive director emeritus at Stroud Water Research Center. B. Currinder is a PhD candidate in ecology at the University of California, Davis. His research investigates population and community dynamics of lakes and ponds in the Sierra Nevada mountains. A. Aufdenkampe is a senior environmental scientist at LimnoTech, leading teams to develop and synthesize biogeochemical and hydrological data, models, and software to support scientifically-sound decision making. He was formerly a senior scientist at the Stroud Water Research Center where he oversaw the Organic and Isotope Geochemistry laboratory group. B. Fisher is an associate professor at Minnesota State University, Mankato. Her teaching and research focus on soil health, regenerative agriculture, climate change, and water quality. She works at the intersection of environmental science and biogeochemistry and mineralogy in soil and aqueous environments.

Journal of Threatened Taxa is indexed/abstracted in Bibliography of Systematic Mycology, Biological Abstracts, BIOSIS Previews, CAB Abstracts, EBSCO, Google Scholar, Index Copernicus, Index Fungorum, JournalSeek, National Academy of Agricultural Sciences, NewJour, OCLC WorldCat, SCOPUS, Stanford University Libraries, Virtual Library of Biology, Zoological Records.

NAAS rating (India) 5.64

Print copies of the Journal are available at cost. Write to:
The Managing Editor, JoTT,
c/o Wildlife Information Liaison Development Society,
3A2 Varadarajulu Nagar, FCI Road, Ganapathy, Coimbatore,
Tamil Nadu 641006, India
ravi@threatenedtaxa.org & ravi@zooreach.org

Journal of Threatened Taxa

www.threatenedtaxa.org

ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

November 2024 | Vol. 16 | No. 11 | Pages: 26063–26186
Date of Publication: 26 November 2024 (Online & Print)
DOI: 10.11609/jott.2024.16.11.26063-26186

Articles

Endemicity and diversity of birds of the Kuvempu University Campus, Shivamogga District, Karnataka: an updated checklist
– M.N. Harisha & B.B. Hosetti, Pp. 26063–26077

Unregulated wild orchid trade in Manipur: an analysis of the Imphal Valley markets from the Indo-Burma hotspot
– Kamei Kambuikhonlu Kabuini & Maibam Dhanaraj Meitei, Pp. 26078–26088

Watershed survey of streams in western Bhutan with macroinvertebrates, water chemistry, bacteria and DNA barcodes
– Juliann M. Battle, Bernard W. Sweeney, Bryan Currinder, Anthony Aufdenkampe, Beth A. Fisher & Naimul Islam, Pp. 26089–26103

Communications

Indian Leopard predation on the sub-adult Himalayan Griffon Vulture (Accipitridae: Accipitriformes)
– Soumya Sundar Chakraborty, Debal Ray, Apurba Sen, P.J. Harikrishnan, Nabi Kanta Jha & Rounaq Ghosh, Pp. 26104–26109

Diet composition and diet choice of Lesser Mouse-tailed Bat *Rhinopoma hardwickii* (Gray, 1831) (Rhinopomatidae: Chiroptera)
– Pawan Kumar Misra, Sayma Farheen, Shaktivardhan Singh & Vadamalai Elangovan, Pp. 26110–26115

DNA barcoding and distribution of *Osteobrama peninsularis* (Teleostei: Cyprinidae) in India
– Boni Amin Laskar, Asha Kiran Tudu, Shibananda Rath & Laishram Kosygin, Pp. 26116–26123

Diving into diversity: aquatic beetles of Sukhna Wildlife Sanctuary, Chandigarh, India
– Karmannye Om Chaudhary, Pp. 26124–26130

Review

An updated checklist of snakes (Reptilia: Squamata) in northeastern India derived from a review of recent literature
– Bijay Basfore, Manab Jyoti Kalita, Narayan Sharma & Ananda Ram Boro, Pp. 26131–26149

Viewpoint

Decades of IUCN recommendations for biocontrol of invasive pest on the Guam cycad: you can lead policy-makers to conservation proposals but you cannot make them follow
– Thomas E. Marler, Anders J. Lindström, L. Irene Terry & Benjamin E. Deloso, Pp. 26150–26162

Short Communications

Photographic record of Kashmir Gliding Squirrel *Eoglaucomys fimbriatus* (J.E. Gray, 1837) from the Gurez Valley, Jammu & Kashmir, India
– G. Mustufa Lone, Bilal A. Bhat, Mir Shabir Hussain & Arif Nabi Lone, Pp. 26163–26166

Winter population of raptor species in the Vellalore dump yard of Coimbatore City, India
– V. Balaji & R. Venkitachalam, Pp. 26167–26171

Notes

Phenotypic variations in Mindoro Warty Pig *Sus oliveri* (Cetartiodactyla: Suidae)
– John Carlo Redeña-Santos, Anna Pauline O. de Guia, Nikki Heherson A. Dagamac & Fernando García Gil, Pp. 26172–26175

First photographic evidence of the Chinese Pangolin *Manis pentadactyla* (Linnaeus, 1758) in Raimona National Park, Assam, India
– Dipankar Lahkar, M. Firoz Ahmed, Bhanu Sinha, Pranjal Talukdar, Biswajit Basumatary, Tunu Basumatary, Ramie H. Begum, Nibir Medhi, Nitul Kalita & Abishek Harihar, Pp. 26176–26179

***Habenaria spencei* (Orchidaceae): rediscovery other than its type locality and new distribution record to Karnataka, India**
– Shreyas Betageri & Katrahalli Kotresha, Pp. 26180–26184

Book Review

Fairies of the day and angels of the night
– Chitra Narayanasami, Pp. 26185–26186

Publisher & Host

WILD zoo reach
Zoo Outreach Organisation

Threatened Taxa