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Original Article

A survey of freshwater Tardigrada of the Mississippi River and U.S. driftless area

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Abstract: The Mississippi River is the largest river in the United States of America (U.S.). While the Mississippi River provides a habitat for many animal species, much of the river has not been explored for microscopic organisms, like zooplankton, microfauna, and tardigrades. In the current study, we collected sixty freshwater samples from the Mississippi River and other freshwater systems in the U.S. Driftless Area. A total of eighty-five tardigrades were collected and four different species of tardigrades were identified using morphological analyses. The results presented here are the first peer-reviewed records of tardigrade species documented from the Mississippi River and other freshwater systems in the U.S. Driftless Area. The tardigrade species *Grevenius granulifer*, *Pseudobiotus kathmanae*, and *Thulinius augusti* were collected from the Mississippi River. The tardigrade species *Dactylobiotus* cf. *dispar* and *Thulinius augusti* present new biogeography records for the state of Iowa. These records update our knowledge of tardigrade biogeography and ecology in North America.

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Introduction

Freshwater systems are found across the globe, but the reality is that only three percent of the water on Earth is freshwater, and two and a half percent is locked in ice or not usable (Bureau of Reclamation California-Great Basin, 2020). Freshwater is a limited resource but a heavily used one. Rivers only make about half of one percent of the surface freshwater of the planet (USGS, 2019). The Mississippi River is the largest and second-longest river in the United States of America. It is also the 15th largest river in the world. The Mississippi River watershed is the fourth-largest in the world and drains approximately 40 percent of the United States (NPS, 2025). Due to its size, the Mississippi River is home to a diverse array of animal species. At least 25 percent of all freshwater fish species in North America are found in the Mississippi River (NPS, 2025). The flyway for birds enables approximately 60 percent of North American bird species to migrate through (NPS, 2025). The Upper Mississippi River alone provides habitat for approximately 145 amphibian and reptile species (NPS, 2025).

Aquatic invertebrates can serve as indicators of freshwater quality (Elbert and McNabb, 1967). The Mississippi Museum of Natural Science (MMNS) Freshwater Invertebrate Collection contains more than 93 species of crustaceans and 45 species of mollusks (MDWFP, 2025). There are at least 38 species of mussels known from the Upper Mississippi River, and 60 species of mussels in the Lower Mississippi River (NPS, 2025). Mussels were extensively used in the button industry, underscoring the value of resources in the Mississippi River (Anthony and Downing, 2001). Many macroinvertebrates have been extensively researched in the Mississippi River (Tucker et al., 1993; Ries et al., 2016; Newton et al., 2022), but research on the microscopic invertebrates of the Mississippi River has been relatively sparse (Burdis and Hoxmeier, 2011; Vander Vorste et al., 2025).

Freshwater systems contain diverse microinvertebrate communities. Microinvertebrates, such as copepods (Copepoda), ostracods (Ostracoda), water fleas (Branchiopoda), rotifers (Bdelloidea),

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nematodes (Chromadorea), and tardigrades (Eutardigrada), have been found in freshwater systems around the world (Pennak, 1978; Bertolani, 1981; Zullini and Ricci, 1980; Külköylüoĝlu and Vinyard, 2000; Bruno et al., 2005; Finn et al., 2012; Zullini, 2021). Many of these microfauna and zooplankton are essential to the freshwater food web. They are responsible for maintaining water quality, nutrient cycling, and fish populations (Schenone et al., 2021). They have also been explored for their potential to combat harmful algal blooms, which can be detrimental to fish populations (Nam et al., 2016).

Freshwater tardigrades have historically been understudied in the U.S. Many regions and states lack documentation of freshwater tardigrade species (Kaczmarek et al., 2016; Staley et al., 2018). Newer records of U.S. freshwater tardigrade species have been documented in Kansas, Missouri, and Tennessee (Beasley et al., 2009; Bertolani et al., 2014; Staley et al., 2018). To date, there have not been any previous peer-reviewed records of freshwater tardigrade species from the Mississippi River (Kaczmarek et al., 2016; Miller and Perry, 2019).

The current research aimed to investigate the freshwater tardigrade species of the Upper Mississippi River and the U.S. Driftless Area. The main hypothesis was that we would find freshwater tardigrade species previously undocumented from the Mississippi River. We also hypothesized that we would identify freshwater tardigrade species not documented in Illinois, Iowa, or Wisconsin. The results presented in this study update our knowledge of freshwater tardigrade taxa in the Mississippi River, U.S. Driftless Area, and North America.

Materials and Methods

Sixty samples of water and sediment were collected from freshwater systems in March 2025 in the U.S. Driftless Region, including the Dubuque Harbor (Dubuque, IA) (N=10), Mississippi River (Dubuque, IA) (N=10), Woodward Wetland Pond (Dubuque, IA) (N=10), Galena River (Galena, IL) (N=10), Little Platte River (Platteville, WI) (N=10), and Rountree Branch (Platteville, WI) (N=10) (Figs. 1-2). Samples

Figure 1. General locations for sampling within the U.S. Driftless Area of Illinois, Iowa, and Wisconsin. (Locations = black stars).

were collected using two-ounce plastic cups with lids and labeled with a unique sample code. Ecological data, including photographs and coordinates, were collected using an iPhone SE (2020).

Three replicates of two milliliter (mL) aliquots were taken from each sample. Each aliquot was expelled into a container 2 in (50.8 mm) long and 1 in (25.4 mm) wide. Abundance counts of tardigrades were taken for each subsample using a Nikon 82074 dissecting microscope at 20-40X. Tardigrades were collected with an Irwin loop and placed in CMCP-10 mounting medium on microscope slides. A coverslip was placed on the mounting medium, and a permanent marker was used to place a dot on the coverslip at the right and left sides of the tardigrade to indicate its location on the slide (Miller, 1997).

After the mounting medium on the microscope slides dried, the slides containing tardigrades were viewed at 400X-1,000X with an Olympus CH30 compound microscope with phase contrast settings. The Olympus CH30 was equipped with a Dino-Eye AM4023X camera for imaging purposes. Tardigrades were also imaged at 200X-400X using an Invitrogen EVOS^{TM} FL color fluorescence microscope. Specimens were imaged with a fluorescence microscope using transmitted light, DAPI (360 nm excitation, 447 nm emission), GFP (470 nm

Figure 2. Sampling sites of freshwater systems included in the current study. (A) Woodward Wetland Pond (IA), (B) Mississippi River (IA), (C) Galena River (IL), and (D) Rountree Branch (WI).

excitation, 525 nm emission), and RFP (530 nm excitation, 593 nm emission) filters. Images of each specimen from the DAPI, GFP, and RFP filters were joined together to create an overlay image. Tardigrades were identified using morphological keys and world literature (Murray, 1907; Guidetti et al., 2006; Chang et al., 2007; Marley et al., 2008; Kaczmarek and Michalczyk, 2010; Pilato and Binda, 2010; Kaczmarek et al., 2012; Bertolani et al., 2014; Bartels et al., 2016; Tumanov, 2018; Gąsiorek et al., 2019; Pogwizd and Stec, 2020; Gąsiorek, 2024; Degma and Guidetti, 2024; Mapalo, unpublished). Voucher specimens of tardigrades collected in the current study were donated to collections at the National Mississippi River Museum and Aquarium in Dubuque, Iowa.

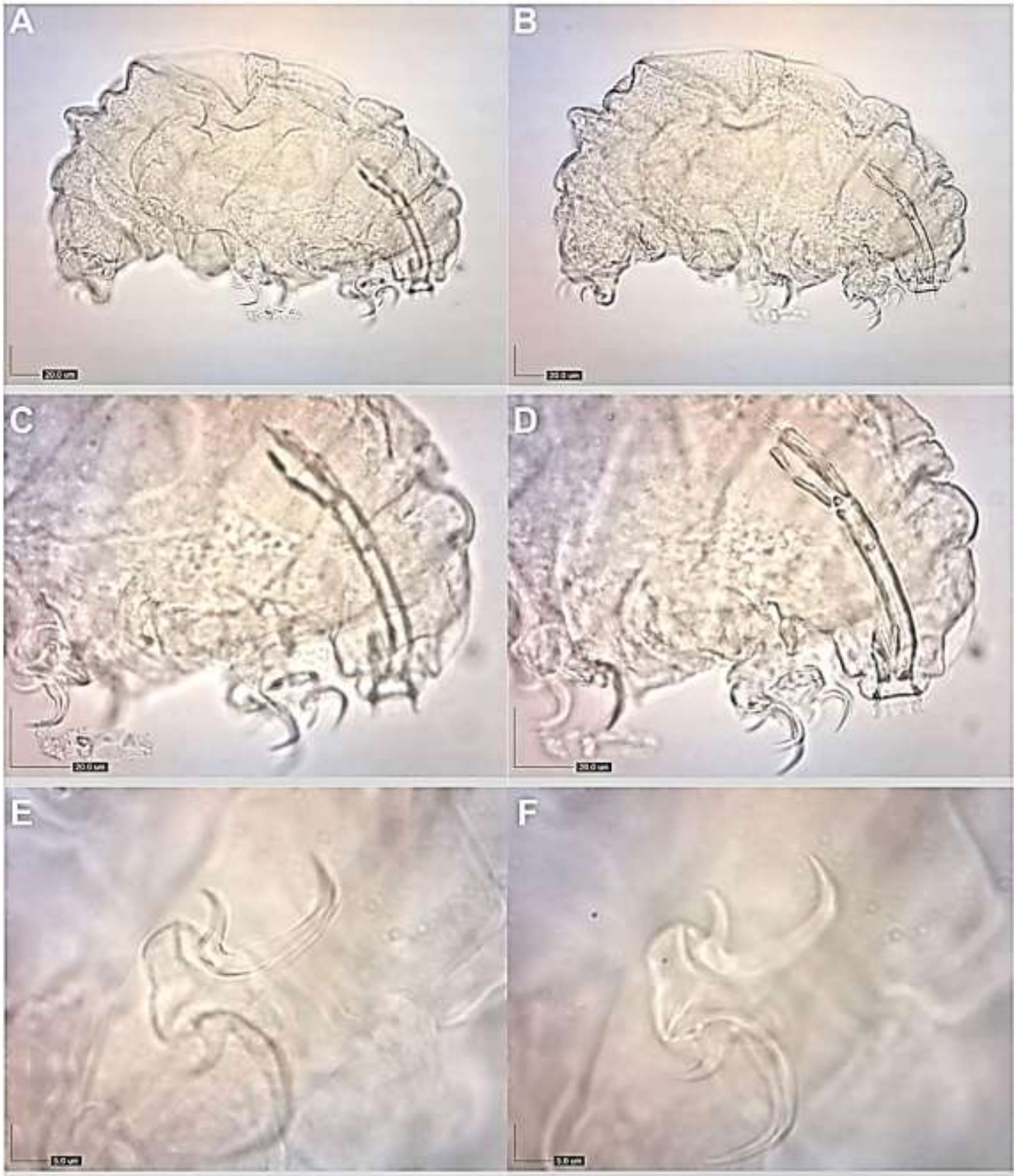

Figure 3. *Dactylobiotus* cf. *dispar* from Woodward Wetland Pond in Dubuque, Iowa. (A) First view at 200X, (B) Second view at 200X, (C) First view at 400X, (D) Second view at 400X, (E) Claws at 1,000X with focus on accessory point of the primary branch, and (F) Claws at 1,000X with focus on the cuticular bar (Scale bars are 20 micrometers for A-D, and 5 micrometers for E-F).

Statistical analyses were utilized to investigate tardigrade abundance in freshwater samples. A One-Way ANOVA and Tukey's Post Hoc analysis were conducted in RStudio to evaluate any statistically significant variance or differences in tardigrade abundance between sampling sites (RStudio Team, 2020). Only P-values of ≤ 0.05 were considered to be significant. The count data are provided in the supplemental file.

Results

A total of 180 subsamples were examined under the microscope. Tardigrades were present in 48 out of 180 subsamples (26.67%). Overall, tardigrades were present in 34 of the 60 samples (56.67%). A total of 85 tardigrades were collected. Of the 85 tardigrades, 69 (81.18%) could be identified. Tardigrades that were not identifiable were undergoing a molting phase in which internal structures (i.e., macroplacoids and

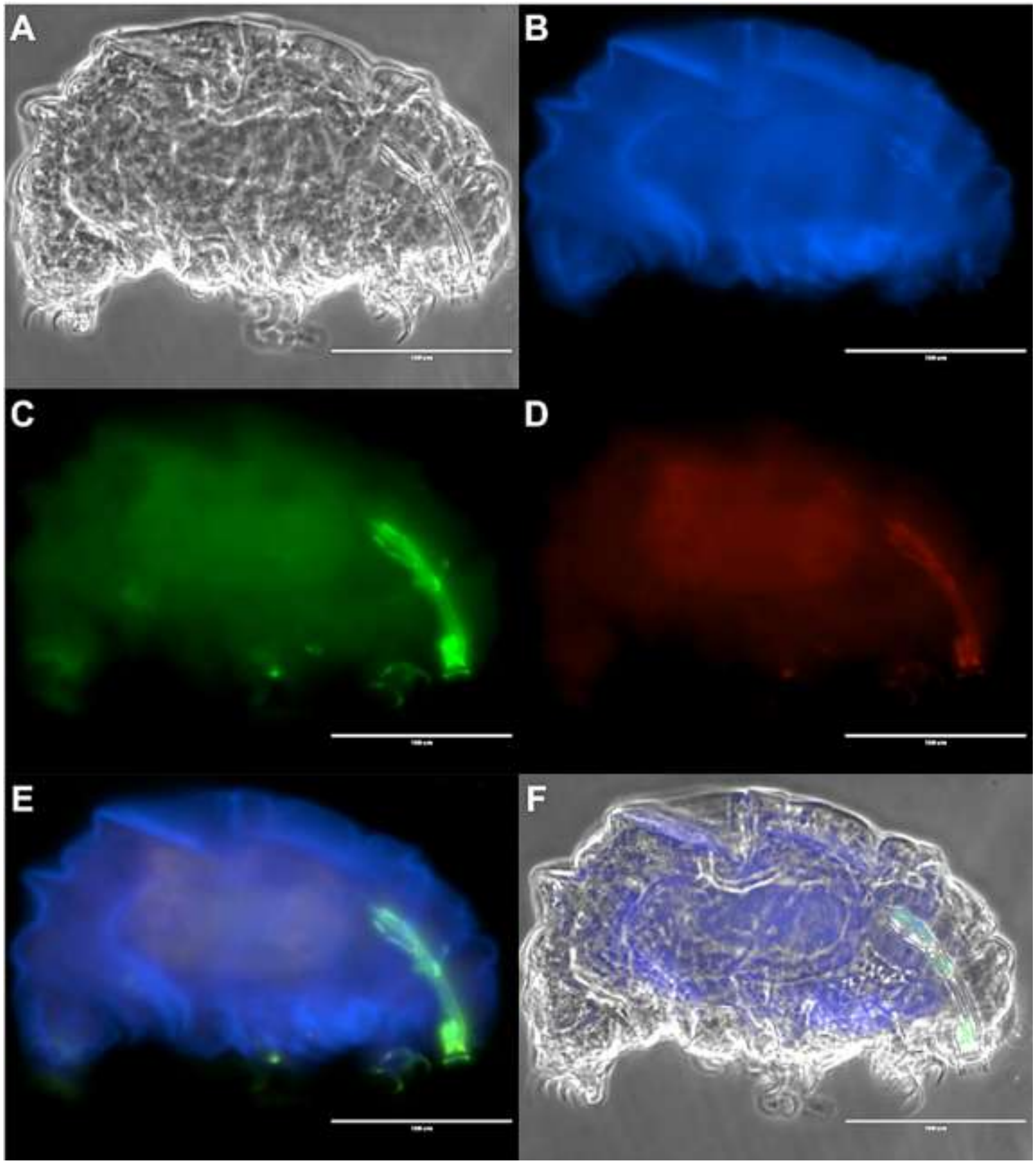

Figure 4. *Dactylobiotus* cf. *dispar* from Woodward Wetland Pond in Dubuque, Iowa. (A) Black and white transmitted light micrograph, (B) DAPI filter, (C) GFP filter. D – RFP filter, (E) Fluorescence overlay of DAPI, GFP, and RFP filters, and (F) Fluorescence overlay combined with transmitted light micrograph (Scale bars are 100 micrometers).

buccal tubes) could not be seen for identification, or they were not extended enough to view the entire body and claws in sufficient detail.

In total, thirty-five tardigrades were collected from Illinois, twenty-three tardigrades were collected from Iowa, and twenty-seven tardigrades were collected from Wisconsin freshwater systems. The One-Way ANOVA revealed significant variance within the dataset (P=0.04). Tukey's Post Hoc analysis did not yield significant differences in tardigrade abundance between sampling sites; however, a few comparisons were very close to significant. The Galena River samples yielded more tardigrades than the Dubuque Harbor (P=0.06), Mississippi River (P=0.07), and Little Platte River (P=0.10) samples.

Tardigrade taxa identified in the current study include *Dactylobiotus* cf. *dispar* Murray, 1907, *Grevenius granulifer* Thulin, 1928, *Pseudobiotus*

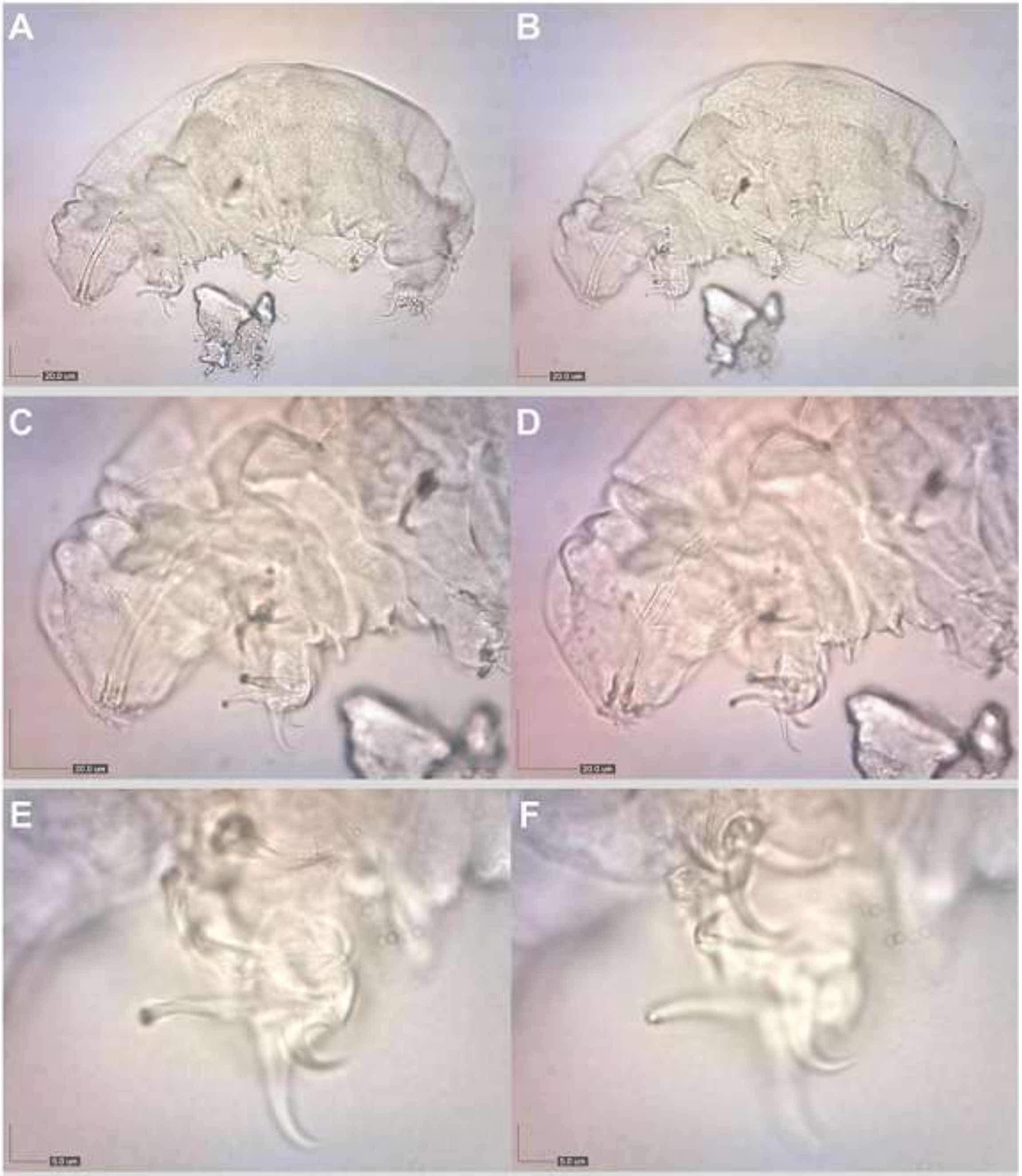

Figure 5. *Thulinius augusti* from the Mississippi River in Dubuque, Iowa. (A) First view at 200X, (B) Second view at 200X, (C) First view at 400X with focus on the first pair of claws, (D) Second view at 400X with focus on buccal tube and macroplacoids, (E) First view of claws at 1,000X, and (F) Second view of claws at 1,000X (Scale bars are 20 micrometers for A-D, and 5 micrometers for E-F).

kathmanae Nelson, Marley, & Bertolani, 1999, *Thulinius augusti* Murray, 1907, *Ramazzottius* sp. Binda & Pilato, 1986, *Isohypsibius* sp. Thulin, 1928, and *Microhypsibius* sp. Thulin, 1928. Voucher specimens were used to identify *D*. cf. *dispar* in the Woodward Wetland Pond in Dubuque, Iowa, which marks a new record for the state of Iowa (Figs. 3-4) (Kaczmarek et al., 2016; Miller and Perry, 2019). The identification of *T. augusti* in samples from the Mississippi River presents a new record for the state of Iowa (Fig. 5) (Kaczmarek et al., 2016; Miller and Perry, 2019). The tardigrade species *G. granulifer* (Figs. 6-7), *P. kathmanae* (Figs. 8-9), and *T. augusti* were found in the Mississippi River samples, and therefore are the first known tardigrade species of the Mississippi River.

The *Ramazzottius* sp. and *Microhypsibius* sp. were found in the Dubuque Harbor, which has water

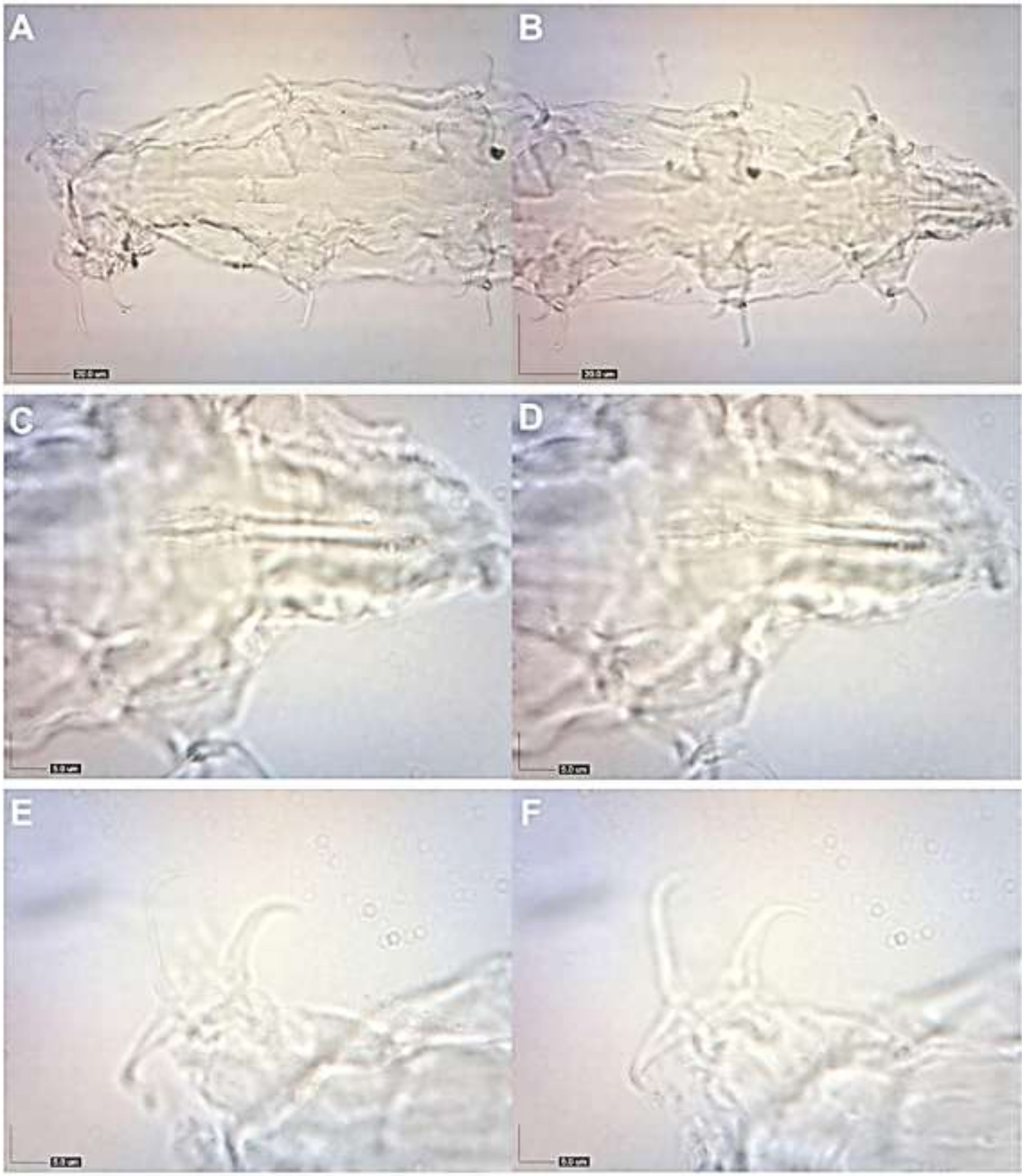

Figure 6. *Grevenius granulifer* from the Mississippi River in Dubuque, Iowa. (A) Posterior end at 400X, (B) Anterior end at 400X, (C) First view of buccal tube and macroplacoids at 1,000X, (D) Second view of buccal tube and macroplacoids at 1,000X, (E) First view of fourth pair of claws at 1,000X, and (F) Second view of fourth pair of claws at 1,000X (Scale bars are 20 micrometers for A-D, and 5 micrometers for E-F).

flowing into it from the Mississippi River. *Isohypsibius* sp. and *Microhypsibius* sp. were collected in the Rountree Branch, meaning this is the first record of the genus *Microhypsibius* in Iowa and Wisconsin, and the first record of the genus *Isohypsibius* in Wisconsin. Coordinates and ecological information of new biogeography records of tardigrade species are included in Table 1. Additionally, this is the first known peer-reviewed report of *D.* cf. *dispar*, *G. granulifer*, and *P. kathmanae* exhibiting autofluorescence at the wavelengths tested (Figs. 4, 7, 9) (Bartels et al., 2024).

Discussions

The current study drastically sets a foundation for our knowledge of freshwater tardigrade species of the Mississippi River and U.S. Driftless area. While this report documents tardigrade species from the Upper

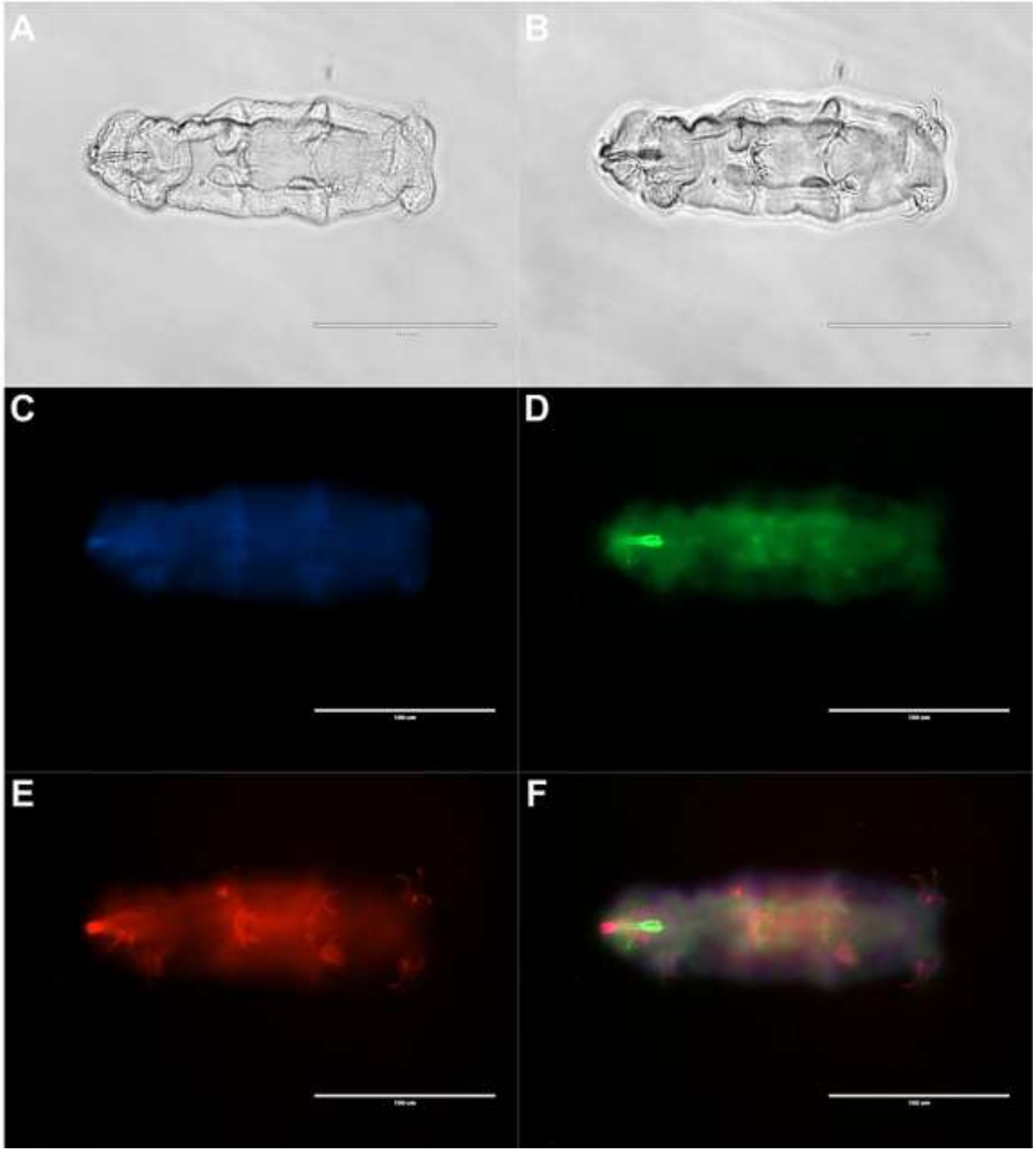

Figure 7. *Grevenius granulifer* from the Rountree Branch in Platteville, Wisconsin. (A) Black and white transmitted light micrograph, (B) Second view of black and white transmitted light micrograph, (C) DAPI filter. D – GFP filter, (E) RFP filter, and (F) Fluorescence overlay of DAPI, GFP, and RFP filters (Scale bars are 100 micrometers).

Mississippi River, over half of the Mississippi River remains unexplored by tardigrade researchers. The Lower Mississippi River (~1,600 km) has no reports of freshwater tardigrade species (Kaczmarek et al., 2016; Miller and Perry, 2019). This indicates substantial gaps in our understanding of freshwater ecosystems and microcosms of the Mississippi River.

Although freshwater tardigrade species have been documented from various U.S. freshwater systems (Beasley et al., 2009; Bertolani et al., 2014; Staley et al., 2018), we still lack an understanding of their roles in these ecosystems. Many freshwater tardigrade species, such as *Grevenius granulifer* and *Pseudobiotus kathmanae*, have been documented from multiple areas in the world (Marley et al., 2008; Kaczmarek et al., 2016; Tumanov, 2018; Gąsiorek, 2024), but there is a lack of detailed documentation of their diet, life cycles, cryptobiotic abilities, and symbiotic relationships with other organisms. Studies evaluating water quality (e.g., pH, salinity, dissolved

Table 1. Tardigrade species, city (state), coordinates, and freshwater environment of new biogeography records.

Tardigrade Species	**City (State)**	**Coordinates**	**Freshwater Environment**
Dactylobiotus cf. *dispar*	Dubuque (IA)	42°29’47”N; 90°39’43”W	Woodward Wetland Pond
Dactylobiotus cf. *dispar*	Platteville (WI)	42°43’32”N; 90°28’45”W	Rountree Branch
Grevenius granulifer	Dubuque (IA)	42°29’42”N; 90°39’25”W	Mississippi River
Grevenius granulifer	Galena (IL)	42°24’37”N; 90°25’52”W	Galena River
Grevenius granulifer	Platteville (WI)	42°43’23”N; 90°31’43”W	Little Platte River
Grevenius granulifer	Platteville (WI)	42°43’32”N; 90°28’45”W	Rountree Branch
Pseudobiotus kathmanae	Dubuque (IA)	42°29’42”N; 90°39’25”W	Mississippi River
Pseudobiotus kathmanae	Galena (IL)	42°24’37”N; 90°25’52”W	Galena River
Pseudobiotus kathmanae	Platteville (WI)	42°43’23”N; 90°31’43”W	Little Platte River
Pseudobiotus kathmanae	Platteville (WI)	42°43’32”N; 90°28’45”W	Rountree Branch
Thulinius augusti	Dubuque (IA)	42°29’42”N; 90°39’25”W	Mississippi River
Thulinius augusti	Platteville (WI)	42°43’23”N; 90°31’43”W	Little Platte River

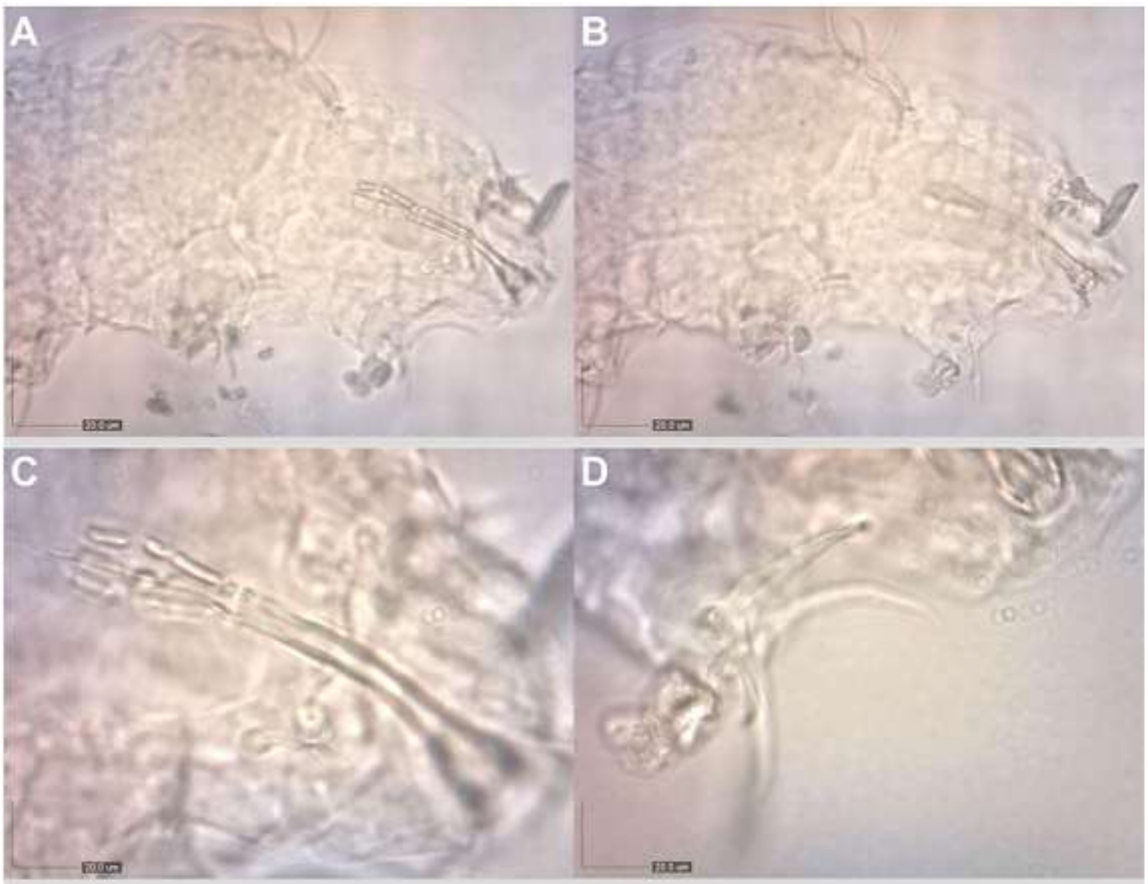

Figure 8. *Pseudobiotus kathmanae* from the Mississippi River in Dubuque, Iowa. (A) First view of anterior end at 200X, (B) Second view of anterior end at 200X, (C) Buccal tube and macroplacoids at 400X, and (D) First pair of claws at 400X (Scale bars are 20 micrometers).

oxygen) and tardigrade abundance could help elucidate freshwater tardigrade habitat preferences (Troell and Jönsson, 2023).

The tardigrade species *D. dispar* has been documented in a few studies in the U.S., with the closest record being from Missouri (Schuster and Grigarick, 1965; Strayer et al., 1994; Lehmann et al., 2007; Staley et al., 2018). The species *D. haplonyx* has been documented in North Carolina, but our specimens have a narrower buccal tube width than *D. haplonyx* (Bartels et al., 2016). One species, *Dactylobiotus kansae*, has been documented from Kansas, but no DNA has been reported, and it has pharyngeal cuticular bars absent in our species (Beasley et al., 2009; McCowen et al., 2018). The only DNA reference sequences on GenBank for

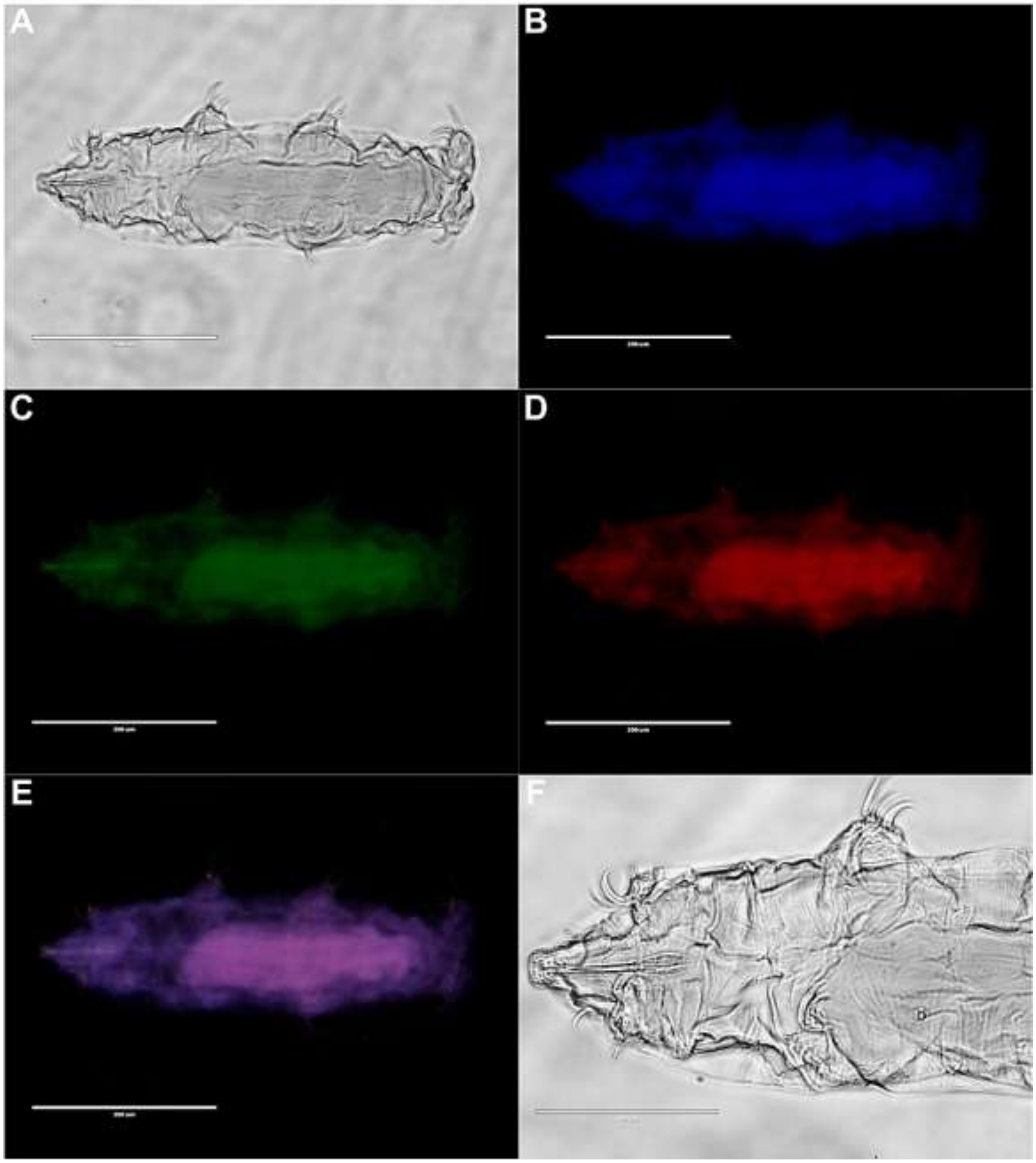

Figure 9. *Pseudobiotus kathmanae* from the Galena River in Galena, Illinois. (A) Black and white transmitted light micrograph, (B) DAPI filter, (C) GFP filter, (D) RFP filter, (E) Fluorescence overlay of DAPI, GFP, and RFP filters, and (F) Black and white transmitted light micrograph with focus on macroplacoids (Scale bars are 200 micrometers for A-E, and 100 micrometers for F).

Dactylobiotus species of the U.S. are of *Dactylobiotus ambiguus* Murray, 1907, from an unpublished report from Jacksonville, Alabama, and *Dactylobiotus grandipes* from Lake Tahoe in El Dorado County, California (Guidetti et al., 2022; Chen et al., unpublished; NCBI, 2025). Murray (1907) did not depict *D. ambiguus* as having accessory points on the claws, and also depicted *D. dispar* as having accessory points; our specimens have accessory points. Our *D.* cf. *dispar* specimens also have considerably shorter claws on the fourth pair of legs than those of *D. grandipes*, which has been designated as the type species of the genus *Dactylobiotus* (Kaczmarek and Michalczyk, 2010).

Thulinius augusti has previously been documented in Illinois but not in Iowa or Wisconsin (Puglia, 1964; Kaczmarek et al., 2016; Miller and Perry, 2019). The species *Pseudobiotus kathmanae* has been documented in the states of New York and Tennessee (Nelson et al., 1999; Strayer et al., 2014; Kaczmarek et al., 2016; Miller and Perry, 2019). The current study includes the first peer-reviewed report of freshwater tardigrade species documented from the Mississippi River, and the states of Iowa and Wisconsin (Kaczmarek et al., 2016; Miller and Perry, 2019). This report also significantly updates our knowledge of tardigrade biogeography within multiple freshwater systems in the U.S. Driftless Area. Further studies investigating U.S. freshwater tardigrade biodiversity and biogeography are recommended.

These records can be useful for education and conservation purposes. Without knowing which tardigrade species are present in the Mississippi River and freshwater systems of the U.S. Driftless Area, we cannot know which species should be considered as a conservation concern. Although microscopic invertebrates are often overlooked in conservation, some tardigrade species, such as *Novechiniscus armadilloides* Schuster, 1975, are considered endemic to specific U.S. regions (Kaczmarek et al., 2016; Loeffelholz et al., 2024b). The rarer a tardigrade species is, the more likely it is to be vulnerable or endangered. According to the International Union for Conservation of Nature's Red List of Threatened Species, there are no current records of vulnerable or endangered Tardigrada species (IUCN, 2024).

Many of the limited genetic records of tardigrades from the U.S. come from specimens sent to European universities for DNA extractions, PCR, and DNA sequencing (Nelson et al., 2020; Morek et al., 2021; Guidetti et al., 2022; Momeni et al., 2023), pointing to the insufficient interests and funding for U.S. universities and museums to study tardigrade biodiversity of North America. DNA barcoding of tardigrades from the Mississippi River and other U.S. freshwater systems would most likely reveal new species and previously undocumented biodiversity. Given the popularity of *Hypsibius exemplaris* Gąsiorek, Stec, Morek & Michalczyk 2018, a freshwater tardigrade species that has commonly been used for genetic, molecular, and cryptobiotic tolerance experiments (Boothby, 2018; Smith et al., 2018; Mapalo et al., 2024; Loeffelholz et al., 2024a), it seems the scientific community could greatly benefit from discovering, isolating, and culturing freshwater species of tardigrades from the Mississippi River. Successful cultivation of these species could make them more accessible to molecular biologists conducting research to discover novel genes, proteins, and molecules that may eventually be useful for combating cancer and other diseases (Hashimoto et al., 2016; Jönsson, 2019).

Acknowledgements
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Ethics approval and consent to participate: The study was conducted within national regulations on the use of microscopic invertebrates for biological research.

Availability of data and material: The count data is included in Supplemental File 2.

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