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Sampling the Bacterial Flora of Freshwater Mussels

Freshwater Mussels Are Ecosystem Barometers

Mussels comprise a very important and diverse segment of freshwater stream ecosystems in the United States. Approximately 300 different species and subspecies of freshwater mussels are native to North America, and more than 70% are categorized as endangered, threatened, or of special concern. As filter-feeding animals, they are important biological indicators of the health of aquatic ecosystems. Because each species requires a host fish as part of their life cycle, mussels are both directly and indirectly susceptible to changes in their environment.

Native Species Under Siege by Exotic Zebra Mussels

In recent years, introduction of the zebra mussel *(Dreissena polymorpha)* has posed a major new threat to native mussel populations in some large river systems. Unlike native mussel species, this exotic invader does not require an intermediate host; instead, it is capable of attaching to and proliferating on virtually any solid substrate--including other mussels. Native species perish as a result of being deprived of oxygen and food. Zebra mussels multiply rapidly and appear to have a high degree of spatial tolerance. In 1996, densities of zebra mussels in the lower Ohio River (mile 814) exceeded 14,000/m, with consequent mortality of native species exceeding 30%. As the zebra mussel advances in affected river systems, the prognosis for native populations is poor. This is especially true for those native species that are threatened and endangered and those having a relatively limited geographic range. Currently, there is no effective means for control of zebra mussels in an open system such as a river.

Mussel Relocation Project Raises Important Questions

A concerted effort was initiated in 1995 by the U.S. Fish and Wildlife Service and other Federal, State, and private partners to selectively collect and hold mussels from areas of zebra mussel infestation in large rivers. Individuals of native populations are collected, maintained, and propagated in certain Federal facilities, including fish hatcheries, with the goal of reintroduction into their native habitat at an appropriate future date. During planning of this relocation project, questions arose regarding the potential for transmission of pathogenic microorganisms from mussels to fish or from fish to mussels. The information presented here is part of ongoing studies to answer such questions.

Ohio River Native Species Studied

Freshwater mussels native to the Ohio River were collected near Muskingum Island (river mile 175), adjacent to Wood County, West Virginia. Animals were collected in November 1995 and in June and September 1996 by brail hook (10 ft long, with 38 chains each holding four dovetail hooks). The brail

was checked and mussels were removed every 5 min. No effort was made to collect certain species; instead, individuals of all species were collected until the desired numbers were obtained. The study was designed for two purposes: (1) to determine if we could isolate any salmonid pathogenic bacteria from the mussels and (2) to evaluate changes in the bacterial flora of the mussels over a 30-day period. Information on sampling techniques and methods used to answer these questions is presented here. The 30-day period was selected because mussels destined for hatcheries must first undergo a 30-day quarantine period to prevent introduction of zebra mussels to those hatcheries.

Methods

In the area of the three collections, water depths ranged from 1 to 7 m; water temperatures were 15.6, 25, and 20 °C, respectively. Water samples were obtained at the time of mussel collection.

In November 1995, 40 mussels were collected and then transported in Ohio River water to the National Fish Health Laboratory (NFHL) for subsequent bacteriological assessment. The following species were represented: threeridge (*Amblema plicata*; 22), monkeyface (*Quadrula metanevra*; 4), Ohio pigtoe (*Pleurobema cordatum*; 2), pimpleback (*Q. pustulosa*; 4), mapleleaf (*Q. quadrula*; 4), and threehorn wartyback (*Obliquaria reflexa*; 4).

During the June and September 1996 collections, 75 mussels were obtained at each collection. The mussels were transported in Ohio River water to the NFHL, where they remained overnight. On the following day (day 0), 15 animals were assayed. The remaining mussels then were transferred to reservoir water at the Leetown laboratory. After 24 h, another 15 mussels were sampled (day 1). Subsequently, post-transfer to reservoir water, 15 mussels were sampled each day on days 3, 15, and 30. In June, we collected 46 threeridge, 10 threehorn wartyback, 8 Ohio pigtoe, 7 monkeyface, and 1 each elephantear (*Elliptio crassidens*), washboard (*Megalonaias nervosa*), pimpleback, and pink heelsplitter (*Potamilis alatus*). In September, we collected 68 threeridge, 1 Ohio pigtoe, and 3 each threehorn wartyback and mapleleaf.

Length, width, and weight were recorded for each mussel and all tissue within the shell was aseptically removed. Tissues were separated into two samples: (1) gut, and (2) all other tissues combined. Tissue samples (two from each mussel) were placed into preweighed sterile stomacher bags. The bags and contents were weighed, and sterile phosphate-buffered saline (PBS; pH 7.2) was added to each bag to yield a 1:2 dilution of tissue. Tissues then were homogenized in a stomacher for 60 s and a portion of the contents transferred to a sterile tube for ease of handling. Three additional 10-fold PBS dilutions were made, resulting in 1:20, 1:200, and 1:2000 tissue dilutions.

A quantity of 25 μl of each dilution was used to inoculate primary isolation media: the high-nutrient media blood agar (BA) and brain heart infusion agar (BHIA), and the low-nutrient medium cytophaga agar (CYTO). Each are general purpose growth media. The BA and BHIA plates were incubated at 20-22 °C for 48 h, and the CYTO plates were incubated at 15 °C for 4-5 days. An additional CYTO plate, supplemented with 30 μg/mL neomycin sulfate, was inoculated and incubated at 37 °C for selective isolation of the fish pathogen Cytophaga columnaris. After all plates were incubated, the lowest-dilution plates that allowed accurate counting of single bacterial colonies were selected for enumeration. These numbers then were converted to colony-forming units per gram of tissue (cfu/g). Total counts were recorded, as well as the cfu/g of each of the different bacterial colony types. Representative colony types were subcultured onto fresh media, and standard taxonomic and biochemical techniques were used to identify the bacteria.

Most Bacteria were Nonfermenters and Motile Aeromonads

The tissue dilutions selected were very effective in producing single, isolated bacterial colonies to count and transfer. Very few plates were lost to overgrowth with fungal contamination; therefore, it was not necessary to incorporate antifungal agents in the primary isolation media. Bacteria were isolated from all mussel tissues assayed. The distribution of bacteria was somewhat equal between the two tissue samples: the overall mean total bacteria count for gut tissue was 1.729 10^5 cfu/g and 1.556 10^5 cfu/g for the combined tissues samples. The temporally spaced bacterial sampling studies seem to indicate that total counts remain stable. Evaluation of bacterial species distribution will be done when taxonomic studies are complete.

Freshwater mussel anatomy does not lend itself easily to dissection and removal of individual tissues. During our sampling, the gut was removed first in an effort to reduce contamination of the remaining tissues with gut contents. We have not determined what organ(s) of our "other tissues" samples yielded the highest concentration of bacteria. Overall, we isolated about 1,153 bacteria for identification. The predominant bacterial groups represented were the nonfermenting organisms and members of the motile *Aeromonas* spp. Approximately 527 (45.7%) of the total bacteria were nonfermenters and about 300 (26.0%) were motile Aeromonads. The remainder included a few Enteric bacteria and some Gram-positive bacteria (not recognized fish pathogens). *Cytophaga columnaris* was isolated from one mussel on day 0 sampling in June 1996. Subsequent sampling--including sampling of mussels that had been in reservoir water for 24 h--did not isolate additional *C. columnaris*.

Conclusions

Based on our preliminary classifications of the isolated bacteria, only *C. columnaris* is considered a primary pathogen of salmonid fish. Our results indicate that this bacteria is not present after the 30-day quarantine period, assuming the water they are quarantined in does not contain this bacterium. Although the motile *Aeromonas* spp. and some members of the nonfermenters are capable of producing disease in salmonids, they are primarily considered to be opportunistic pathogens; furthermore, most are ubiquitous in the aquatic environment, including hatchery waters, and many can be routinely isolated as part of the flora of healthy fish.

The animals we studied were collected under West Virginia State collecting permits and no species collected is currently listed on any Federal protected lists.

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