The Effect of Disinfection Strategies on Transmission of *Aeromonas salmonicida* and *Yersinia ruckeri* in a Recirculating Aquaculture System

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ABSTRACT

Continuous addition of *Aeromonas salmonicida* (which causes furunculosis) or *Yersinia ruckeri* (which causes enteric redmouth disease, or ERM) broth cultures to recirculating aquaculture systems, without fish, resulted in the presence of these pathogens in the fluidized sand biofilters. Disinfection of the recirculating systems, except biofilters, with 200 ppm sodium hypochlorite and flushing biofilters for 24 hours with filter-sterilized spring water (FSSW) did not prevent outbreaks of furunculosis or enteric redmouth disease after stocking Arctic char (*Salvelinus alpinus*) or rainbow trout (*Oncorhynchus mykiss*), respectively. Disinfection of the entire recirculating systems with 10 ppm Chloramine-T following outbreaks of furunculosis or ERM, or after addition of broth cultures prevented transmission of ERM in three trials and in two of three trials with furunculosis. Within 75 days of stocking Atlantic

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salmon (*Salmo salar*) with subclinical furunculosis in the recirculating system with rainbow trout, *A. salmonicida* could be cultured from the mucus of rainbow trout and from the fluidized sand biofilters. Removal of salmon and trout and disinfection of the recirculating system with 10 ppm Chloramine-T prevented a furunculosis outbreak when Arctic char were stocked into the system. However, if the recirculating system was only drained and refilled after removal of salmon and trout, furunculosis occurred within 7 days of stocking char.

INTRODUCTION

Infectious diseases can be a major cause of mortality in intensive recirculating aquaculture (Noble and Summerfelt 1996). Precautions such as use of specific-pathogen-free fish stocks, a clean water supply, proper sanitation, and other biosecurity procedures are practiced to help reduce the risk of disease outbreaks. In recirculation aquaculture systems, biofilters used for ammonia removal may be an additional reservoir of pathogens. If an infectious disease occurs in a recirculation system, the pathogen may become established in biofilters and infect newly stocked fish. In a previous unpublished study, results suggested that *Flavobacterium branchiophila* (bacterial gill disease, BGD) became established in fluidized sand biofilters of two experimental recirculating systems. When the systems were built and biofilters functioning, rainbow trout (*Oncorhynchus mykiss*) that had never experienced BGD were stocked into both systems and maintained disease free for 3 months. Rainbow trout that had recovered from a BGD outbreak were stocked with the original rainbow trout and within 7 days BGD occurred in both systems. Trout were then removed from both systems and no trout stocked for 7 days in order to allow *F. branchiophila* to be flushed from the system. However, when the system was restocked with healthy trout, BGD occurred within 21 days suggesting that the bacterium may have colonized the biofilters. The present research was initiated 1) to determine if *Aeromonas salmonicida* or *Yersinia ruckeri* would persist in fluidized sand biofilters and become a source of infection for newly stocked salmonids, and, 2) to test the efficacy of disinfection with 10 ppm Chloramine-T or 200 ppm sodium hypochlorite.

MATERIALS AND METHODS

Recirculating System

All tests were conducted in two identical recirculating systems located at the USGS National Fish Health Research Laboratory (NFHRL; Kearneysville, WV, USA) (Figure 1). Water flowed from the 1.5-m^3 culture tank into a drum filter (100 μm screen size) to remove solids, and was then pumped at 8 L/min up through each of 6 fluidized sand biofilters, each 2.5 m high, and 0.17 m in diameter. Water leaving the biofilters fell back to the culture tank through two degassing columns each 0.2 m diameter x 1.5 m length and filled with 5-cm Norpac media (NWS Corporation, Roanoke VA, USA) to remove carbon dioxide. Water temperature in the systems ranged from 14 to 17°C and oxygen levels were maintained between 9 to 12 ppm. Spring water (12°C) was added to the systems to provide two complete changes per day. Biofilters could be switched to a separate set of pumps and be isolated from the rest of the recirculating system.

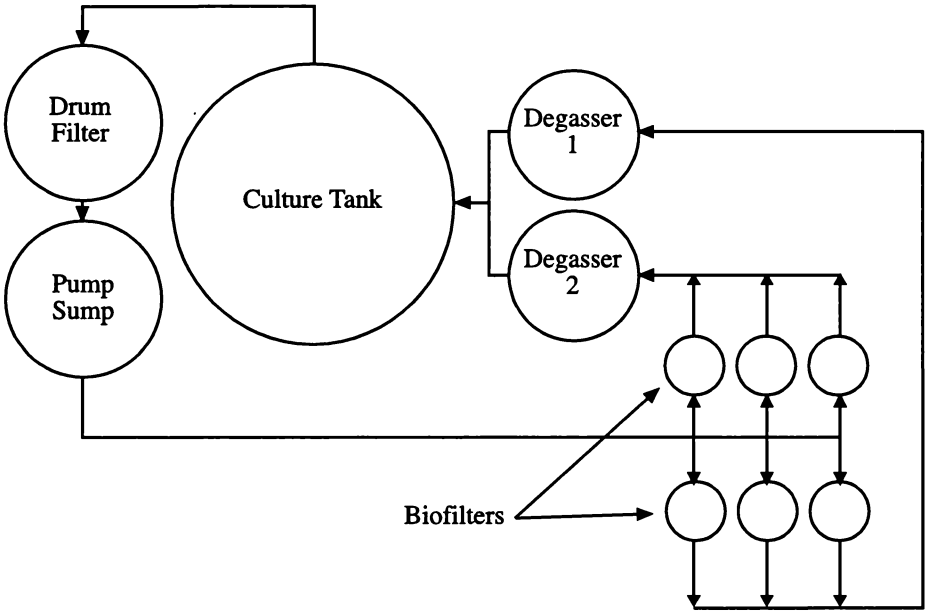

Figure 1. Schematic diagram of fish-culture recirculation system used in bacterial transmission experiments

Bacterial Strains and Media

An *A. salmonicida* strain isolated from Arctic char (*Salvelinus alpinus*) with furunculosis and a *Y. ruckeri* strain isolated from rainbow trout with ERM were used to colonize biofilters and infect stocked char or rainbow trout, respectively. Both isolates had been previously used to produce experimental infections. Isolates were grown in brain heart infusion broth (BHIB; Difco Laboratories Inc., Detroit, MI, USA) for 48 hours at 25°C, and then counted using the drop plate procedure of Miles *et al.* (1938), prior to pumping into the recirculating systems. Coomassie brilliant blue (CBB) agar (Cipriano and Bertolini 1988) was used to detect *A. salmonicida* and Shotts Waltmann (SW) differential medium (Waltman and Shotts 1984) was used to detect *Y. ruckeri.* Suspect *A. salmonicida* colonies on CBB were dark blue, 1 to 2 mm in diameter and were transferred to tryptic soy agar (TSA; Difco Laboratories Inc, Detroit MI, USA) for confirmation as *A. salmonicida salmonicida.*

Colonies on TSA slants were confirmed as *A. salmonicida* if they produced a brown water soluble pigment, were cytochrome oxidase positive, fermentative in O/F glucose (Difco Laboratories Inc, Detroit, MI, USA) and non-motile in a hanging drop. After 48 hours growth on SW, suspect *Y. ruckeri* colonies were 1-2 mm in diameter and surrounded by a zone of precipitation caused by degraded Tween 20 and calcium chloride. Colonies were confirmed as *Y. ruckeri* if they showed an acid slant in triple sugar iron agar slants (Difco Laboratories, Inc, Detroit MI, USA), and a positive slide agglutination test with type one *Y. ruckeri* antiserum (NFHRL, Kearneysville, WV, USA).

Adherence of Pathogens to Active Biofilter Sand

On two occasions, pathogen adherence to functioning (colonized) biofilter sand was determined. Pathogens were grown 24 hours in BHIB at 25°C on a shaker at 125 rpm, after which 1 mL BHIB culture was centrifuged at 10,000 x g. The pellet was washed two times in 1 mL of 0.45 μm filter-sterilized spring water (FSSW), resuspended in 1 mL FSSW, and then stained according to instructions with a Live/Dead Backlight Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA). After checking that most cells were viable (see below) the cell/stain preparation was centrifuged, washed 3 times with FSSW, resuspended in 1 mL of FSSW and mixed with 9 mL of a 1:10 dilution of biofilter sand. The mixture of sand and bacterial cells was inverted once a minute for 10 minutes, then

allowed to settle, and fluid aspirated from the sand. The sand/cell mixture was washed 6 times with FSSW by inverting the sample and allowing sand to settle between washings. The supernatant was then aspirated and the sand was examined under an epifluorescence microscope at 450x. Live cells fluoresced green while dead bacterial cells fluoresced red.

Salmonids

Yearling rainbow trout or Arctic char, stocked at 50 to 60 kg per system (30 to 40 kg/m^3) were used in experiments because rainbow trout are susceptible to *Y. ruckeri* and char are susceptible to *A. salmonicida*. Both salmonids were obtained from the Freshwater Institute (Shepherdstown, WV, USA), which has neither furunculosis nor ERM. However, both species were sampled to ensure they were not infected by these pathogens. Mucus from 25 char were cultured onto CBB agar to detect *A. salmonicida* (Cipriano *et al.* 1992, 1994) and feces from 25 rainbow trout were cultured onto SW agar to detect *Y. ruckeri* (Bullock 2004). All samples were negative.

Pathogen Transmission and Disinfection Trials

Three trials were carried out to determine whether *Y. ruckeri* or *A. salmonicida* could become established in biofilter sand, and to test disinfection methods prior to stocking the systems with either rainbow trout or Arctic char. One gram samples of sand were collected from each biofilter (Bullock *et al.* 1993) and assayed to ensure neither pathogen was present. In trials one and two, broth cultures of test pathogens were added to the systems for 5 days to colonize biofilters. In trial one, sodium hypochlorite (Fisher Scientific, Hampton, NH, USA) disinfection of the system (except for biofilters), and 24-hour flushing of biofilters with FSSW were used to attempt removal of added pathogens. If disease occurred when the system was restocked, fish were removed and a 1-hour 10-ppm Chloramine-T (N-chloro-p-toluene sulfonamide sodium salt, Sigma Chemical Co., St. Louis, MO, USA) treatment of the entire recirculating system was carried out and the system restocked with char or rainbow trout. A concentration of 8.5 ppm Chloramine-T has been shown to be effective in controlling BGD (From 1980) and isolates of *A. salmonicida* were found to be inhibited by 9.0 ppm Chloramine-T (Cipriano *et al.* 1996b). However, efficacy of Chloramine-T for disinfection of *Yersinia ruckeri* has not been reported. In trial two, only the Chloramine-T treatment of the entire system was used to attempt pathogen removal. In

trial three, it was determined whether low numbers of *A. salmonicida* shed from Atlantic salmon (*Salmo salar*), with subclinical furunculosis would result in establishing the pathogen in biofilters, and whether a 1-hour Chloramine-T treatment would remove the *A. salmonicida.*

Trial one – Using a peristaltic pump, 48-hour BHIB broth cultures of either *A. salmonicida* (2.5 x 10^6 to 2.2 x 10^8 cfu/mL) or *Y. ruckeri* (2.8 x 10^8 cfu/mL) were added continuously, at 1 mL/min for 5 days, to the pump intake of the recirculating system (without fish). Once per day for 5 days, a 1-gram sand sample from each of the 6 biofilters was weighed, diluted 1:10 with sterile phosphate buffered saline (PBS), sonicated using the procedure of Bullock *et al.* (1993), and streaked onto culture plates. Each day for 5 days, a 10 μL sample of tank water was also streaked onto culture plates. On day 6, the biofilters were taken off-line. The recirculating system was then disinfected by adding 200 ppm sodium hypochlorite to the culture tank and pumping it throughout the system for 2 hours. The sodium hypochlorite was neutralized with 250 ppm sodium thiosulfate (Univar, Middletown, PA, USA), the system drained, refilled with spring water, and checked with a sodium hypochlorite test strip (Hach Company, Loveland, CO, USA). The biofilters were then continuously flushed for 24 hours with FSSW to attempt removal of added pathogens, after which they were returned on-line to the disinfected system. The day after sodium hypochlorite disinfection of the system and FSSW flushing of biofilters, Arctic char or rainbow trout were added and the system was monitored for an outbreak of furunculosis or ERM. An outbreak was defined by lethargy, loss of equilibrium, mortality, and a bacteriological confirmation of infection. If an outbreak occurred within 6 weeks or sooner after stocking, fish were removed and the system, including biofilters, was disinfected for 1 hour with 10 ppm Chloramine-T. The system was then restocked and monitored for 6 weeks for furunculosis or ERM. During Chloramine-T treatment, total chlorine was measured every 30 minutes in fish-tank water and water entering and leaving biofilters using the DPT method (N,N-dimethyl-p-phenylenediamine; Hach Chemical Co., Loveland, CO, USA). Two trials were carried out with each pathogen.

Trial two – Broth cultures of the test pathogen were again pumped into the system, without trout or char, for 5 days and the pathogen's presence confirmed by culture of biofilter sand and fish-tank water. The entire recirculating system was then disinfected for 1 hour with 10 ppm

Chloramine-T as described, char or trout added, and the system monitored for disease outbreaks for 6 weeks. A single trial was carried out with each pathogen using choramine-T disinfection to remove pathogens.

Trial three – In trials one and two the recirculating systems were subjected to high concentrations of pathogens via high-density cultures. To determine if biofilters could be colonized by a bacterial pathogen from infected fish, two systems were stocked with 50 kg specific-pathogen-free rainbow trout and 8 Atlantic salmon, subclinically infected with *A. salmonicida*. Once per week, 1 sand sample from each of the 6 biofilters and 1 mucus sample from each of 25 rainbow trout from each system were cultured for presence of *A. salmonicida* (Bullock *et al.* 1993; Cipriano *et al.* 1996a). When the pathogen was isolated from biofilter sand and trout mucus, all fish were removed from the systems. One system was disinfected for 1 hour with 10 ppm Chloramine-T and the other was only drained and refilled with FSSW. Char were then stocked in both systems and monitored for furunculosis for 6 weeks or until the disease occurred. A single trial was carried out.

RESULTS

Adherence of *A. salmonicida* and *Y. ruckeri* to Biofilter Sand

On each occasion, color and fluorescence were used to identify the bacteria and indicate whether either bacterial pathogen would attach to colonized biofilter sand. Green fluorescing (live) *A. salmonicida* cells were found attached to sand particles. Only red (dead) scattered *Y. ruckeri* cells were seen and were not attached to sand particles.

Trial One

Arctic char and A. salmonicida – Five-day continuous pumping of *A. salmonicida* resulted in the establishment of the pathogen in all 6 biofilters and tank water both times the trial was carried out. In both tests with *A. salmonicida*, biofilters were still positive for the bacterium after the 24-hour flushing and furunculosis occurred within 3 weeks after char were stocked. When the entire system was treated with 10 ppm Chloramine-T, biofilters were negative for the pathogen after disinfection in the first test and furunculosis did not occur within 6 weeks after char were stocked. However, in the second test, biofilters were also negative after Chloramine-T disinfection, but furunculosis occurred in char within

6 weeks after stocking. The total chlorine concentration in fish-tank water and water entering and leaving biofilters was 9.0 ppm at 30 and 60 minutes of chloramine treatment.

Rainbow trout and Y. ruckeri – The 2.0 x 10^9 cfu/mL *Yersinia ruckeri* cultures that were added to the system also became established in all 6 biofilters of both recirculating systems each time the trial was done. An ERM outbreak did not occur after the biofilters were flushed during the first test, but did occur in the second test. Chloramine-T disinfection of the entire recirculating systems, after disease outbreaks, did prevent ERM outbreaks in both of the two tests, but 1 of 6 biofilters was still positive for *Y. ruckeri* after disinfection in test one. The total chlorine concentration in fish tank water and water entering and leaving biofilters was 9.0 ppm at 30 and 60 minutes of chloramine treatment.

Trial Two

In the single test with each pathogen when the recirculating systems were disinfected immediately after addition of cultures, both pathogens were cultured from biofilters after disinfection but neither furunculosis nor ERM occurred within 6 weeks after char or rainbow trout were stocked. The chlorine concentration in water entering and leaving biofilters was 9.0 ppm during disinfection.

Trial Three

When Atlantic salmon, subclinically infected with *A. salmonicida*, were stocked into both recirculating systems containing rainbow trout, the pathogen could be cultured from biofilters and mucus from rainbow trout (with weekly sampling), but required at least 75 days after stocking infected salmon. Disinfection of one system with 10 ppm Chloramine-T, following removal of trout and salmon, prevented transmission to newly stocked char. Simply draining and refilling the second system after removal of fish resulted in an outbreak of furunculosis to newly stocked char within 8 days.

DISCUSSION

While these studies are not definitive, they show that sodium hypochlorite disinfection of the recirculating systems, except for biofilters, can destroy added *A. salmonicida* and *Y. ruckeri*. However, 24-hour flushing of

biofilters with FSSW after 5-day addition of pathogens does not remove the pathogens and stocking of susceptible salmonids after flushing can result in disease. Furunculosis occurred in both tests of trial one and ERM occurred in one of two tests in trial one.

Cipriano *et al.* (1996b) reported that 9.0 ppm Chloramine-T inhibited *A. salmonicida*, but in this study the efficacy of Chloramine-T disinfection for either pathogen was not consistent. Although ERM did not occur following Chloramine-T disinfection, the pathogen was isolated from one biofilter after disinfection. Conversely, furunculosis did occur after Chloramine-T treatment in test two of trial one even though *A. salmonicida* could not be isolated from biofilters. Failure to isolate *A. salmonicida* from biofilters after Chloramine-T disinfection and the subsequent outbreak of furunculosis is not unexpected. Cipriano *et al.* (1996a) were unable to culture *A. salmonicida* from biofilters during an active furunculosis epizootic. Furunculosis may have occurred after flushing biofilters in both tests of trial one and in one test after Chloramine-T disinfection of the entire system because *A. salmonicida* was protected by biofilm attachment to sand particles and other surfaces (Carballo *et al.* 2000). Cells of *Y. ruckeri* did not attach to sand particles and ERM occurred in only one of two trials after biofilter washing and no outbreaks occurred after disinfection of the systems with Chloramine-T. However, this pathogen may have also been protected by biofilms on system surfaces (Coquet *et al.* 2002). In any case, it is evident that a single 1-hour 10-ppm Chloramine-T treatment will not reliably remove either pathogen.

These studies clearly show that washing biofilters with FSSW or a single 10-ppm Chloramine-T treatment is not reliable in removing two bacterial pathogens of salmonids from a small-scale recirculating system. Using a higher concentration of Chloramine-T or multiple treatments are alternative approaches. The use of an appropriate detergent before Chloramine-T and paying special attention to dead spots such as crevices would likely be more effective. Disinfection with 200 ppm sodium hypochlorite was an effective method, and should still be considered reliable (Piper *et al.* 1982). Efficacy of sodium hypochlorite would also be increased by first using an appropriate detergent. Regardless of the method used, the entire recirculating system, including biofilters, should be disinfected, with subsequent reestablishment of functioning biofilters.

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