Received: 18 August 2023 | Accepted: 30 August 2024

DOI: 10.1111/ivb.12441

ORIGINAL ARTICLE

Invertebrate Biology WILEY

Bacteriological analysis of unionid hemolymph collected from freshwater mussel populations in the Pacific northwestern United States

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Funding information
United States Fish and Wildlife Service, Grant/Award Number: F19AC00507

Abstract

Native freshwater mussel (Unionidae) mortality events have been occurring with increased frequency in recent decades, with few investigations into potential etiological agents. In the western United States, no surveys have been published regarding the bacteria associated with unionid mussels. Herein, we examine locations of known mussel mortality events in the Chehalis River (Washington), in the Crooked River (Oregon), and Owyhee River (Oregon). Mussel populations considered healthy were sampled in the Skookumchuck River (Washington) for comparison. A variety of bacteria were isolated from these populations, and most notably, *Acinetobacter* spp. were identified from 82% of moribund individuals of *Gonidea angulata* in the Owyhee River. Future work evaluating whether *Acinetobacter* spp. are pathogenic to freshwater mussels could be valuable in unraveling the factors associated with these enigmatic mortality events.

KEYWORDS
Acinetobacter, *Anodonta*, *Gonidea angulata*, *Margaritifera falcata*, mussel mortality events, Oregon, Washington

1 | INTRODUCTION

Whereas North America is known to host nearly a third of unionid mussel species worldwide, the contiguous western United States (excluding Alaska) has relatively low mussel diversity, comprising just three genera—*Margaritifera falcata* (Gould 1850) (Western Pearlshell), *Gonidea angulata* (Lea 1838) (Western Ridged Mussel), and species of *Anodonta*. Although there are fewer species than in the eastern United States, freshwater mussels are still an important component of western freshwater ecosystems and occur in large numbers in lotic systems, with some tens of thousands of individuals in intact beds in certain locations. However, recent analysis of occurrence data at the watershed scale has revealed that freshwater mussels in Oregon and Washington have enigmatically declined in their distribution as compared with their historical range (Blevins et al., 2017).

Causes of widespread mussel decline are not well established in the western United States as compared with the eastern United States, although declines have been reported in the historical literature, dating back slightly more than a century (Hannibal, 1912). Documented effects include habitat loss and water abstraction (Hannibal, 1912), sedimentation (Vannote & Minshall, 1982), and instream mining (Krueger et al., 2007). Other presumed threats

include habitat degradation, declines in host fish, poor water quality, the introduction of non-native invasive species, and drought (Blevins et al., 2017; Waller & Cope, 2019). Yet, these threats are generally more associated with the longer term decline of populations than the sudden mass mortality of a mussel bed, as seen more recently in rivers in the western United States.

Recent mass mortality events, in which large numbers of mussels or proportions of mussel beds suddenly die within a short period of time (in some cases as quickly as a month or two), have been increasingly reported from the western United States. These events appear to be occurring both rapidly, as well as chronically, sometimes with recurring mortality events each year, continually depleting remaining mussels within once large beds. Such events have been observed or inferred at sites spanning multiple western states, in watersheds along the Pacific coast to inland Idaho (Blevins et al., 2020). Despite these recent mortality events in the western United States, not much attention has been given to studying the health of these mussels. Our group has thus far, to our knowledge, completed the only health assessments of mussels from this region resulting in the identification of 557 mostly novel viruses identified from the same mussels examined herein (Goldberg et al., 2023; Richard et al., 2023). However, none of these identified viruses were consistently associated with disease (Richard et al., 2023).

Across the entire United States, there have been relatively few bacteriological investigations assessing mortality events using culture-based (Jenkinson & Ahlstedt, 1987; Leis et al., 2019; Leis, Dziki, Richard, et al., 2023; Leis, Dziki, Standish, et al., 2023; Sparks et al., 1990; Starliper et al., 2011; Thiel, 1987) or metagenomic-based methods (Richard et al., 2021) to identify the bacteria associated with dying mussels. In this investigation, we examined the bacterial communities associated with mussel hemolymph from three rivers in the western United States, including during active mortality events.

2 | METHODS

Freshwater mussels were collected by hand from locations in Washington and Oregon listed below with collection details (Figure 1; refer to Richard et al., 2023 for a detailed description). At all sites, mussels were characterized as moribund if they were abnormally not burrowed and resting on top of the surface, with little to no response to touch; mussels were considered healthy if they were burrowed and responded to touch.

2.1 | Collection locations and accounts

2.1.1 | Chehalis River (near Oakville, Washington), September 26, 2018

Washington Department of Fish and Wildlife staff first reported an observation of a sudden mass mortality of freshwater mussels in the lower Chehalis River of southwest Washington in 2015. Since 2015, observations of large numbers of shells of three species of freshwater mussel (*M. falcata*, *G. angulata*, and *Anodonta* sp.) have been reported extending >80 river km upstream from this location. In September 2018, individuals of *M. falcata* ($n = 9$; mean length

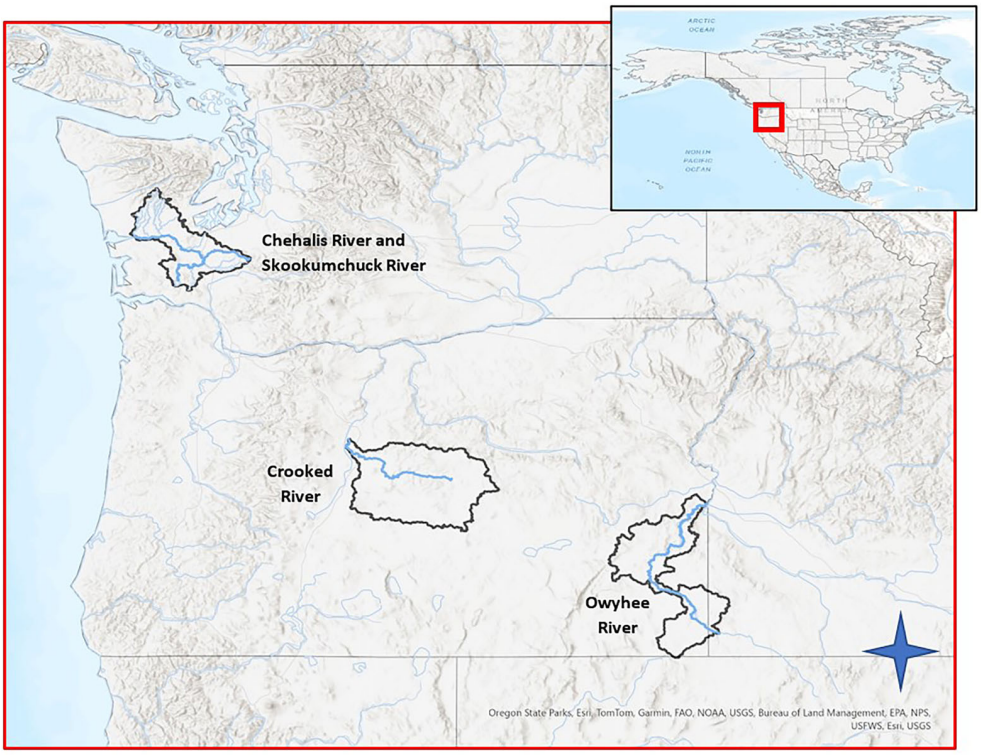

FIGURE 1 Sites where mussels were sampled in the Pacific Northwest of the United States.

101.3 mm) were collected from within a single bed in the Chehalis River, centrally located within the long stretch of the river where the die-off remains ongoing. Collected mussels were found still burrowed although some were gaping, slow to respond, and, thus, were considered moribund. A large number of shells were also observed for each species.

2.1.2 | Skookumchuck River (near Centralia, Washington), September 26, 2018

The Skookumchuck River, a tributary to the Chehalis River near Centralia, Washington, was surveyed in 2017 and 2018 to assess whether the mass mortality events were also observed in tributaries. Many large, healthy beds have been found in the Skookumchuck River over the last several years, and no evidence of dying mussels was observed. In September 2018, individuals of *M. falcata* ($n = 5$; mean length 100.3 mm) were collected for comparison with mussels collected from the Chehalis River.

2.1.3 | Crooked River (near Terrebonne, Oregon), October 8, 2018

In 2014, members of the Pacific Northwest Native Freshwater Mussel Workgroup reported an observation of a sudden mass mortality of *G. angulata* in the Crooked River at Smith Rock State Park near Terrebonne, Oregon. Many thousands of shells were reported at that time. The site was revisited multiple times over Summer 2018, when both normally burrowed and apparently sick or moribund mussels (not burrowed) were observed. Since 2018, the annual presence of fresh empty shells and mussels that are not burrowed indicates that the mass mortality event remains ongoing.

Specimens of *G. angulata* and *Anodonta* sp. were collected in October 2018. Both apparently healthy ($n = 14$; mean length 98.3 mm) and moribund ($n = 6$; mean length 101.5 mm) specimens of *G. angulata*, as well as apparently healthy ($n = 3$; mean length, 54.8 mm) specimens of *Anodonta* sp., were collected from within the same bed.

2.1.4 | Owyhee River (near Rome, Oregon), August 20, 2019

A citizen report of a large number of mussel shells in the Owyhee River in 2017, ~35 km downstream from Rome, Oregon, initiated surveys and sampling in 2018 and 2019. In August 2019, we collected samples during a float trip between Rome and the Birch Creek Historic Ranch downstream, covering ~80 river km. Throughout much of this stretch of river, beds of live individuals of *G. angulata* were observed, although a large number of shells, as well as many unburrowed mussels, were also observed. In 2019, we collected moribund specimens of *G. angulata* ($n = 11$) for analysis.

2.2 | Hemolymph collection

Mussels were wrapped in wet paper towels, placed on ice, and shipped overnight to the U.S. Fish and Wildlife Service La Crosse Fish Health Center in La Crosse, Wisconsin, for sampling. Hemolymph was collected from the anterior adductor muscle with a 1-ml syringe and 25-gauge needle from each mussel. Approximately 100 µl of hemolymph was streaked with a disposable, sterile inoculation loop onto Tryptic Soy Agar (TSA). Bacteriology samples were analyzed using methods similar to those described in Leis et al. (2019); Leis, Dziki, Richard, et al. (2023); and Leis, Dziki, Standish, et al. (2023). Briefly, the plates were incubated at 22°C for 7–14 days, and unique isolates were placed into a microcentrifuge tube by use of a sterile loop. Then, the DNA for each isolate was extracted with the Prepman Ultra Sample Preparation Reagent (Thermofisher). The *16S* rRNA gene for each bacterial isolate was amplified with polymerase chain reaction (PCR) using the primers in Leis et al. (2019), followed by Sanger sequencing by Eton Biosciences (Union, New Jersey). Sequences were then assembled and edited with Geneious v11.1.5 (https://www.geneious.com) and identified through BLAST searches in the NCBI database (https://www.ncbi.nlm.nih.gov/).

2.3 | Statistics

Potential associations between bacterial genera and health status of *M. falcata* were evaluated using Fisher's exact test. Moribund individuals of *M. falcata* from the Chehalis River ($n = 9$) were compared with apparently healthy individuals of *M. falcata* from the Skookumchuck River ($n = 5$), with a significance level of $\alpha = .05$. Similarly, apparently healthy individuals of *G. angulata* from the Crooked River ($n = 14$) were compared against moribund individuals of *G. angulata* from the Crooked River ($n = 6$) and the Owyhee River ($n = 11$) using Fisher's exact test. All genera of bacteria were evaluated for both species of mussel; we also looked at patterns of prevalence in mussels that yielded no isolates. We used t-tests to compare the average number of isolates per individual between apparently healthy versus moribund mussels in specimens of *M. falcata*. For *G. angulata*, analysis of variance (ANOVA) with Tukey HSD was used to compare average number of isolates between three groups: healthy mussels from the Crooked River, moribund mussels from the Crooked River, and moribund mussels from the Owyhee River.

2.4 | Histopathology

Histopathology was completed using the same methods described in Knowles et al. (2022), with the samples being collected from the same mussels examined for the presence of bacteria. Samples from the Owyhee River were also stained with Gram (Brown and Hopps).

3 | RESULTS

There were 36 bacterial genera identified from a total of 48 mussels across four rivers in three watersheds in the western United States, of which 14 genera were single incident isolates (Table 1; Appendix S1). Sampling during September of 2018 compared moribund individuals of *M. falcata* from a site with recurring mortalities (Chehalis River) with a nearby site that lacked known mortalities (Skookumchuck River). It was revealed that 89% (8 of 9) of the mussels from the Chehalis River harbored bacteria in their hemolymph compared with 20% (1 of 5) from the Skookumchuck River. Furthermore, *Bacillus* (in 4 of 9 mussels) and *Pedobacter* (4 of 9) were the most prevalent bacteria identified from mussels in the Chehalis River. From the sampling that occurred in the Crooked River, bacteria were isolated from 100% (3 of 3) of the apparently healthy individuals of *Anodonta* sp., from 64% (9 of 14) of the apparently healthy individuals of *G. angulata*, and from 83% (5 of 6) of the moribund individuals of *G. angulata*. The most prevalent bacterial genera isolated from mussels at this site was *Bacillus*, occurring in 30% (7 of 23) of the mussels overall, in 29% (4 of 14) of the apparently healthy individuals of *G. angulata*, in 33% (2 of 6) of the moribund individuals of *G. angulata*, and in a single (1 of 3) apparently healthy individual of *Anodonta* sp. Additionally, a noteworthy fish pathogen, *Yersinia ruckeri*, was identified from a moribund individual of *G. angulata* from the Crooked River. The most compelling association overall was identified during a mortality event in the Owyhee River, with *Acinetobacter* spp. identified from the hemolymph of 82% (9 of 11) of moribund individuals of *G. angulata*, although it is important to note that we were unable to sample healthy mussels from this river for comparison. *Pseudomonas* was the next most prevalent (4 of 11; 36%) bacterial genus identified from mussels in the Owyhee River.

Moribund individuals of *M. falcata* from the Chehalis River yielded significantly more bacterial isolates per mussel (2.89 ± 1.83, mean ± standard deviation [SD]) than did healthy individuals of *M. falcata* from the Skookumchuck River (0.6 ± 0.89 isolates per mussel, t-test, $p = .024$). Healthy individuals of *G. angulata* from the Crooked River yielded an average of 2.29 ± 2.49 isolates per mussel, whereas moribund individuals of *G. angulata* from the Crooked River yielded 2.33 ± 2.50 isolates per mussel and moribund individuals of *G. angulata* from the Owyhee River yielded 3.00 ± 2.14 isolates per mussel. No relationships among these groups were significant (ANOVA, $p = .737$). Of all bacterial genera, the higher prevalence of *Acinetobacter* in moribund individuals of *G. angulata* was the only statistically significant relationship between bacterial genus and mussel health status (Fisher's exact test, $p = .001$). All other statistical comparisons using Fisher's exact test to evaluate the relationship between bacterial genera and health status were nonsignificant (including those evaluating the prevalence of mussels yielding no bacterial isolates). In histopathological examinations, bacteria were only observed within lumina of the gastrointestinal tract, with no evidence of systemic bacterial infection.

4 | DISCUSSION

Several bacterial genera were identified from mussels in the northwestern United States that were previously identified from other bacteriological evaluations of mussel hemolymph. *Aeromonas* and *Pseudomonas* have frequently been identified from both healthy and moribund mussels of a variety of species (Leis et al., 2019; Leis, Dziki, Richard, et al., 2023; Leis, Dziki, Standish, et al., 2023; Richard et al., 2021). The genus *Bacillus* has been found more frequently in healthy mussels, although this relationship was not statistically significant (Leis, Dziki, Standish, et al., 2023). Most of the bacterial genera identified from mussels in the western United States may be part of the typical microbiome associated with healthy mussels, and some could potentially be beneficial to the mussel, as mentioned in Leis et al. (2019). The only discernable pattern from our sampling effort was the presence of *Acinetobacter* spp. from 82% of the moribund individuals of *G. angulata* sampled from the Owyhee River (Table 1). This prevalence is quite high and similar to the prevalence of *Yokenella* observed during a mortality event in the Clinch River in the southeastern United States (Leis et al., 2019; Leis, Dziki, Richard, et al., 2023; Leis, Dziki, Standish, et al., 2023; Richard et al., 2021). The significance of the high prevalence of *Acinetobacter* sp. is not immediately apparent because we were unable to sample healthy mussels from this location. A majority of the isolates from *G. angulata* were most similar molecularly to *A. johnsonii* and *A. lwoffii* (Appendix S1), both of which have been considered pathogens of fish and humans (Cao et al., 2018; Kozińska et al., 2014). *Acinetobacter schindleri*, *A. beijerinckii*, and *A. soli* were also identified as single incident isolates (Appendix S1). Species within the genus *Acinetobacter* are generally considered ubiquitous saprophytes that have been isolated from a variety of conditions, although some species are noteworthy as potential human pathogens, some of which are nosocomial (Al Atrouni et al., 2016). Furthermore, *Acinetobacter* spp. were occasionally identified from healthy mussels in the upper Midwest, not identified from the 2017 mortality event in the Clinch River, and not identified from a mortality event in the Tennessee River (Leis et al., 2019; Leis, Dziki, Richard, et al., 2023; Leis, Dziki, Standish, et al., 2023; Starliper et al., 2011). Whether the presence of *Acinetobacter* is an incidental finding that is insignificant (e.g., saprophytes that are invading dying mussels), the source of infection or a possible bioindicator of the presence of a stressor warrants future study (refer to Leis et al., 2019). Furthermore, in vivo assays exposing samples of *G. angulata* to species of *Acinetobacter*, notably *A. johnsonii* and *A. lwoffii*, could be valuable in determining pathogenicity.

Previous research showed a statistically significant association between *Yokenella regensburgei* and moribund individuals of *Ortmanniana* (= *Actinonaias*) *pectorosa* during mortality events in the southeastern United States (Leis et al., 2019; Leis, Dziki, Richard, et al., 2023; Richard et al., 2021) as well as in multiple species during a mortality event in the Midwest (Leis, Dziki, Standish, et al., 2023). However, during our investigation of mortality events in the Pacific Northwest, this bacterium was not identified, indicating that some unidentified factors may link the mortality events where this microbe is found.

TABLE 1 Bacteria isolated from hemolymph of freshwater mussels from the northwestern United States. In the records of *Gonidea angulata* from the Crooked River, bacteria prevalence from moribund mussels is reported in parentheses after the prevalence for healthy mussels.

Sampling date	Location	Mussel species	Health status	Number sampled	Identification	Prevalence
9/26/2018	Chehalis River, WA	*Margaritifera falcata*	Moribund	9	*Aeromonas*	3 of 9
					Arthrobacter	1 of 9
					Bacillus	4 of 9
					Buttiauxella	1 of 9
					Exiguobacterium	1 of 9
					Micrococcus	2 of 9
					Paenarthrobacter	1 of 9
					Paenibacillus	2 of 9
					Pedobacter	4 of 9
					Pseudomonas	1 of 9
					Sporosarcina	1 of 9
					Staphylococcus	3 of 9
					Viridibacillus	1 of 9
					No isolates	1 of 9
	Skookumchuck River, WA	*Margaritifera falcata*	Healthy	5	*Acinetobacter*	1 of 5
					Buttiauxella	1 of 5
					Pseudomonas	1 of 5
					No isolates	3 of 5
10/8/2018	Crooked River, OR	*Anodonta* sp.	Healthy	3	*Aeromonas*	1 of 3
					Bacillus	1 of 3
					Chryseobacterium	1 of 3
					Deefgea	1 of 3
					Deinococcus	1 of 3
					Domibacillus	1 of 3
					Flavobacterium	1 of 3
					Massilia	1 of 3
					Pseudomonas	2 of 3
					Shewanella	1 of 3
		Gonidea angulata	Healthy (Moribund)	20	*Arthrobacter*	2 of 14 (1 of 6)
					Achromobacter	1 of 20
					Aeromonas	2 of 14 (1 of 6)
					Bacillus	4 of 14 (2 of 6)
					Brevibacterium	1 of 20
					Buttiauxella	2 of 20
					Clostridium	(1 of 6)
					Deefgea	1 of 20
					Enterobacter	1 of 20
					Flavobacterium	2 of 20
					Nesterenkonia	(1 of 6)
					Oerskovia	1 of 20
					Paenibacillus	1 of 20
					Paenisporosarcina	1 of 20
					Pararheinheimera	1 of 20
					Pseudarthrobacter	2 of 14 (1 of 6)
					Pseudomonas	1 of 14 (1 of 6)
					Shewanella	(2 of 6)

(Continues)

TABLE 1 (Continued)

Sampling date	Location	Mussel species	Health status	Number sampled	Identification	Prevalence
					Variovorax	1 of 20
					Yersinia	(1 of 6)
8/20/2019	Owyhee River, OR	*Gonidea angulata*	Moribund	11	*Acinetobacter*	9 of 11
					Aeromonas	2 of 11
					Chryseobacterium	3 of 11
					Comamonas	2 of 11
					Enterobacter	1 of 11
					Enterococcus	1 of 11
					Exiguobacterium	1 of 11
					Paenibacillus	1 of 11
					Pseudomonas	4 of 11
					Rheinheimera	2 of 11
					Shewanella	1 of 11
					Wautersiella	1 of 11

Nonetheless, continuing to sample mussels for *Y. regensburgei* is important in understanding the associations and connections between mussel mortalities and this relatively obscure bacterium. Similarly, future work determining whether *Acinetobacter* species can consistently be isolated with high prevalence from mortality events in populations of *G. angulata* could provide further support for this association, similar to what has been reported for *Y. regensburgei*.

One limitation of this study was the use of only one media type (TSA) to evaluate the hemolymph of these mussels. However, a comparison of the bacteria cultured on TSA and bacteria identified through metagenomic analysis of the microbiome showed similar bacteria faunas (Richard et al., 2021). Although use of TSA as a culture medium appears to be adequate, future bacteriological investigations using additional media types or amplicon-based sequencing of each sample could be valuable in identifying additional bacterial genera that cannot grow on TSA.

The capacity for statistical inference from this dataset was inherently limited by the sampling design and the availability of mussels in various health states among sites. The only statistically significant association observed in this study was that of the bacterial genus *Acinetobacter* associated with moribund individuals of *G. angulata*, but this relationship is difficult to interpret because apparently healthy mussels for comparison came from a different river than those infected with *Acinetobacter* sp. The observed pattern could reflect site-specific differences in mussel bacterial microbiomes, but further inference is challenging.

The isolation of *Y. ruckeri* from individuals of *G. angulata* from the Crooked River in Oregon highlights the importance of biosecurity when rearing mussels in propagation facilities. This bacterium has also been identified from other mussel populations in the upper Mississippi River and can have regulatory implications if identified from hatchery populations (Leis et al., 2019; USFWS and AFS Bluebook, 2014). Furthermore, another important fish pathogen, *Aeromonas salmonicida*, has been identified from various mussel populations (Leis et al., 2019; Richard et al., 2021). Some practices to consider when mussels are brought into a facility for restoration efforts could include the depuration of mussels, isolation of mussels from fish populations, disinfection of effluent, and separation of equipment used in the rearing of these invertebrates and captive fish to prevent transfer of potential pathogens.

Understanding factors influencing mussel health is critical to interpreting the sudden mass mortality events affecting freshwater mussel species, which occur each year and span major river basins in the Pacific Northwest. Because mortality of other aquatic species has not been observed at these sites, and because these die-offs can appear suddenly or chronically, continued sampling could be beneficial in determining the extent to which bacteria factor into such events. Outside of the high prevalence of *Acinetobacter* species in moribund individuals of *G. angulata* in the Owyhee River, no other connections could be made between health status and the presence of bacteria or viruses in these dying mussels (Richard et al., 2023). The causal factors associated with the mussel mortality events investigated herein remain enigmatic.

ACKNOWLEDGMENTS

We would like to thank John Fisher (National Conservation Training Library; United States Fish and Wildlife Service; Shepherdstown, WV) for assistance obtaining references. We would also like to thank Dave Banks and Kirk Handley (Oregon Department of Fish and Wildlife) for assistance in collecting samples. This research was funded by the United States Fish and Wildlife Service, grant number F19AC00507. The use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. The findings and conclusions in this article are those of the authors and the U.S. Geological Survey and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are reported in this manuscript and supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Leis, E. M., Dziki, S., Blevins, E., Waller, D. L., Richard, J. C., Knowles, S., & Goldberg, T. L. (2024). Bacteriological analysis of unionid hemolymph collected from freshwater mussel populations in the Pacific northwestern United States. *Invertebrate Biology*, *143*(4), e12441. https://doi.org/10.1111/ivb.12441