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Further bacteriological analysis of annual Pheasanshell mussel (Ortmanniana pectorosa) mortality events in the Clinch River (VA/TN), USA reveals a consistent association with Yokenella regensburgei

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REGULAR ARTICLE

Running head: Bacteria Associated With Pheasantshell Mortality

FURTHER BACTERIOLOGICAL ANALYSIS OF ANNUAL PHEASANTSHELL MUSSEL (*ACTINONAIAS PECTOROSA*) MORTALITY EVENTS IN THE CLINCH RIVER (VA/TN), USA, REVEALS A CONSISTENT ASSOCIATION WITH *YOKENELLA REGENSBURGEI*

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ABSTRACT

Pheasantshell mussels (*Actinonaias pectorosa*) in the Clinch River (Tennessee/Virginia, USA) have declined dramatically in recent years. The bacterium *Yokenella regensburgei* was first isolated with high prevalence from Pheasantshell during the peak of a 2017 mortality event, but it was not identified after mortality subsided a few months later. Since 2017, Pheasantshell mortality in the Clinch River has occurred each autumn. We extended the investigation of culturable bacterial communities in the Clinch River during mussel mortality events in 2018, 2019, and 2020 and examined the spatial and temporal distribution of bacterial genera among Pheasantshells, as well as other unionid mussels. We identified *Yokenella regensburgei* each year, almost exclusively during active mortality events. The significance of *Y. regensburgei* remains unclear, but the continued association of this bacterium with mussel mortality events warrants further study.

KEY WORDS - *Actinonaias pectorosa*, Pheasantshell mussel, Unionidae, *Yokenella regensburgei*, Clinch River, Virginia, mortality event

INTRODUCTION

Large-scale mortality events and declines in mussel populations have occurred with increasing frequency in recent decades (Strayer et al. 2004). Clear explanations, such as toxic spills, have been identified in some cases, but in others, disease has been suspected but not confirmed (Neves 1987; Haag 2019). Beginning in summer 2016, biologists observed a mass mortality event affecting numerous mussel species in the Virginia and Tennessee portions of the Clinch River. Subsequent investigations revealed that mortality recurred seasonally from late summer to early autumn. While many species were observed dead or moribund, the Pheasantshell (*Actinonaias pectorosa*) was affected disproportionately. Pheasantshell initially were among the most abundant species in the Clinch River, but population sizes declined 50–80% across multiple sites after mortality events (Richard 2018; Cope et al. 2021). In response to the mortality event in the Clinch River and a contemporaneous multi-species mortality event in Big Darby Creek, Ohio, a research group was formed to study the events and gather baseline data to identify potential pathogens (Leis et al. 2018). The group reported a picorna-like virus from a Wabash Pigtoe (*Fusconaia flava*) in the upper Mississippi River (Goldberg et al. 2019), 17 novel viruses, including a densovirus associated with moribund mussels in the Clinch River (Richard et al. 2020), and a novel gonadotropic microsporidian (Knowles et al. 2022). They also conducted molecular- and culture-based evaluations of the bacterial composition of hemolymph from several river systems in the eastern USA (Leis et al. 2019; Richard et al. 2021).

In a previous study, we examined culturable bacteria associated with a 2017 mussel mortality event in the Clinch River (Leis et al. 2019). We identified many bacterial genera, but only *Yokenella regensburgei* was detected with high prevalence in Pheasantshell while mortalities were occurring, and it was not present a few months later after mortality subsided.

This bacterium was previously identified from a mussel mortality event in the Tennessee River (Starliper et al. 2011), but whether it plays a direct role in such events remains unknown. Since 2017, episodic mortality of Pheasantshell has continued each autumn in the Clinch River. We investigated bacterial communities in the Clinch River during mussel mortality events in 2018, 2019, and 2020 and examined the spatial and temporal prevalence of bacterial genera among Pheasantshells and other unionid species.

METHODS

We collected samples from live and moribund mussels at seven sites in the Clinch River in 2018, 2019, and 2020 (Fig. 1, Table 1). After observing mussel mortality in autumn 2016 and 2017, we established a series of sampling sites within and upstream of the zone of observed mortality and began sampling in summer 2018. We sampled six sites monthly from August to October 2018. High rainfall forced us to abandon planned sampling events in November and December 2018. In 2018, we sampled Pheasantshell and Mucket (*Actinonaias ligamentina*); the annual Pheasantshell mortality event began in September and no moribund Muckets were observed (Table 1). In 2019, we observed a mortality event that began in September and sampling occurred at Sycamore Island while the event was ongoing in October. We sampled moribund Pheasantshells and apparently healthy individuals of Mucket, Pocketbook (*Lampsilis ovata*), Threeridge (*Amblema plicata*), Kidneyshell (*Ptychobranchus fasciolaris*), Wavyrayed Lampmussel (*Lampsilis fasciola*), and Purple Wartyback (*Cyclonaias tuberculata).* We observed mortality in October 2020 and collected targeted samples consisting of moribund Pheasantshells combined from three adjacent sites (Speers Ferry, Sycamore Island, and Clinchport). Later in the month, we also sampled moribund Pheasantshell, Mucket, and Cumberlandian Combshell (*Epioblasma brevidens*) from Sycamore Island.

In 2018 and 2019, we collected hemolymph from the anterior adductor muscle of each mussel by slightly opening the shell with a child nasal speculum, placing a stopper between the shells, and drawing out a hemolymph sample with a 1-mL syringe and 25-gauge needle. After collecting each sample, we immediately plated and streaked approximately 100 µL of hemolymph onto sterile Tryptic Soy Agar (TSA) culture plates (Becton Dickinson, Le Pont de Claix, France). Plates were shipped overnight to the US Fish and Wildlife Service La Crosse Fish Health Center, La Crosse, WI. We incubated the plates at 21ºC for seven to 14 days. After incubation we used a sterile, disposable loop to remove morphologically unique colonies from each plate, placed them in a microcentrifuge tube, and extracted DNA using the PrepMan™ Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts). We subjected the extracted DNA to 16S rRNA gene PCR using the same primers used by Leis et al. (2019), followed by Sanger sequencing (Eton Biosciences, Union, New Jersey). We then edited and assembled the sequences *de novo* using the default parameters in Geneious v11.1.5 (https://www.geneious.com/download/previous-versions/#geneious-r11-dot-1, accessed August 19, 2022), and we identified resulting contig sequences through megaBLAST searches in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed August 19, 2022). In 2020, moribund mussels were wrapped in wet towels and sent on ice to the La Crosse Fish Health Center for processing as described above. Because Pheasantshell was the primary species observed in moribund condition, we used Fisher’s exact tests to examine whether there were nonrandom associations between frequently observed bacterial genera and healthy or moribund Pheasantshell samples. For each bacterial genus present in ≥6 Pheasantshell, as well as for the condition of “no bacterial growth observed”, we set up a 2x2 contingency table with categories of bacteria presence/absence and healthy/moribund mussels. Pheasantshell samples within the moribund and healthy groups were pooled across all sites and dates from the study. The results of each Fisher’s exact test indicate whether there was a statistically significant association between the presence of a particular bacterial genus and Pheasantshell health status.

RESULTS

We examined a total of 91 mussels (67 Pheasantshell, 15 Mucket, 1 Cumberlandian Combshell, 1 Purple Wartyback, 2 Wavyrayed Lampmussel, 1 Kidneyshell, 2 Threeridge, and 2 Pocketbook), including 49 healthy and 42 moribund individuals, from the Clinch River during 2018, 2019, and 2020. Bacteria were isolated from 80% (73 of 91) of the mussels sampled; 18 mussel samples yielded no bacterial isolates. All the cultured colonies were identifiable, except for two isolates from Muckets sampled on August 16, 2018 and October 25, 2018.

Across all sampling seasons, we identified 190 isolates belonging to 46 bacterial genera from 91 individual mussel hemolymph samples (49 apparently healthy, 42 moribund; Appendix A1). Most bacterial genera were observed only rarely, with 39 of the 46 genera present in ≤3 individual mussels and one present in four individuals (Appendix A1). The six most common genera identified were (in order of decreasing abundance): *Yokenella*, *Bacillus*, *Microbacterium*, *Pseudomonas*, *Aeromonas*, and *Acinetobacter*. The most common isolates for healthy mussels were *Bacillus* (27%; 13 of 49), *Microbacterium* (20%; 10 of 49) and *Pseudomonas* (16%; 8 of 49), with all other genera present in ≤4 individuals. The most common isolates for moribund mussels were *Yokenella* (57%; 24 of 42), *Aeromonas* (26%; 11 of 42) and *Bacillus* (14%; 6 of 42), with all other genera present in ≤4 individuals. *Yokenella* was observed in only three healthy individuals, while *Aeromonas* was never observed in healthy individuals. The prevalence of *Yokenella* and *Aeromonas* was significantly higher in moribund versus healthy Pheasantshell (Fisher's exact test; $P < 0.0001$ and $P = 0.0021$, respectively; Table 2). The prevalence of the other four most common genera and the prevalence of samples yielding no bacterial isolates were not significantly different between moribund and healthy Pheasantshell (Table 2).

We observed *Yokenella regensburgei* each year during active mortality events in the Clinch River. Sequences identified as *Y. regensburgei* shared >99.3% similarity and were between 636 and 1375 bp long (Appendix A1). In 2018, *Y. regensburgei* was present in Pheasantshell at Speers Ferry, Sycamore Island, Wallen's Bend and Kyle's Ford, all of which are sites where moribund mussels were observed (Table 1). The bacterium was not isolated from apparently healthy Muckets sampled at these sites or from any samples collected at Artrip, an upstream site where Pheasantshell mass morality has not been observed. All detections of *Y. regensburgei* in Pheasantshell occurred during periods of active mortality, except for one isolation from Wallen's Bend on August 16, 2018, which preceded our first observations of mortality by several weeks.

In 2019, *Y. regensburgei* was isolated only from Pheasantshell but not from six other mussel species; active mortality of Pheasantshell was also observed (Table 1). In 2020, during sampling that targeted moribund mussels, *Y. regenburgei* was isolated from 86% of Pheasantshell at three sites on October 7 and from 89% of Pheasantshell at Sycamore Island on October 20. *Yokenella regensburgei* also was isolated from moribund Mucket and Cumberlandian Combshell on October 20 (Table 1).

Aeromonas was detected only in 2020, when it was present in 11 of 18 moribund mussels collected. In one of these samples, two *Aeromonas* isolates were the only bacteria cultured, while in the remaining 10 samples containing *Aeromonas*, it co-occurred with *Yokenella*.

The prevalence of *Bacillus* spp. did not differ between apparently healthy mussels (44%; 16 of 36) and moribund mussels (17%; 6 of 36; Fisher's exact text, $P = 0.1986$).

DISCUSSION

The consistent association of *Y. regensburgei* with mussel mortality events and moribund mussels was one of the strongest and most conspicuous patterns of bacterial occurrence in our samples. We isolated *Y. regensburgei*, generally with high prevalence, during mortality events in every year of our study, and it was previously isolated during a mortality event in 2017 (Leis et al. 2019). Furthermore, it was rarely identified when mortality events were not occurring or at sites where mortality has not been observed (Artrip). The only occurrence of *Y. regensburgei* outside of a mortality event was its detection in an apparently healthy Pheasantshell on August 16, 2018, at Wallen's Bend; this may have represented an incipient occurrence at the onset of mussel mortality, which was observed a few weeks later at this site.

Yokenella regensburgei, along with predominantly *Hafnia alvei*, was identified from Ebonyshell (*Reginaia ebenus*) during mortality events in the Tennessee River, Alabama (2006 and 2008), and *H. alvei* was previously identified from the Clinch River (Starliper et al. 2008, 2011). *Hafnia alvei* and *Y. regensburgei* both are enteric bacteria that share similar biochemical characteristics, which makes separation of the two species uncertain using traditional laboratory diagnostic systems (Lo et al. 2011). It is unclear whether Starliper et al. (2011) used molecular or biochemical techniques to identify *Y. regensburgei* and *H. alvei*. Furthermore, the Analytical

Profile Index database (API, Biomérieux, Marcy-l'Étoile, France, https://www.biomerieux-diagnostics.com/sites/clinic/files/9308960-002-gb-b-apiweb-booklet.pdf, accessed December 5, 2022) used by Starliper et al. (2008) would have been unable to identify *Y. regensburgei* because that species is not included in the database, but *H. alvei* is included. Due to this limitation, it is possible that *Y. regensburgei* was present at higher prevalence during the Tennessee River mortality event. Our molecular methods should have allowed accurate separation of the two species, but neither we nor Richard et al. (2021) detected *H. alvei* in samples of mussel hemolymph from the Clinch River.

Despite the consistent association of *Y. regensburgei* with mussel mortality events, its role in these events is unclear. At least two scenarios could explain this association. The first is that this bacterium is pathogenic. Preliminary histopathology work does not support pathogenicity (S. Knowles, unpublished data), but additional research is needed to confirm this result. The second is that *Y. regensburgei* opportunistically colonizes mussels that are stressed and of compromised health due to a separate insult, such as exposure to environmental toxins or degraded water quality (see Leis et al. 2019). Richard et al. (2021) found a shift in bacterial communities of mussel hemolymph when mussels exhibit signs of apparent disease (see Richard et al. 2020). An important question is whether there is a relationship between *Y. regensburgei* and Clinch densovirus 1 or other viruses identified from the Clinch River (Richard et al. 2020). For example, are these organisms pathogenic, or does a separate environmental factor (e.g., toxins, thermal stress, changes in water chemistry or algal communities) result in an immunocompromised state that allows unchecked bacterial growth and viral replication? Another important question is whether *Y. regensburgei* is consistently associated with mussel mortality events in other watersheds. Future work evaluating the importance of this bacterium would involve the development of a diagnostic assay to rapidly identify *Y. regensburgei* in mussels, which could also be used to search for potential environmental sources or reservoirs and to better understand the seasonality of its occurrence. Additionally, *in vivo* infection trials are needed to evaluate pathogenicity of *Y. regensburgei* to Pheasantshell and other mussel species, alone and in combination with other factors.

Although the prevalence of *Bacillus* spp. did not differ significantly between healthy and moribund mussels, there was a suggestive trend of higher prevalence in healthy mussels, a trend also noted by Leis et al. (2019). Members of *Bacillus* have several characteristics which, hypothetically, could be considered beneficial to freshwater mussels (see Leis et al. 2019). The lack of a significant difference in the prevalence of *Bacillus* between healthy and moribund mussels could be due to the persistence of these bacteria in moribund mussels after the onset of disease. Additional studies are needed to evaluate potential associations of *Bacillus* spp. with mussel health.

The strong pattern of co-occurrence between *Aeromonas* and *Yokenella* in 2020 is intriguing because it also was observed by Richard et al. (2021) (which included samples from Clinch River mussels in 2017–2018) and Leis et al. (2019) (which included samples from Clinch River mussels in 2017). Both of those studies found high *Aeromonas spp.* and *Yokenella* prevalence associated with moribund mussels from mortality sites, and the two genera often co-occurred in samples. However, Richard et al. (2021) found high *Aeromonas spp.* prevalence in 2018 samples from Clinch River mussels, while we observed *Aeromonas spp.* only in samples collected in 2020. It is possible these discrepancies are due to differences between metagenomics versus culture-based techniques, differences in sampling strategy, or other factors. Gill et al. (2022) observed an increase in potentially pathogenic *Aeromonas* in gut samples from Plain

Pocketbook (*Lampsilis cardium*) after experimental exposure to mixed agricultural contaminants. It is possible that the *Aeromonas* represents late-stage opportunistic infections of individuals previously stressed by pathogens, contaminants, or other stressors. Future field studies and experimental infection challenges would aid our understanding of the role of these bacteria in mussel mortality events.

DATA AVAILABILITY STATEMENT: Data for this study are available at Leis et al. 2022 https://doi.org/10.5066/P9SARYP3, accessed December 5, 2022.

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FIGURE LEGENDS

Figure 1. Locations where hemolymph samples were collected from mussels in the Clinch River. Inset map shows location of the study area in southwestern Virginia and northeastern Tennessee, USA.

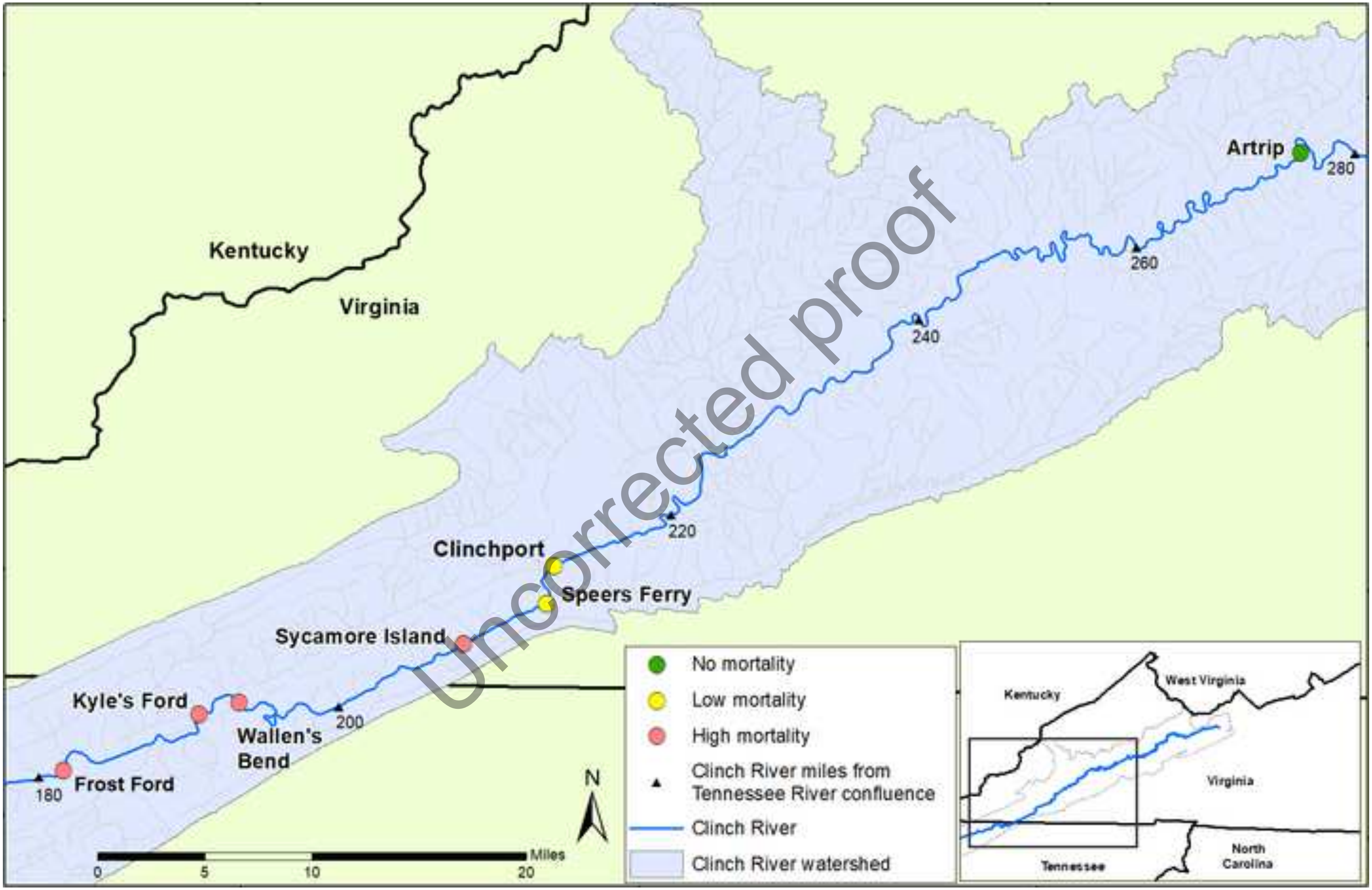
Table 1. Isolation and prevalence of *Yokenella regensburgei* in Clinch River mussels from 2018 to 2020. *A. ligamentina* = *Actinonaias ligamentina*; *A. pectorosa* = *Actinonaias pectorosa*; *A. plicata* = *Amblema plicata*; *P. fasciolaris* = *Ptychobranchus fasciolaris*; *L. fasciola = Lampsilis fasciola; C. tuberculata = Cyclonaias tuberculata; E. brevidens = Epioblasma brevidens; L. ovata = Lampsilis ovata*. *N* = number of individuals sampled.

Sampling month	Location	Mussel species (*N*)	Active mortality observed	Prevalence of *Yokenella*
August 2018	Sycamore Island	*A. pectorosa* (2)	No	0%
		A. ligamentina (2)	No	0%
	Wallen Bend	*A. pectorosa* (2)	No	50%
		A. ligamentina (2)	No	0%
	Kyles Ford	*A. pectorosa* (2)	No	0%
		A. ligamentina (2)	No	0%
	Frost Ford	*A. pectorosa* (2)	No	0%
		A. ligamentina (2)	No	0%
September 2018	Artrip	*A. pectorosa* (3)	No	0%
	Speers Ferry	*A. pectorosa* (3)	No	0%
	Sycamore Island	*A. pectorosa* (5)	Yes	60%
	Wallen Bend	*A. pectorosa* (5)	Yes	40%
	Kyles Ford	*A. pectorosa* (6)	Yes	33%
October 2018	Artrip	*A. pectorosa* (3)	No	0%
	Spears Ferry	*A. pectorosa* (4)	Yes	25%
		A. ligamentina (1)	No	0%
	Sycamore Island	*A. pectorosa* (6)	Yes	0%
		A. ligamentina (1)	No	0%
	Kyles Ford	*A. pectorosa* (6)	Yes	17%
October 2019	Sycamore Island	*A. pectorosa* (2)	Yes	50%
		A. ligamentina (4)	No	0%
		L. ovata (2)	No	0%
		A. plicata (2)	No	0%
		P. fasciolaris (1)	No	0%
		L. fasciola (2)	No	0%
		C. tuberculata (1)	No	0%
October 2020	Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa* (7)	Yes	86%
	Sycamore Island	*A. pectorosa* (9)	Yes	89%
		A. ligamentina (1)	Yes	100%
		E. brevidens (1)	Yes	100%

Table 2. Prevalence of the six most common bacterial genera and samples yielding no bacterial isolates in moribund and healthy Pheasantshell (*Actinonaias pectorosa*) mussels collected from 2018 to 2020. An asterisk (*) indicates statistically significant differences in prevalence between healthy and moribund mussels (Fisher's exact test, $P \leq 0.002$).

	*Yokenella**	*Aeromonas**	*Bacillus*	*Pseudomonas*	*Microbacterium*	*Acinetobacter*	No isolates
Healthy (N = 33)	9.1%	0.0%	24.2%	21.2%	21.2%	9.1%	27.3%
Moribund (N = 34)	64.7%	26.5%	11.8%	11.8%	8.8%	8.8%	11.8%

Figure 1


Kentucky
Virginia
Artrip
280
260
240
220
Clinchport
Speers Ferry
Sycamore Island
Kyle's Ford
Wallen's Bend
200
Frost Ford
180
N
0
5
10
20
Miles
● No mortality
● Low mortality
● High mortality
▲ Clinch River miles from Tennessee River confluence
Clinch River
Clinch River watershed
Kentucky
West Virginia
Virginia
Tennessee
North Carolina

Appendix A1**.** Bacteria identified from mussels in the Clinch River. Isolate refers to the number assigned to the mussel sample (number) and the isolate identification (letter). Location refers to the sampling locations as defined on Figure 1. Mussel species abbreviations are as follows: *A. ligamentina = Actinonaias ligamentina*; *A. pectorosa = Actinonaias pectorosa*; *A. plicata = Amblema plicata; P. fasciolaris = Ptychobranchus fasciolaris; L. fasciola = Lampsilis fasciola; C. tuberculata = Cyclonaias tuberculata; E. brevidens = Epioblasma brevidens; L. ovata = Lampsilis ovata.* Genus and species refer to the identifications of bacteria made through BLAST searches in the NCBI database (see text). Percent similarity represents the similarity of the isolate sequence to sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed August 19, 2022). Length refers to the number of contiguous base pairs in the sequence for each isolate. Accession number refers to the top match or matches identified through the BLAST search of the NCBI database.

Isolate	Location	Mussel species	Genus	Species	Percent similarity	Length	Accession #
8/16/2018							
1B	Sycamore Island	*A. ligamentina*	*Microbacterium*	*testaceum*	100	901	MK618600.1
26A	Wallen's Bend	*A. ligamentina*	*Exiguobacterium*	*antarcticum/acetylicum*	100	817	MK478815.1 / MH719376.1
26B	Wallen's Bend	*A. ligamentina*	Unidentifiable				-
26C	Wallen's Bend	*A. ligamentina*	*Bacillus*	*mycoides*	100	882	CP037992.1
26D	Wallen's Bend	*A. ligamentina*	*Bacillus*	*pumilus/zhangzhouensis*	100	890	MK696261.1 / MK131335.1
27A	Wallen's Bend	*A. ligamentina*	*Bacillus*	*megaterium/aryabhattai*	100	893	MK027252.1 / MK618612.1
45A	Kyle's Ford	*A. ligamentina*	*Bacillus*	*cereus/wiedmannii/thuringiensis*	100	778	MK696545.1 /MK696254.1 / MK696253.1
45C	Kyle's Ford	*A. ligamentina*	*Pseudoxanthomonas*	*japonensis/mexicana*	99.6	842	KX588601.1 / KF135463.1
45D	Kyle's Ford	*A. ligamentina*	*Novosphingobium*	*barchaimii*	99.8	1212	KM019861.1

45E	Kyle’s Ford	*A. ligamentina*	*Fictibacillus*	*phosphorivorans/nanhaiensis*	100	895	MG547923.1 / MG049786.1
45F	Kyle’s Ford	*A. ligamentina*	*Bacillus*	*cereus/mycoides/pseudomycoides/anthracis*	99.7	766	MK285635.1 / MG694513.1 / MG198676.1 / MH261153.1
45G	Kyle’s Ford	*A. ligamentina*	*Bacillus*	*mycoides*	99.9	816	MK719967.1
45I	Kyle’s Ford	*A. ligamentina*	*Pseudomonas*	*rhodesiae*	100	907	MG571697.1
47A	Kyle’s Ford	*A. ligamentina*	*Acinetobacter*	*guillouiae/lwoffii*	100	899	MK070050.1 / MH930396.1
47B	Kyle’s Ford	*A. ligamentina*	*Acinetobacter*	*guillouiae/johnsonii*	99.9	904	MH144279.1 / MG788346.1
47C	Kyle’s Ford	*A. ligamentina*	*Curtobacterium*	sp.	99.2	587	MK704290.1
47D	Kyle’s Ford	*A. ligamentina*	*Microbacterium*	*oxydans*	99.7	906	KX083528.1
1D	Sycamore Island	*A. ligamentina*	*Streptomyces*	sp.	99.8	908	MH053444.1
2A	Sycamore Island	*A. ligamentina*	*Bacillus*	*megaterium*	100	781	MH071287.1
2B	Sycamore Island	*A. ligamentina*	*Microbacterium*	*marinum*	99.8	901	MF373495.1
2D	Sycamore Island	*A. ligamentina*	*Agrococcus*	*terreus*	99.8	790	MH934923.1
10A	Sycamore Island	*A. pectorosa*	*Arthrobacter*	sp.	99.9	707	MG860243.1
10B	Sycamore Island	*A. pectorosa*	*Pseudarthrobacter*	*defluvii/siccitolerans/scleromae*	100	708	MH910272.1 / MF681877.1 / KY496253.1
13A	Sycamore Island	*A. pectorosa*	*Microbacterium*	sp.	90.4	463	HM352378.1

13B	Sycamore Island	*A. pectorosa*	*Bacillus*	*pumilus/zhangzhouensis*	100	1413	MH045860.1 / MG651573.1
17A	Wallen's Bend	*A. pectorosa*	*Pantoea*	sp.	99.9	900	MH769349.1
17B	Wallen's Bend	*A. pectorosa*	*Yokenella*	*regensburgei*	99.7	705	KR537290.1
17D	Wallen's Bend	*A. pectorosa*	*Microbacterium*	*oleivorans*	99.9	919	HF952706.1
17E	Wallen's Bend	*A. pectorosa*	*Yokenella*	*regensburgei*	100	788	LC383918.1
18A	Wallen's Bend	*A. pectorosa*	*Bacillus*	*pumilus/zhangzhouensis*	100	810	MK696262.1 / MK131335.1
18B	Wallen's Bend	*A. pectorosa*	*Curtobacterium*	*luteum/citreum*	100	845	MF959445.1 / LT986192.1
18C	Wallen's Bend	*A. pectorosa*	*Bacillus*	*pumilus/zhangzhouensis*	100	890	MK696261.1 / MK131335.1
33A	Kyle's Ford	*A. pectorosa*	*Acinetobacter*	*soli*	100	823	MK241870.1
34A	Kyle's Ford	*A. pectorosa*	*Bacillus*	*cereus/thuringiensis*	100	864	KT241012.1 / FJ463780.1
34C	Kyle's Ford	*A. pectorosa*	*Pantoea*	*dispersa*	100	872	MF681826.1
34C	Kyle's Ford	*A. pectorosa*	*Erwinia*	sp.	100	872	MG681219.1
34D	Kyle's Ford	*A. pectorosa*	*Bacillus*	*cereus/bingmayongensis/ pseudomycoides*	100	875	MK285635.1/ MK120869.1 / MH578628.1
34E	Kyle's Ford	*A. pectorosa*	*Arthrobacter*	*pascens/globiformis*	99.9	902	HQ530516.1 / HQ455821.1
8/23/2018							
49A	Frost Ford	*A. ligamentina*	*Bacillus*	*megaterium*	99.3	667	LC269278.1

49B	Frost Ford	*A. ligamentina*	*Bacillus*	*toyonensis/cereus/thuringiensis*	100	816	MK611646.1 / MK592620.1 / MK583935.1
49C	Frost Ford	*A. ligamentina*	*Bacillus*	*cereus/mycoides/pseudomycoides*	99.9	1368	MK285635.1 / MG694513.1 / MG198676.1
49D	Frost Ford	*A. ligamentina*	*Bacillus*	*idriensis*	99.4	964	MK240437.1
49E	Frost Ford	*A. ligamentina*	*Lysinibacillus*	*sphaericus/xylanilyticus/boronitolerans*	99.9	893	MG928532.1 / MG905851.1 / MF111565.1
49F	Frost Ford	*A. ligamentina*	*Bacillus*	*thuringiensis*	98.5	1103	EU161995.1
49G	Frost Ford	*A. ligamentina*	*Bacillus*	*pumilus*	99.8	509	MK521054.1
49H	Frost Ford	*A. ligamentina*	*Bacillus*	*mycoides*	100	743	MK217080.1
49I	Frost Ford	*A. ligamentina*	*Bacillus*	*altitudinis/pumilus/stratosphericus*	99.1	980	MK521060.1 / MH118525.1 / MH910298.1
63A	Frost Ford	*A. pectorosa*	*Bacillus*	*thuringiensis*	99.3	1049	JF421247.1
63B	Frost Ford	*A. pectorosa*	*Bacillus*	*thuringiensis*	98.5	1104	MK491010.1
63C	Frost Ford	*A. pectorosa*	*Bacillus*	*megaterium/aryabhattai*	99.6	826	MH244337.1 / MH177254.1
63D	Frost Ford	*A. pectorosa*	*Lysinibacillus*	*parviboronicapiens/sphaericus*	99.8	889	KY038722.1 / MF111586.1
63E	Frost Ford	*A. pectorosa*	*Lysinibacillus*	*sphaericus/fusiformis*	98.3	514	JQ835283.1 / JQ834699.1
63F	Frost Ford	*A. pectorosa*	*Exiguobacterium*	*acetylicum/indicum*	99.7	732	MK606065.1 / MK294298.1
63G	Frost Ford	*A. pectorosa*	*Nocardiopsis*	*alba*	100	802	MH333283.1

9/26/2018

15A	Speers Ferry	*A. pectorosa*	*Acinetobacter*	*radioresistens*	100	908	MK127547.1
15B	Speers Ferry	*A. pectorosa*	*Citricoccus/Micrococcus*	sp.	100	773	MF063312.1 / JQ072036.1
15C	Speers Ferry	*A. pectorosa*	*Pseudomonas*	*monteilii*	99.7	310	MH484604.1
15D	Speers Ferry	*A. pectorosa*	*Pseudomonas*	*putida*	100	669	MK064155.1
17A	Speers Ferry	*A. pectorosa*	*Bacillus*	*cereus/bingmayongensis/ pseudomycoides*	99.9	878	MK285635.1 / MK120869.1 / MH578628.1
17B	Speers Ferry	*A. pectorosa*	*Novosphingobium*	*lindaniclasticum*	99.9	816	MK318596.1
17C	Speers Ferry	*A. pectorosa*	*Bacillus*	*pseudomycoides*	100	1398	MG905900.1
17D	Speers Ferry	*A. pectorosa*	*Pseudomonas*	*putida*	98.6	808	MK064155.1
26A	Sycamore Island	*A. pectorosa*	*Gordonia*	*hongkongensis/terrae*	100	1139	MK277458.1 / MH518251.1
26B	Sycamore Island	*A. pectorosa*	*Pontibacter*	sp.	95.6	158	KY814729.1
32A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	808	LC383918.1
36A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.8	939	LC383918.1
37A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	100	882	LC383918.1
37B	Sycamore Island	*A. pectorosa*	*Gordonia*	*hongkongensis/terrae*	100	1163	MK277458.1 / MH518251.1

37C	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	100	872	LC383918.1
44A	Wallen's Bend	*A. pectorosa*	*Hannaella*	*oryzae*	99.3	425	NG_063522.1
44B	Wallen's Bend	*A. pectorosa*	*Sphingobium*	sp.	99.8	1345	DQ413165.1
44C	Wallen's Bend	*A. pectorosa*	*Microbacterium*	*chocolatum*	100	639	MH748626.1
44D	Wallen's Bend	*A. pectorosa*	*Sphingobium*	*yanoikuyae*	100	603	CP033230.1
46A	Wallen's Bend	*A. pectorosa*	*Bacillus*	*toyonensis*	99.8	1225	KU179349.1
46B	Wallen's Bend	*A. pectorosa*	*Microbacterium*	*flavenscens*	100	692	JQ958839.1
46C	Wallen's Bend	*A. pectorosa*	*Bacillus*	*cereus/wiedmannii/thuringiensis*	100	718	MK696545.1 / MK696254.1 / MK696253.1
47A	Wallen's Bend	*A. pectorosa*	*Yokenella*	*regensburgei*	99.8	794	LC383918.1
49A	Wallen's Bend	*A. pectorosa*	*Oceanobacillus*	*massiliensis*	99.9	887	HQ586893.1
49B	Wallen's Bend	*A. pectorosa*	*Microbacterium*	*oxydans/foliorum*	99.5	894	MK696389.1 / MK696388.1
49C	Wallen's Bend	*A. pectorosa*	*Pseudomonas*	*putida/guariconensis*	100	794	MK534000.1 / MK318649.1
50A	Wallen's Bend	*A. pectorosa*	*Yokenella*	*regensburgei*	100	694	LC383918.1
50B	Wallen's Bend	*A. pectorosa*	*Oceanobacillus*	*massiliensis*	100	766	KU727149.1

50C	Wallen’s Bend	*A. pectorosa*	*Pseudomonas*	*cichorii*	99.9	878	KU923374.1
59A	Kyle's Ford	*A. pectorosa*	*Yokenella*	*regensburgei*	99.3	947	KR537290.1
59B	Kyle's Ford	*A. pectorosa*	*Chromobacterium*	*haemolyticum*	99.4	869	LT628074.1
63A	Kyle's Ford	*A. pectorosa*	*Pseudomonas*	*monteilii/plecoglossicida/ putida*	100	863	MH997643.1 / MH997642.1 / MH997641.1
63B	Kyle's Ford	*A. pectorosa*	*Acinetobacter*	*schindleri*	100	1399	CP025618.2
63C	Kyle's Ford	*A. pectorosa*	*Pseudomonas*	*parafulva*	99.9	1398	CP009747.1
63D	Kyle's Ford	*A. pectorosa*	*Pseudomonas*	*monteilii/putida*	100	1384	KU862315.1 / KT259326.1
64A	Kyle's Ford	*A. pectorosa*	*Acinetobacter*	*schindleri*	100	1201	MG461629.1
64B	Kyle's Ford	*A. pectorosa*	*Acinetobacter*	sp.	99.8	829	MG517420.1
64C	Kyle's Ford	*A. pectorosa*	*Serinibacter*	sp.	98.9	368	LC203065.1
66A	Kyle's Ford	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	891	KT260720.1
66B	Kyle's Ford	*A. pectorosa*	*Rhizobium/ Agrobacterium*	sp./*larrymoorei*	99.2	1351	JQ660121.1 / JN084151.1
67A	Kyle's Ford	*A. pectorosa*	*Microbacterium*	*proteolyticum*	99.9	903	MK281612.1
70A	Kyle's Ford	*A. pectorosa*	*Acinetobacter*	sp.	99.9	1385	MG517420.1
70B	Kyle's Ford	*A. pectorosa*	*Pseudomonas*	sp.	99.6	1333	MK680062.1
10/25/2018							
006A	Artrip	*A. pectorosa*	*Knoellia*	*flava*	100	640	MG205530.1
016A	Speers Ferry	*A. pectorosa*	*Cellulosimicrobium*	*cellulans*	99.9	915	MH718838.1
016B	Speers Ferry	*A. pectorosa*	*Massilia*	*timonae*	100	901	MF592282.1
016C	Speers Ferry	*A. pectorosa*	*Bacillus*	*pumilus/aerius/stratosphericus/altitudinis*	99.9	885	MH261170.1/MK629822.1 /

016D	Speers Ferry	*A. pectorosa*	*Bacillus*	*safensis/altitudinis/pumilus*	100	1412	MH261171.1 /MH261156.1 MK285608.1/MK241863.1 / MG597491.1
021A	Speers Ferry	*A. ligamentina*	*Cutibacterium*	*acnes*	100	418	CP033842.1
023A	Speers Ferry	*A. pectorosa*	*Bacillus*	*zhangzhouensis/pumilus*	100	881	MK583949.1 / MH819709.1
027A	Speers Ferry	*A. pectorosa*	*Actinotalea*	*solisilvae*	100	832	NR_159882.1
028A	Speers Ferry	*A. pectorosa*	*Yokenella*	*regensburgei*	100	850	LC383918.1
028B	Speers Ferry	*A. pectorosa*	*Bacillus*	*aerius/altitudinis/pumilus*	100	839	MG996801.1 / MK521068.1 / MK521063.1
028gA	Speers Ferry	*A. pectorosa*	*Yokenella*	*regensburgei*	100	831	LC383918.1
044A	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*kribbensis/protegens*	100	768	MK240439.1 / LT628144.1
044B	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*kribbensis/reinekei/syringae/fluorescens*	100	819	MK240439.1 / MK392138.1 / CP024646.1 / CP022313.1
049A	Sycamore Island	*A. ligamentina*	Unidentifiable				-
049B	Sycamore Island	*A. ligamentina*	*Knoellia*	*flava*	99.9	845	MG205530.1
049C	Sycamore Island	*A. ligamentina*	*Pseudarthrobacter*	*oxydans/siccitolerans*	100	812	MG694475.1 / MF681913.1

049D	Sycamore Island	*A. ligamentina*	*Microbacterium*	*phyllosphaerae*	98.7	832	KC355288.1
049E	Sycamore Island	*A. ligamentina*	*Bacillus*	*pumilus*	99	799	MH261008.1
051A	Sycamore Island	*A. pectorosa*	*Bacillus*	*cereus/bingmayongensis/pseudomycoides/mycoides/subtilis*	100	793	MK285635.1 / MK120869.1 / MH578628.1 / MH422001.1 / MH422001.1
051B	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*putida*	99.6	550	MH298490.1
051C	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*taiwanensis/putida/parafulva*	100	686	MK598329.1 / CP030750.1 / MH304303.1
057A	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*helmanticensis*	100	664	MK070159.1
057B	Sycamore Island	*A. pectorosa*	*Bacillus*	*gibsonii*	100	759	MH910172.1
068A	Kyle's Ford	*A. pectorosa*	*Knoellia*	*flava*	99.7	770	MG205530.1
068B	Kyle's Ford	*A. pectorosa*	*Rhodococcus*	sp.	100	790	KY922741.1
068C	Kyle's Ford	*A. pectorosa*	*Arthrobacter*	*halodurans/aurescens*	100	705	MK424278.1 / KR611860.1
068D	Kyle's Ford	*A. pectorosa*	*Rhodococcus*	*gingshengii/erythropolis*	100	886	MH938043.1 / MK371078.1
068E	Kyle's Ford	*A. pectorosa*	*Microbacterium*	sp.	99.4	1392	JQ977333.1
069A	Kyle's Ford	*A. pectorosa*	*Microbacterium*	*aureliae*	99.7	992	MK226317.1
069B	Kyle's Ford	*A. pectorosa*	*Terrabacter*	*terrigena/koreensis/tumescens*	100	732	MF681978.1 / NR_134212.1 / JQ342911.1

073A	Kyle's Ford	*A. pectorosa*	*Curtobacterium*	*oceanosedimentum/luteum/herbarum/citreum*	100	760	MK618608.1 / MK618607.1 / MK618606.1 / MK618605.1
073B	Kyle's Ford	*A. pectorosa*	*Microbacterium*	*lemovicicum*	100	721	CP031423.1
074A	Kyle's Ford	*A. pectorosa*	*Leucobacter*	*tardus*	99.7	770	NR_042694.1
077A	Kyle's Ford	*A. pectorosa*	*Yokenella*	*regensburgei*	100	824	LC383918.1
077B	Kyle's Ford	*A. pectorosa*	*Plantibacter*	*flavus*	99.9	1230	LN774386.1
10/4/2019							
16A	Sycamore Island	*P. fasciolaris*	*Lysinibacillus*	*fusiformis*	99.9	917	MT605500.1
19A	Sycamore Island	*L. fasciola*	*Curtobacterium*	*ammoniigenes*	99.9	809	KP296215.1
21A	Sycamore Island	*A. ligamentina*	*Falsirhodobacter*	*halotolerans*	100	701	LN774250.1
26A	Sycamore Island	*A. plicata*	*Bacillus*	*paramycoides*	100	390	MT647568.1
26B	Sycamore Island	*A. plicata*	*Bacillus*	*cereus*	100	934	LR215149.1
28A	Sycamore Island	*A. pectorosa*	*Oerskovia*	*paurometabola*	100	1266	KX034798.1
28B	Sycamore Island	*A. pectorosa*	*Bacillus*	*aryabhattai*	99	1336	MK519060.1
28C	Sycamore Island	*A. pectorosa*	*Agrococcus*	*terreus*	99.9	856	MH934923.1
28D	Sycamore Island	*A. pectorosa*	*Microbacterium*	*paludicola*	99.8	536	MT733951.1
29A	Sycamore Island	*A. pectorosa*	*Acinetobacter*	sp.	75	803	AY922105.1

29B	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.6	1312	MT505131.1
29C	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.8	1345	MT505131.1
31A	Sycamore Island	*L. ovata*	*Staphylococcus*	*hominis* subsp. *novobiosepticus*	100	1262	MT585539.1
33A	Sycamore Island	*A. plicata*	*Micrococcus*	*yunnanensis*	100	855	MT033093.1
33B	Sycamore Island	*A. plicata*	*Staphylococcus*	*hominis* subsp. *novobiosepticus*	100	840	MT544813.1
33C	Sycamore Island	*A. plicata*	*Agrococcus*	*terreus*	99.5	913	JN585711.1
33D	Sycamore Island	*A. plicata*	*Micrococcus*	*yunnanensis*	100	836	LT978429.1
33E	Sycamore Island	*A. plicata*	*Staphylococcus*	*epidermidis*	99.4	883	KM972394.1
35A	Sycamore Island	*A. ligamentina*	*Micrococcus*	*yunnanensis*	99.9	828	KF758820.1
40A	Sycamore Island	*A. ligamentina*	*Exiguobacterium*	*aurantiacum*	99.4	837	MT225757.1
40B	Sycamore Island	*A. ligamentina*	*Staphylococcus*	*epidermidis*	100	1348	MT613456.1
40C	Sycamore Island	*A. ligamentina*	*Citricoccus*	*zhacaiensis*	100	1139	MG025801.1
40D	Sycamore Island	*A. ligamentina*	*Massilia*	*oculi*	99.9	1308	CP029343.1
10/7/2020							
1A	Speers Ferry/	*A. pectorosa*	*Yokenella*	*regensburgei*	99.8	1335	MT505131.1

1B	Sycamore Island/ Clinchport Speers Ferry/	*A. pectorosa*	*Kocuria*	*rhizophila*	100	847	MT377849.1
2A	Sycamore Island/ Clinchport Speers Ferry/	*A. pectorosa*	*Aeromonas*	*veronii*	100	910	MT384380.1
2B	Sycamore Island/ Clinchport Speers Ferry/	*A. pectorosa*	*Aeromonas*	*allosaccharophila*	100	887	MN216261.1
3A	Sycamore Island/ Clinchport Speers Ferry/	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	1372	MT505131.1
3B	Sycamore Island/ Clinchport Speers Ferry/	*A. pectorosa*	*Yokenella*	*regensburgei*	100	819	MT505139.1
4A	Sycamore Island/ Clinchport Speers Ferry/	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	1342	MT505131.1

	Sycamore Island/ Clinchport						
4B	Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa*	*Aeromonas*	*veronii*	100	1375	MT384380.1
5A	Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa*	*Chryseobacterium*	sp.	96.8	704	KU360139.1
5B	Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa*	*Yokenella*	*regensburgei*	99.8	1372	MT505131.1
5C	Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa*	*Yokenella*	*regensburgei*	100	904	MT505139.1
6A	Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	1362	MT505131.1
6B	Speers Ferry/	*A. pectorosa*	*Yokenella*	*regensburgei*	99.7	1362	MT505131.1

7A	Sycamore Island/ Clinchport Speers Ferry/ Sycamore Island/ Clinchport	*A. pectorosa*	*Yokenella*	*regensburgei*	99.7	1375	MT505131.1
10/20/2020							
ALI-1A	Sycamore Island	*A. ligamentina*	*Yokenella*	*regensburgei*	99.9	698	MT505139.1
ALI-1B	Sycamore Island	*A. ligamentina*	*Aeromonas*	*hydrophila*	100	387	MK880338.1
APE-1A	Sycamore Island	*A. pectorosa*	*Aeromonas*	*hydrophila*	100	549	MK880338.1
APE-1B	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	791	MT505139.1
APE-2A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	100	636	MT505139.1
APE-2B	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*poae*	100	758	MT631989.1
APE-2C	Sycamore Island	*A. pectorosa*	*Aeromonas*	*veronii*	100	760	MT384380.1
APE-3A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	100	700	MT505139.1
APE-3B	Sycamore Island	*A. pectorosa*	*Aeromonas*	*caviae*	100	578	MN481052.1
APE-4A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.7	762	MT505131.1

APE-4B	Sycamore Island	*A. pectorosa*	*Aeromonas*	*veronii*	97.7	303	CP012504.1
APE-6A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.7	653	MT505131.1
APE-6B	Sycamore Island	*A. pectorosa*	*Stentrophomonas*	*pavanii*	95.6	484	MH810095.1
APE-6C	Sycamore Island	*A. pectorosa*	*Pseudomonas*	*putida*	100	811	CP050951.1
APE-7A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.8	1063	MT505131.1
APE-7B	Sycamore Island	*A. pectorosa*	*Aeromonas*	*hydrophila*	99.8	493	MK880338.1
APE-8A	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	100	751	MT505139.1
APE-8B	Sycamore Island	*A. pectorosa*	*Aeromonas*	*aquatilis*	100	765	LT630765.1
APE-9A	Sycamore Island	*A. pectorosa*	*Aeromonas*	*hydrophila*	100	452	MK880338.1
APE-9B	Sycamore Island	*A. pectorosa*	*Yokenella*	*regensburgei*	99.9	794	MT505139.1
EBR-1A	Sycamore Island	*E. brevidens*	*Yokenella*	*regensburgei*	88	673	MT505131.1
EBR-1B	Sycamore Island	*E. brevidens*	*Aeromonas*	*caviae*	100	467	MN481052.1
EBR-1C	Sycamore Island	*E. brevidens*	*Flavobacterium*	*tructae*	99.9	759	NR_133749.1