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BACTERIAL PATHOGEN CONTAGION STUDIES AMONG FRESHWATER BIVALVES AND SALMONID FISHES

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ABSTRACT A part of the conservation efforts of native freshwater bivalves is a relocation program whereby animals are collected and moved to a safe refuge for maintenance and propagation. With the rearing of two different hosts, mussels and fish, on the same facility there is a question of the possibility for contagion of pathogens. The studies presented here are part of a continuing effort to address the concerns of contagion. Freshwater bivalves collected throughout the 1997 season were cultured for fish pathogens. Counts of total bacteria on cytophaga medium ranged between 1.07×10^5 and 4.99×10^5 cfu/g of mussel soft tissues. The predominate groups of bacteria were motile *Aeromonas* spp. and *Pseudomonas* spp., both of which include members that are opportunistic pathogens to salmonid fishes. No primary fish pathogens were cultured; however, cells with correct morphology for *Renibacterium salmoninarum*, cause of bacterial kidney disease, were detected from mussel soft tissues in all six trials using the direct fluorescent antibody test. Groups of mussels were subjected to 24-h waterborne challenges using bacteria cultured from healthy fish; no mortality occurred to any of the animals. Another group exposed to the fish pathogen *Aeromonas salmonicida* also showed no effects of the challenge; however, susceptible fish became infected and died after the fish were added to cohabit with this group of mussels.

KEY WORDS: Freshwater bivalves, salmonid, pathogen, disease, contagion, bacteria, cohabitation

INTRODUCTION

Over 70% of the approximately 300 species and subspecies of freshwater bivalves (*Unionidae*) native to North America are categorized as endangered, threatened, or of special concern (Williams et al. 1993). Bivalves are particularly susceptible to impacts from environmental changes, not only because they are sessile, filter-feeding animals, and adverse effects placed on their intermediate fish hosts that are necessary for glochidia development also may indirectly affect them.

A number of factors have been documented as contributors to the decline in numbers of freshwater bivalves. Human disturbance in the terrestrial environment, such as agriculture and development, result in siltation that may impair growth and respiration and lead to suffocation (Ellis 1936, Kat 1982). Also, habitat alteration from dredging or dam construction can result in changes in flow or temperature and also affect movement of the intermediate fish host (Fuller 1974; Keller and Zam 1990). In recent years, the zebra mussel (*Dreissena polymorpha*) has become a major threat to native bivalve populations in large river systems (Herbert et al. 1991; Nalepa 1994). Zebra mussels are able to proliferate and compete very favorably against native animals (Gillis and Mackie 1994). Their high spatial tolerance allows them to colonize in great numbers, leading to mortality of native bivalves by impeding feeding and respiration (Griffiths et al. 1991, Haag et al. 1993, Leach 1993, Mackie 1991). In 1996, densities of zebra mussels in the lower Ohio River (near river mile 814) exceeded 14,000 per square meter, and mortality to the native populations exceeded 30% (P. Morrison, Ohio River Islands National Wildlife Refuge, U.S. Fish and Wildlife Service, pers. comm.).

In the mid 1990s, the U.S. Fish and Wildlife Service (USFWS) along with other federal, state, and private partners initiated conservation efforts. One such program was to isolate native animals from impending zebra mussel infestation from selected large rivers. Under this program, individuals are collected and relocated to safe refugia for maintenance and propagation with the hope of future successful reintroduction. These refugia, which are free of zebra mussels, include salmonid fish-rearing hatcheries, which may culture such species as rainbow (*Oncorhynchus mykiss*) and brook (*Salvelinus fontinalis*) trout. With the rearing of these two hosts (mussels and fish) on the same facility, there exists the possibility for contagion of pathogens either by shared water or via contaminated equipment such as boots, nets, and buckets. The question of contagion is particularly relevant, because one of the two hosts (freshwater mussels) is originating from a natural environment and might be exposed to pathogens of both mussels and fish that could be introduced to a hatchery along with their relocation. All of the recognized primary bacterial pathogens of salmonids involve, to varying degrees of significance, horizontal transmission, and, hence, there is a potential to develop disease in fish that might be exposed to pathogens via contamination or by some vector. It can be speculated that bivalves may serve as a pathogen vector. On the other hand, it is not known if certain microbial flora of healthy resident fish might pose a disease threat to mussels once they are exposed to the pathogen, and they are reared in intensive culture situations. There are no single pathogens described that produce disease in both salmonid fishes and freshwater bivalves. There is a wealth of knowledge on diseases to salmonids, but there are few reports of diseases and epizootics that occur in native freshwater bivalves. However, it has been demonstrated that freshwater mussels may serve as a vector for fish pathogens, because *Flavobacterium columnare*, the cause of columnaris disease, was isolated from a single *Amblema plicata* that was collected from the Ohio river (Starliper et al. 1998).

Presented here are studies that are part of a continuing effort to evaluate the potential for contagion of bacterial pathogens. We report on efforts to isolate salmonid bacterial pathogens from freshwater bivalves that were collected from their natural environment (without quarantine), and to produce disease or mortality

experimentally in bivalves using the representative bacterial flora from healthy fish and two fish bacterial pathogens.

MATERIALS AND METHODS

Freshwater Bivalves and Fish

All bivalves were collected by brailing between August and November 1997, and this duration was representative of that year's sampling season. Duration of sampling seasons varies from one year to another, depending upon water temperature and when animals emerge from the river bottom. The collection site was between river miles 175 and 177 at Muskingum Island of the Ohio River, which is near Boaz, Wood County, West Virginia. This region of the river is not easily accessible by land, and the only public use is pleasure boat traffic. The site supports 28 species of freshwater mussels, including two federally listed species (*Lampsillis abrupta* and *Cyprogenia stegaria*). The island and its underwater acreage, including the mussel beds, are protected from development, because it is within the Ohio River Islands National Wildlife Refuge. No harvesting or collecting of mussels is allowed on the refuge except for scientific or management purposes.

Bivalves used to evaluate the presence of fish pathogenic bacteria were collected at 2 to 3 week intervals during this season. Twenty animals were collected on each of six dates (trials 1–6, respectively), except for trial 6, when only eight were collected because of low water temperature. Upon collection, animals were kept cool and moist and shipped overnight by commercial carrier to our laboratory for bacteriological analysis the following day. They were not placed in other water in the interim between collection and analysis. Species and physical data for the bivalves represented in this study are presented in Table 1. Also, the distribution data for *Amblema plicata*, which was the species most frequently collected during the season, are given in Table 2. Ohio River surface water temperatures (°C) were recorded at the time and collection location for each trial.

Two hundred animals, representing six species, were used as subjects for bacterial challenges with flora isolated from healthy fish: *A. plicata* (123), *Quadrula metanevra* (33), *Q. quadrula* (21), *Obliquaria reflexa* (10), *Q. pustulosa* (9), and *Pleurobema cordatum* (4). Physical data of the animals used in the challenge studies are not presented; however, mean values were very similar to those animals used for fish pathogen isolation, which are presented in Tables 1 and 2. These animals were quarantined for 30 days at the Ohio River Islands National Wildlife Refuge (Parkersburg, WV) to eliminate any zebra mussels (Gatenby et al. 1998). They were then transported to the Leetown Science Center, where they were acclimated to (over 2–3 hours) and maintained in pathogen-free spring water (12 °C) delivered via a flow through system.

The fish added to the tanks to cohabit with mussels following their (mussels) challenge were the Nashua strain of brook trout (*Salvelinus fontinalis*), about 50 g each, and the Shasta strain of rainbow trout (*Oncorhynchus mykiss*), also about 50 g each. The fish were certified as fish pathogen-free through biyearly fish health inspections by the U.S. Fish and Wildlife Service's Fish Health Unit, Lamar, Pennsylvania. The fish were maintained in the same water as described for the mussels; all *in vivo* studies were also done using the same water source.

Collection and Processing of Tissues from Bivalves

Tissues were collected and prepared using a procedure similar to that developed by Starliper et al. (1998). The external shell surface of each animal was cleaned by brushing with 200-ppm chlorine, rinsed in deionized water, and allowed to dry. An oyster knife was used to pry open the shell valves, and the adductor muscles were cut. All soft tissues were excised from the shells and were separated into two samples. One consisted primarily of digestive tract tissues, denoted "gut" (e.g., stomach, intestine), while all remaining soft tissues, denoted "OT" or other tissues, (e.g., mantle, gill, foot, lymph) comprised the second. The gut sample was removed first, and effort was made not to contaminate the remaining tissues with bacterial contents from the gut. Soft tissue samples were placed in preweighed, sterile stomacher bags. The bags with tissues were weighed, and the difference of the two weights was the weight of the tissue sample.

Isolation and Growth of Bacteria

For bacterial isolation, the tissue samples were diluted in sterile 0.1% peptone- 0.05% yeast extract (PEP-YE). To each sample, a volume (mL) of PEP-YE equal to the tissue weight (g) was added yielding a 1:2 dilution of tissues. This was homogenized for 120 seconds using a Model 80 stomacher (Seward Medical, London SE1 1PP, UK). A portion of the supernatant from each homogenate was transferred to a sterile tube for ease of handling and three serial tenfold dilutions were prepared from this, also in PEP-YE. Four drops (0.025 mL each) from all dilutions were applied to the surface of each bacteriological medium. After the drops had been adsorbed, plates were incubated at the appropriate temperature and duration specified in the appropriate reference materials (see Media employed). Following incubation, colonies were enumerated by counting the lowest dilution with single colonies, and this was converted to a standard colony forming units per g of tissue (cfu/g). For the selective and/or differential media used for specific

TABLE 1.

Mean values for physical data on freshwater bivalves from the Ohio River assayed for presence of bacterial fish pathogens. Animals were collected at six different times (trials 1–6) during the 1997 collection season.

Species	No.	Length (mm)	Width (mm)	Weight (g)	% Soft Tissue[a]	Gut/OT %[b]
Amblema plicata	88	100.9	74.4	238.5	12.5	61/39
Quadrula quadrula	7	74.1	58.4	123.0	10.5	62/38
Obliquaria reflexa	5	58.8	46.8	75.2	11.2	64/36
Pleurobema cordatum	3	77.0	65.3	142.9	11.7	57/43
Quadrula metanevra	3	67.3	57.0	107.6	12.0	61/39
Quadrula pustulosa	1	57.0	54.0	63.1	15.2	53/47
Ellipsaria lineolata	1	45.0	34.0	25.7	10.9	56/44

[a] % Soft tissue = the percentage of the total weight that is comprised of soft tissue.
[b] Gut/OT % = the percentage of the total weight of soft tissue in gut and OT samples.

TABLE 2.

Mean values of physical data for *Amblema plicata* collected during the 1997 sampling season.

Trial	No. Animals	Length (mm)	Width (mm)	Weight (g)	Soft Tissue (%)[a]	Gut/OT Ratio[b]
1	13	105.8	76.9	269.9	11.9	59/41
2	18	102.4	76.5	247.6	11.6	59/41
3	18	101.9	76.9	252.4	12.1	62/38
4	20	87.9	64.3	153.7	12.7	61/39
5	17	108.6	78.7	283.2	13.9	64/36
6	2	112.5	80.5	294.7	13.1	63/37

[a] Percentage of the total weight of the bivalves that were soft tissues used for isolation of bacteria.
[b] Percentage of the total soft tissues used for: Gut (*f*) and OT samples for isolation of bacteria.

isolation of pathogens, suspect colonies that were picked to fresh media had their identity confirmed as to that particular pathogen, or not. Biochemical tests employed for bacterial characterizations were described in the appropriate reference papers or with standard biochemical characterization (Koneman et al. 1988; MacFaddin 1980). The sets of 1:2 dilutions were used to prepare smears on microscopic slides for detection of *Renibacterium salmoninarum* cells using the direct fluorescent antibody test (FAT; Bullock et al. 1980) and commercially available FITC-conjugated antiserum prepared in goats to the whole cells (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). One hundred microscopic fields of view were observed from each stained homogenate at 1,000× magnification using a Reichert Diastar Model 420 microscope with a halogen light source (Cambridge Instruments Inc., Buffalo, NY). An *R. salmoninarum*-positive kidney tissue from a diseased salmon served as a control slide for reference to the correct cell morphology.

Isolation of *A. salmonicida* from fish that died as a result of cohabitation with *A. plicata* that were previously challenged with the bacterium was done using a sterile inoculation loop to collect kidney tissue, and this was used to inoculate primary isolation plates. The medium for isolation of *A. salmonicida* was tryptic soy agar (TSA; Difco Laboratories, Detroit, MI) supplemented with 0.01 % coomassie brilliant blue (CBB; Cipriano and Bertolini 1988). The identities of suspect, blue colonies were confirmed as *A. salmonicida* with standard biochemical characterization tests.

Bacteria used for the waterborne challenges of bivalves were isolated from rainbow trout at the White Sulphur Springs National Fish Hatchery, White Sulphur Springs, WV. This facility was selected, because mussels are currently being held there, and the resident populations of fish are healthy and well maintained; therefore, they would be representative hosts of normal flora bacteria that relocated mussels encounter, either via the fish as a source or from their common water supply. Mucus and kidneys from 100 randomly selected rainbow trout were used to inoculate BHIA and CYTO plate media (described in Media employed). The resulting bacterial growth on the primary isolation media was observed, and the most frequent colony types, on the basis of morphology, were noted. Subcultures of the predominate bacterial types were established by transferring representative single colonies onto fresh plates. These isolates were used to challenge the groups of freshwater bivalves.

Media Employed

Thirteen media were employed to isolate bacteria, including fish pathogens from mussels. These media are routinely used by fish health personnel to culture environmental bacteria commonly found on fish or in aquatic environments and for isolation of specific pathogens. Two were used to determine total bacterial counts, brain heart infusion agar (BHIA; Difco Laboratories, Detroit, MI) and *Cytophaga* agar (CYTO), a medium of reduced nutrient concentration (Anacker and Ordal 1959); plates were incubated at 22 °C for 48 hours. An additional set of CYTO plates were incubated at 15 °C for culture of *Flavobacterium psychrophilum*, cause of bacterial coldwater disease of salmonid fishes (Bernardet et al. 1996). The cooler incubation temperature is favorable for growth of *F. psychrophilum*, and it also retards the growth of other, environmental bacteria that facilitate isolation. Three media were used for Gram-positive bacteria. Rogosa SL (ROGO; Difco Laboratories, Detroit, MI) was used for *Lactobacillus* and related genera. Azide blood agar base (ABA; Difco Laboratories, Detroit, MI) containing 5% sheep erythrocytes (Bio-Whittaker, Walkersville, MD) was used for other species, including *Streptococcus* spp. and *Staphylococcus* spp. Incubation of ROGO and ABA plates was at 28 °C for 3 days. A third, the selective medium (SKDM) described by Austin et al. (1983), was used for isolation of *R. salmoninarum*, cause of bacterial kidney disease (Bullock and Herman 1988). To enhance growth of this bacterium, the sterilized SKDM was cooled and supplemented with 1% filter sterilized culture metabolite (Evelyn et al. 1990) before pouring plates. Following incubation at 15 °C for up to 4 weeks, suspect *R. salmoninarum* colonies (Sanders and Fryer 1980) were suspended in 0.5 mL PEP-YE; 100 µl of this was placed on a microscopic slide and air dried. The dried smears were subjected to the direct fluorescent antibody test, as previously described. Other media used were for isolation and enumeration of Gram-negative fish pathogenic bacteria and unless indicated, were incubated at 28 °C for 2 to 3 days. Two were for isolation of *Yersinia ruckeri*, cause of enteric redmouth disease. They were the differential medium described by Waltman and Shotts (SW; 1984) and the differential and selective medium of Rodgers (ROD; 1992). Suspect colonies were transferred to TSA. Two media were for *Aeromonas* spp. One was CBB for *A. salmonicida*, cause of fish furunculosis; these plates were incubated at 22 °C; suspect blue colonies were transferred to TSA. The other medium (SGAP-10C) was for growth and enumeration of motile *Aeromonas* spp. (Huguet and Ribas 1991, Jenkins and Taylor 1995). Although other organisms may grow on SGAP-10C, such as *Pseudomonas fluorescens*, they are easily distinguished from *Aeromonas* spp. by colony characteristics and additional biochemical tests, such as fermentation of glucose. *Pseudomonas* isolation agar (PIA; Difco Laboratories, Detroit, MI) was for growth of *Pseudomonas* spp., of which many members of this or related genera are commonly found in aquatic environments or as part of the flora of healthy fish. *Edwardsiella* isolation medium (EIM; Shotts and Waltman 1990) is both differential and selective and was used for *Edwardsiella ictaluri*, cause of enteric septicemia of

catfish, and *E. tarda*, a potential pathogen often found in warm- and cold-blooded animals. Selective *Cytophaga* agar (SCA; Hawke and Thune 1992) was employed as a selective medium for *Flavobacterium columnare*, these plates were incubated at 37 °C for additional selection.

Challenge Procedures: Mussels and Fish

For the bacterial challenges of mussels, 20 tanks (27-L each) were used, each containing 10 mussels. The 200 animals, listed previously, were distributed equally by species among the 20 groups. The animals were allowed to acclimate in the tanks for 2 d before challenge. Sixteen of the tanks received bacteria: this included 14 that were of the predominate normal flora from rainbow trout from the White Sulphur Springs Hatchery, seven that were originally isolated on BHIA, and seven from CYTO. Another two tanks were challenged with the known fish pathogens *A. salmonicida* and *R. salmoninarum*, and there were four control groups exposed, one each for the four sterile media used to grow the challenge bacteria. The challenge strain of *A. salmonicida* originated from a furunculosis-diseased Atlantic salmon (*Salmo salar*) from Vermont, and the *R. salmoninarum* isolate (ATCC33209) was from a Chinook salmon (*Oncorhynchus tshawytscha*) from Oregon. The normal flora bacteria were each grown in a 200-mL quantity of either BHI broth or CYTO broth, the same medium that was used for original isolation. *Aeromonas salmonicida* was grown in 200 mL of TS broth, and *R. salmoninarum* was grown in 200 mL of KDM2 broth. Cultures were grown for 48 hours, except for *R. salmoninarum*, which was grown for 14 days. A viable cell count was done for each culture, and this was determined by preparing sets of serial tenfold dilutions in the homologous broth medium, and drop inoculating known quantities on plate media. Colonies were quantified, and the number of cfu/mL of tank water at the start of the challenges was calculated. To perform a challenge, the water supply to the tank was turned off, the culture was poured and mixed in, and exposure was for 24 hours. Then, the water was turned on and allowed to rinse for 24 hours; then to each tank, 10 fish were added: five rainbow and five brook trout. The mussels and fish were then observed for a period of 21 days for mortality and development of abnormal signs or pathology. Kidneys of fish that succumbed were cultured onto the appropriate medium, and subsequent bacterial growth was identified to confirm re-isolation of the bacterium used to challenge the mussels.

RESULTS

Physical data on the animals collected for isolation of bacteria are presented in Tables 1 and 2. The predominate bivalve species collected for the six trials was *A. plicata*, accounting for 81.5 % of the total. The average weight of the animals varied, depending on species, and ranged from 25.7 g for the single *E. lineolata* to 294.7 g for the *A. plicata*. However, regardless of over-all size, the percentage of the total weight comprised of soft tissues ranged from 10.5 to 15.2 %. Of the total soft tissues, the ratios of our portioned gut to OT samples were also similar among the hosts. The percentage of soft tissues collected as gut samples ranged between 53 to 64 %, with the paired OT samples comprising the balance of the total soft tissues. With exception of trial 4, the distribution of *A. plicata* physical data remained relatively similar throughout the season (Table 2). In five of the trials, the mean weights of the *A. plicata* ranged from 247.6 to 294.7g; whereas, with trial 4, the average weight was less, 153.7 g.

Results of the bacteriological analyses including enumeration of bacterial flora, numbers and qualitative characterization of suspect colonies, DFAT staining for *R. salmoninarum* in tissue homogenates and Ohio River surface water temperatures at the time and location of collection for the six trials are given in Table 3. Total bacteria counts using the routine growth media BHIA and

TABLE 3.

Mean bacterial counts (cfu/g), colonies selected and characterization results of suspect fish pathogenic bacteria isolated from freshwater bivalves sampled six times (Trials 1–6) during the 1997 collection season. The data are mean or summary values for the number of animals per trial. Trials 1–5 had 20 animals each, trial 6 had eight.

Medium[a]	Trial 1 (29 °C[b])	Trial 2 (27 °C)	Trial 3 (24 °C)	Trial 4 (21 °C)	Trial 5 (20 °C)	Trial 6 (10 °C)
BHIA	1.07×10^5	2.90×10^5	2.31×10^5	2.10×10^5	2.85×10^5	2.01×10^4
CYTO at RmT	1.81×10^5	4.99×10^5	2.91×10^5	4.29×10^5	2.73×10^5	1.07×10^5
SGAP-10C	3.81×10^4	8.39×10^4	3.77×10^4	2.03×10^4	8.07×10^4	2.95×10^3
PIA	8.37×10^3	5.41×10^4	7.61×10^3	6.69×10^3	2.01×10^4	3.36×10^3
CYTO at 15 °C	NS[c]	NS	1 (0)[d]	14 (0)	NS	NS
SW/ROD	18 (0)	12 (0)	21 (0)	21 (0)	21 (0)	10 (0)
CBB	3 (0)	12 (0)	11 (0)	13 (0)	12 (0)	7 (0)
EIM	6 (0)	10 (0)	7 (0)	8 (0)	7 (0)	8 (0)
SCA	NS	NS	NS	NS	NS	NS
ROGO	NG	2, 1.2×10^{2}[e]	NG	NG	2, 8.0×10^1	1, 8.0×10^1
ABA	3, 5.90×10^4	1, 3.20×10^2	NG	NG	NG	NG
SKDM	9 (0)	NS	9 (0)	14 (0)	12 (0)	12 (0)
DFAT	3: 3, 1, 40[f]	2: 1, 1	2: 1, 5	3: 2, 1, 1	8: 2, 1, 1, 1, 1, 1, 1, 1	1: 1

[a] Media for total counts: BHIA, brain heart infusion agar; CYTO, *Cytophaga* agar; SGAP-10C, for motile *Aeromonas* spp.; PIA, *Pseudomonas* isolation agar. Gram-negative media: CYTO@15 °C, for *Flavobacterium psychrophilum;* SW/ROD, two media for *Yersinia ruckeri;* CBB, for *A. salmonicida;* EIM, *Edwardsiella* isolation medium; SCA, for *F. columnare.* Gram-positive media: ROGO, primarily for *Lactobacillus* spp.; ABA, azide blood agar; SKDM, for *Renibacterium salmoninarum;* DFAT, direct fluorescent antibody test for *R. salmoninarum* of 1:2 dilution of each tissue.

[b] Temperature of Ohio River surface water at time and location of collection.

[c] NS = bacterial growth, but no characteristic colonies present. NG = no growth.

[d] Number of suspect colonies picked for biochemical characterization or for SKDM, DFAT for *R. salmoninarum* (number positive).

[e] Number of samples with growth, mean cfu/g of those with growth.

[f] Number of tissues positive: number of positive cells per 100 microscopic fields of each positive sample.

CYTO remained quite similar throughout the sampling season, with exception of trial 6 for which counts were less, presumably because of the colder water temperature. The average bacterial counts on BHIA ranged from 2.01×10^4 to 2.90×10^5 cfu/g of soft tissue and for CYTO, the range was 1.07×10^5 to 4.99×10^5 cfu/g. The range for *Aeromonas* spp. isolated on SGAP-10C was 2.95×10^3 to 8.39×10^4 cfu/g; whereas, for *Pseudomonas* spp., counts on PIA were between 3.36×10^3 and 5.41×10^4 cfu/g of soft tissue. All of the *Aeromonas* spp. were presumptively identified as being of the motile *Aeromonas* spp. group, because the only member of this group that is nonmotile and considered significant with regard to fish disease is *A. salmonicida* and none of the suspect blue colonies transferred and characterized off CBB were nonmotile. The sum of bacterial counts off SGAP-10C and PIA accounted for a large portion of the total bacteria. The water temperature was lowest when animals were collected for trial 6, not only had the animals burrowed beneath the surface of the river bottom and limited the number collected but also resulted in the lowest counts for total bacteria, motile *Aeromonas* and *Pseudomonas*. Conversely, the highest cfu/g of tissue for these three groups of bacteria were all recorded on trial 2, when the water temperature was near the highest at 27 °C. The proportion of the total bacteria that was comprised of motile *Aeromonas* and *Pseudomonas* was also highest from trial 2.

A number of Gram-negative colonies suspected of being pathogenic for fish were selected off the media CYTO at 15 °C, SW/ROD, CBB, and EIM (Table 3). Suspect colonies were identified based on meeting criteria set forth in the specific references. After transfer and biochemical characterization, none was confirmed as being a fish pathogen. There were no characteristic *F. columnare* colonies cultured from any tissues of any of the trials. In a previous year this bacterium was isolated from an *A. plicata* that came from the same location in the Ohio River (Starliper et al. 1998). The selective Gram-positive isolation media, ROGO and ABA, supported very minimal growth relative to that of the media used for Gram-negatives. Bacterial growth was present on ROGO medium in three of six trials, but from only five of the 96 tissue samples assayed. The counts of the tissues with growth ranged between 8.0×10^1 and 1.2×10^2 cfu/g. Tissues from two of the trials showed growth using ABA medium and these were from only four of 80 samples with averages of 5.90×10^4 and 3.20×10^2 cfu/g for trials 1 and 2, respectively. Isolates selected from the ABA culture plates were also identified as α–hemolytic *Lactobacillus* and were catalase negative, and, therefore, differed from *Carnobacterium piscicola* (formerly *Lactobacillus piscicola*) that has been previously noted to cause disease and low mortality primarily in postspawning rainbow trout (Starliper et al. 1992). Only in trial 2 was there any bacterial growth of any tissue samples on both ROGO and ABA. Serological based observation of the 1:2 tissue homogenates for *R. salmoninarum* using the DFAT showed positive fluorescent cells of correct morphology from every trial. Trial 5 had the largest number of tissues (8 of 40) with at least one cell detected within 100 microscopic fields of view. The number of cells detected in those positive tissue samples was either one or two for most, but 40 cells were seen in 100 fields from one *Q. quadrula* OT sample. However, no bacterial colonies yielding fluorescent cells having correct morphology for *R. salmoninarum* were noted on SKDM primary isolation medium from these or any other tissue homogenates. One suspect bacterial colony from SKDM growth plates did yield excellent positive fluorescence, but the individual cells were too large to be considered of correct *R. salmoninarum* cell morphology; this was from a *Q. metanevra* OT homogenate.

At the start of the 24-hour bacterial challenges, the average number of viable bacteria was 5.31×10^6 cfu/mL of tank water for the seven groups of freshwater bivalves exposed to the selected normal flora bacteria isolated from fish and grown in CYTO media. There was an average of 4.89×10^6 cfu/mL of challenge tank water in the seven groups of animals exposed to the normal flora bacteria isolated and grown in BHI medium. There was 1.98×10^6 cfu/mL of tank water in the group exposed to *A. salmonicida* and for *R. salmoninarum*, there was 3.53×10^5 cfu/mL. During the actual 24-hour waterborne exposures, there was no mortality experienced in the bivalves. The only bivalve death in the duration of the study, a *Q. quadrula*, occurred on day 8 of the 21-day cohabitation with fish and was in the group exposed to the TSB medium control. There was no mortality in the brook or rainbow trout in any groups that were placed to cohabit with mussels previously challenged with the 14 bacterial types cultured from the White Sulphur Springs National Fish Hatchery. Also, there were no deaths of fish in the *R. salmoninarum* group or the four control groups exposed to bacteriological media only. However, in the group of animals challenged with *A. salmonicida*, mortality in brook trout began on day 8 of cohabitation, two more died on day 15, and the remaining one on day 21. One rainbow trout died on day 21. Of these dead fish, there were no external lesions produced, but there was extensive internal pathology indicative of a systemic, Gram-negative bacterial infection. There were elevated amounts of red, ascitic fluid, hemorrhaging of internal organs, and the hind gut was filled with yellow, pus-like material. Kidney tissues of dead fish inoculated onto CBB plates resulted in heavy growth with presumptive blue *A. salmonicida*. Single colonies were picked, and their identity was characterized as *A. salmonicida* with the following criteria, in addition to blue on CBB: production of brown, water soluble pigment on TSA, K/A on triple sugar iron agar, oxidase positive, nonmotile by the hanging drop method, gelatin liquefaction positive, and a negative ornithine decarboxylase. The greater mortality in brook trout was expected, because they are known to be more susceptible to *A. salmonicida* than are rainbow trout (Cipriano 1982). After the 21-day observation, kidney tissues of the surviving rainbow trout were streak plated onto CBB, and *A. salmonicida* was isolated and the identity confirmed, as previously, from two of the fish. The 10 mussels in this group were comprised of seven *A. plicata* and one each of *Q. metanevra, P. cordatum* and *Q. quadrula*. After the 21 days of cohabitation, the gut and OT soft tissue samples of each of these animals was excised, homogenized, and diluted as previously described with drop inoculation onto CBB plates for isolation of *A. salmonicida*. From these, no suspect colonies were cultured from any of the 20 soft tissue homogenates; therefore, *A. salmonicida* was not re-isolated.

The 10 freshwater bivalves challenged with *R. salmoninarum* included seven *A. plicata*, two *O. reflexa*, and one *Q. pustulosa*. In contrast with the group challenged with *A. salmonicida*, no mortality in fish occurred. After the observation period, the fish kidney tissues of surviving fish were used to prepare smears on microscopic slides for evaluation by DFAT. No fluorescent *R. salmoninarum* cells were detected. At this time, the same was done for the gut and OT tissues of the ten mussels, and one *A. plicata* was positive with two cells of correct morphology for *R. salmoninarum* being detected in 100 microscopic fields viewed. However, it is not known if these cells were alive, beecause the DFAT stains both

live and dead cells. Upon observation of the slides prepared from the mussel tissues, there was a significant amount of small (1μ in diameter) fluorescent particles present that did not have uniform shape and were not confused with intact cells. There were too many, and each was too small to be quantified accurately. It is not known if these entities in some way originated from the *R. salmoninarum* cells used for challenge or if they were artifactual staining. In either case, this is unique, because this is not typically noted on stained slides prepared from fish kidneys, whether the tissue is positive or not for the bacterium.

DISCUSSION

With intensive fish culture and fish health management, the best defense against pathogenic diseases is prevention (Piper et al. 1982). All of the major bacterial pathogens of salmonid fishes may involve horizontal transmission. This might occur among resident individuals within a facility or could result from introduction of a pathogen to resident fish by contamination with a new lot of fish placed into the facility. Relocated fish could be carriers of a pathogen and because of their past exposure may have some innate immunity that would allow them to harbor an organism and not display any obvious signs of disease. Then, when naive fish are exposed to bacteria shed by the carriers, an epizootic may ensue. Because of this potential, it is imperative that fish to be relocated undergo a health inspection to identify pathogens, including those that are not obvious because of a lack of clinical signs. The potential for introduction of pathogens via relocated freshwater bivalves is also of concern. Animals may be originating from open and uncontrolled environments, such as the case with those from the Ohio River, where they might be exposed to wild fish that could be diseased. Bacteria are shed into the environment and the bivalves could uptake bacteria either as a food source or by simply filtering contaminated water. The primary pathogens of salmonid fishes are not known to cause diseases in freshwater bivalves; therefore, animals would be unlikely to become carriers of a pathogen in the sense that susceptible fish can following their survival of an epizootic. Once freshwater bivalves are removed from the source (shedding) of a bacterial pathogen and relocated to a pathogen-free water supply, such as could be the case of quarantine for zebra mussels, the length of time the pathogen remains present and viable in the tissues and could still be infective to fish is not known. This is especially important considering the results of the cohabitation study where mussels were exposed to *A. salmonicida*. In a study by Plusquellec et al. (1994) with two marine bivalves, the mussel *Mytilus edulis* and the oyster *Crassostrea gigas*, retention of the enteric human pathogenic *Salmonella* following artificial exposure and air drying showed retention of viable cells of at least 5 days at 12 to 15 C and as long as 20 days at 10 C. However, when the clam *Mercenaria mercenaria* was artificially exposed to *Escherichia coli* or *S. typhimurium*, and the infected animals were moved to flowing, pathogen-free seawater, they are able to reduce viable cell loads in tissue homogenates within 24 hours by factors of 10^4 and 10^5, respectively, from a starting tissue load of between $1–2 \times 10^5$ cfu/g (Timoney and Abston 1984). A change in bacterial flora was similarly noted within 24 hours in freshwater bivalves that were not initially exposed artificially to bacteria, rather these animals were simply relocated from one water supply to another (Starliper et al. 1998). In this study, the total bacterial count per gram of soft tissue remained quite stable at both water sources, but the most notable change was a relative increase of nonfermenting bacterial types, coinciding with a decrease in other bacterial types after 24 hours of being in the different water supply.

The bivalves that were collected in trial 4 were smaller than those collected in the other five trials (Table 2). The smaller animals were several years younger than the larger ones. When collecting animals from a large environment, such as the case with the Ohio River, a group with size characteristics unique to other groups collected is not unusual. Bivalves are not evenly deposited on the river bottom, rather, they often occur clustered as to age and species, which has to do with the movement of the host and where the juveniles drop from the host. This is particularly relevant with *A. plicata*, because of the variety of fishes that may act as hosts and include many species of the family *Centrarchidae*, sauger (*Stizostedion vitreum*), and the flathead catfish (*Pylodictis olivaris*).

In the study by Starliper et al. (1998), *Flavobacterium columnare*, cause of columnaris disease in many cool and warm water fishes, was isolated from an *A. plicata*. This animal was assayed directly after being removed from the Ohio River and had not been placed into pathogen-free water for any time prior to analysis. The pathogen was not isolated from animals that were collected at the same time, and location but had been in pathogen-free, flow-through water for 24 hours. Alone, information on the isolation of a fish pathogen from a bivalve that could be intended for relocation to a salmonid rearing facility is discouraging. However, before relocation, if they undergo quarantine according to a protocol such as that of Gatenby et al. (1998) to ensure that zebra mussels are not inadvertently spread and if the animals are maintained in pathogen-free water, there is the chance that pathogens may be depurated during the quarantine. This is surmised from the demonstrated rapid change in flora after being moved to a different water supply and because *F. columnare* was not isolated from animals after having been relocated for 24 hours.

The detection of cells of correct morphology for *R. salmoninarum* from the DFAT of mussel tissue homogenates could become a concern for fish health managers of salmonid rearing facilities. This bacterium and the disease it causes, when in a population of fish, presents significant health management problems, because a primary means of pathogen transmission is vertical, in addition to the fact that it is horizontally transmitted. Therefore, presence of any fluorescent bacterial cells of correct morphology noted in fish kidney tissues or ovarian fluids of spawned fish is not good, particularly at those facilities that are involved with egg production and shipment. The nature of the bacterium's being very slow growing and difficult to isolate, especially in low numbers, only adds to the dilemma of fish health managers faced with the situation. When fish health inspections yield only minimal numbers of positive cells using the DFAT or minimal positive results from other serology-based assays in the absence of bacterial isolation or clinical disease signs in fish, management personnel are faced with difficult decisions on the health status of the population. Therefore, if relocated mussels might be determined to be a source of a bacterium that could result in positive cells by DFAT, regardless of a lack of culture isolation, a significant concern is realized. An important topic for further study would be to evaluate if a 30-day quarantine in a clean or different water supply would result in depuration of cells that may yield fluorescence.

In the six trials of the present study, the results using the DFAT and culture on SKDM for detection of *R. salmoninarum* from bivalve tissues were not in agreement; no cells were noted from culture; whereas, there were using the DFAT for tissues. Discrepancies of this sort are not uncommon using various methodologies for detection of this bacterium in fish (Cipriano et al. 1985; Teska et al. 1995). A number of factors contribute to this and include the slow and difficult nature of isolation and growth of *R. salmoni-*

narum, specificity and sensitivity of antisera, the host, and the extent of infection within a population of fish. Furthermore, the DFAT was originally developed as a method for quick, presumptive diagnosis of clinical bacterial kidney disease (Bullock et al. 1980) that would later be confirmed by bacterial culture. The advantage was that a DFAT could be done in hours, as compared to weeks for primary isolation. This affords quick intervention for fish health managers to control the disease and prevent further spread. With heavily infected fish, the two methodologies correlate well, but when used for relatively healthy, pathogen carriers, the agreement may decrease.

In the cohabitation group with mussels previously exposed to *A. salmonicida* and brook and rainbow trout, it is noteworthy that after 21 days, the bacterium was not re-isolated from the mussels' soft tissues. This is interesting, because there were infected and dying fish present in that tank water, and clinically diseased and carrier fish are known to shed viable *A. salmonicida* cells. However, with only four rainbow left at the end, the quantity of bacteria shed might have been too few to maintain bacterial presence within the *A. plicata*. Also, the normal flora of the mussels may have displaced and/or prevented further infection, because to mussels, it is assumed that *A. salmonicida* is merely an environmental organism and not pathogenic.

With the *A. salmonicida* challenge experiment, it is encouraging that after being exposed to a load of viable bacteria in the water that is greater than would occur naturally, the bacterium was not isolated from animals after the challenge's observation period. Again, it would be important to evaluate if the quarantine for 30 days to eliminate zebra mussels is sufficient for native bivalves to concurrently depurate fish pathogens. Additional topics for research are to extend beyond 24 hours the duration between when mussels are removed from bacterial exposure and when susceptible fish are introduced. Furthermore, the bivalves can be exposed to lesser bacterial cell concentrations and a more natural challenge method, those more analogous to what might be encountered in nature and then evaluate contagion. Also, a determination should be made on whether the bacterium enters the soft tissues or merely is contained in the fluid portion outside the soft tissues, but within the shell.

Following the challenge and cohabitation involving *R. salmoninarum* and when fish and mussels tissues were evaluated by DFAT, no cells were detected in fish, and only two were detected from one *A. plicata*. These results were not surprising for this bacterium. Reproduction of experimental bacterial kidney disease in a laboratory by contact exposure is very difficult and requires significant effort and time. Wolf and Dunbar (1959) were able to produce mortality in brook trout by a noninjectable challenge with *R. salmoninarum* only after fish were maintained in tanks with bricks in place as a means for abrasion, then the water level was dropped daily and viable cells were added. Still, it took 96 days for the first death. In another study, IP injection of brook trout with a number of viable cells similar to that per mL of tank water used in the present study, the mean days to death for the group of fish was about 28 days, and the first occurred on about day 25 (Starliper et al. 1997). Because horizontal or contamination infection to fish is difficult and because this bacterium has a limited host susceptibility range that is known to include primarily salmonid fishes, perhaps the chance for mussels to act as vectors is remote. Particularly, if mussels are able to rid the bacterium as noted in the present study when exposed to *R. salmoninarum*, that after 21 days in clean water only two cells were detected by DFAT of the soft tissue homogenates. Furthermore, the high frequency of fluorescent particles in the tissues might be indicative of cellular debris resulting from the bacterial cells being digested by the mussels.

All of the freshwater bivalves in these studies were used, because they are readily available, and they exist in high numbers; however, they may not be species that will be collected as part of the relocation program. Once techniques are developed using common animals and are available, the appropriate studies may then be repeated using minimal numbers of surrogates selected to represent those animals that do fit the criteria for relocation.

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