Effects of Chloramine-T and Hydrogen Peroxide on Nitrification in Fluidized-Sand Biofilters for Cold Water Fish Production

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Introduction

In a previous study using fluidized-sand biofilters it was determined that formalin treatments, at levels commonly used in fish culture, caused no apparent significant effect on biofilter performance when tested under ambient conditions (Heinen et al. 1995). Given that most commonly used therapeutants are biocides it was assumed that they must have some effect on the microbial community within biofilters. Fluidized-sand biofilters are typically designed with excess nitrification capacity (Summerfelt 1996; Summerfelt and Cleasby 1996) in the form of surface area available for microbial colonization. This excess capacity allows fluidized-sand biofilters to nitrify more ammonia and nitrite than they do under normal operating concentrations. Because of this property it was hypothesized that, a change in the microbial community caused by a chemotherapeutant treatment that was not evident when a biofilter was tested under ambient conditions, would become evident when the biofilter was “challenged” with a spike of higher than normal ammonia concentration. Challenging the biofilters under normal conditions should allow for the determination of their maximum instantaneous capacity, which could then be used as a benchmark to compare biofilter performance after exposure to a chemotherapeutant. If a chemotherapeutant treatment caused an impairment of maximum biofilter nitrification capacity that was not apparent under ambient conditions, it should become apparent when the biofilters are challenged. Hence, it was thought that the effect of chemotherapeutants on biofilter nitrification capability might be ascertained through the determination of diminished maximum capacity. With this in mind, an investigation into the effect of chloramine-T and hydrogen peroxide on biofilter efficiency was undertaken.
MATERIALS AND METHODS

All tests were conducted using two identical recirculating systems. Each system contained: 1500-L culture tank; drum filter; pump sump; two degassers with sumps; and six identical biofilters operating in parallel.

Before a given chemotherapeutant test the ambient biofilter water chemistry was analyzed first, then the biofilters were challenged with a spike of ammonium chloride solution (NH₄Cl) approximately four times that of the ambient influent TAN concentration, and then the chemotherapeutant was added to the system immediately after the challenge. Twenty-four hours after each chemotherapeutant treatment, ambient biofilter performance was measured and then the biofilters were again challenged with a spike of ammonium chloride. Parameters measured during these tests were: temperature, pH, dissolved oxygen, TAN, and nitrite-nitrogen. Water quality parameters were all analyzed according to standard methods (APHA, 1989). At least 4-6 weeks were allowed to elapse between tests with a given chemotherapeutant to allow the biofilters time to stabilize from any perturbations caused by previous treatments. Two months were allowed to elapse between the conclusion of one set of chemotherapeutant tests and the onset of tests with the next chemotherapeutant.

Static bath treatments were conducted by turning off the make-up flow to prevent dilution of the chemotherapeutant, and isolating the biofilters in a separate recirculating loop to maintain fluidization. The chemotherapeutant was then added to the static culture tank and the above conditions were maintained for an hour after which normal operating conditions were resumed. In this type of treatment biofilters were exposed to the chemotherapeutant only after normal operations were resumed, at which time the chemotherapeutant would have been diluted by water volume residing in other compartments of the system.

Recycle bath treatments were conducted by leaving all processes in their normal mode with the only difference being that the make-up flow was turned off to prevent dilution of the chemotherapeutant. The chemotherapeutant was then added in aliquots throughout the system. Normal make-up flow operating conditions were resumed after one hour. During recycle bath treatments the biofilters were left connected to the main flow and as such were continually exposed to the chemotherapeutant during treatment.

Single static bath and recycle bath treatments with 9 ppm of chloramine-T were conducted first and then a multiple static bath treatment at 12 ppm consisting of three treatments given on alternate days. The hydrogen peroxide treatment consisted of one static bath treatment at 100 ppm.

Biofilter nitrification efficiency was calculated by subtracting the outlet concentration from the inlet concentration and dividing the difference by the inlet concentration.
The statistical significance of differences between removal efficiencies was determined using a one-tailed Wilcoxon paired-sample test (Zar 1974) on the mean of six biofilters. A non-parametric test was chosen because the data was not distributed normally.

The experimental protocol and methods described are in compliance with Animal Welfare Act (9CFR) requirements and were approved by the Freshwater Institute Institutional Animal Care and Use Committee.

**Results and Discussion**

After the 9 ppm single chloramine-T static bath treatment, ambient ammonia removal (AAR) increased 20% and challenged ammonia removal (CAR) decreased 5%. The AAR decreased 10% and the CAR decreased 9% after the 9 ppm single chloramine-T recycle bath treatment. After the set of multiple 12 ppm chloramine-T static bath treatments there was only a slight decrease in AAR while CAR decreased by 8%.

The 100 ppm single hydrogen peroxide static bath treatment caused almost total impairment of nitrification. Twenty-four hours after treatment the AAR was reduced by 84% and the CAR by 57%.

The primary goal in this research was to determine which of the chemotherapeutants evaluated affect biofilter performance, and how acutely; with the overall concern being the preservation of adequate water quality for fish rearing. As long as adequate water quality can be maintained, minor drops in biofilter efficiency can be tolerated. Within the recirculating aquaculture system used in these experiments, biofilter nitrification efficiency often fluctuates from 5-10% over a period of several days without significant effects on water quality (unpublished data). The authors chose to make the distinction between significant (p<0.05) and highly significant (p<0.01) statistical differences in biofilter efficiency because they felt that only highly significant differences would have a biologically significant effect on biofiltration and the resulting water quality. The effect of changes in nitrification efficiency on TAN concentrations, within the particular recirculating system used in this experiment, can be illustrated using Liao and Mayo's (1972) equation for calculating steady-state concentrations in recirculating systems. For example: assuming an initial TAN removal efficiency of 90%, a 10% decrease in TAN removal efficiency will increase the tank TAN concentration by 12%; a 20% decrease will increase it by 27%; while a 60% decrease will increase it by 170%.

All of the chloramine-T treatments caused a significant reduction of CAR while only the single 9 ppm chloramine-T recycle bath treatment caused a significant reduction of both AAR and CAR. In contrast, Noble and Summerfelt (1996) reported that treatment with 12 ppm of chloramine-T had no effect on biofilters at the Glenwood State Fish Hatchery, Utah.
The single 100 ppm hydrogen peroxide treatment caused significant reduction of both AAR and CAR. As there was limited literature available on the effect of hydrogen peroxide treatments on biofilters, the authors had to rely on anecdotal data for comparison. Bullock and others at the Freshwater Institute observed hydrogen peroxide treatments at 100 ppm to cause a major impairment of biofilter efficiency (unpublished data).

Only the single 9 ppm chloramine-T recycle bath, and the single 100 ppm hydrogen peroxide static bath had a highly significant impact on both AAR and CAR. These treatments should be avoided as they could cause major changes in water quality.

The multiple 12 ppm chloramine-T static bath treatment caused a highly significant effect on CAR only. This treatment could also cause significant impairment of water quality.

Irregardless of treatment type or concentration, chloramine-T and hydrogen peroxide consistently impaired nitrification. The severe impact of hydrogen peroxide would make it suitable for use as a chemotherapeutant in recirculating systems only if completely flushed out of the system before resuming normal operations. Chloramine-T could possibly be used with caution in a static bath treatment at the lowest concentration possible.

The results support the hypothesis that impairment of nitrification in fluidized-sand biofilters can be determined through challenging the biofilters with high concentrations of TAN. In all cases where AAR was significantly impaired, CAR was also significantly impaired.

Acknowledgements

This research was funded by the U.S. Dept. of Agriculture's Agriculture Research Service under agreement 59-1931-3-012 and any opinions, findings, conclusions, or recommendations expressed in this publication are those of The Conservation Fund or the authors and do not necessarily represent those of the USDA.

References


The Potential for the Presence of Bacterial Pathogens in Biofilms of Recirculating Aquaculture Systems

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Recirculating aquaculture offers the greatest potential for successful fish farming since it requires limited resources, and is independent of environmental conditions. However, this form of aquaculture presents a potential unacceptable public health risk. With growing concerns for increased antibiotic resistant organisms, controlling pathogenic microorganisms is paramount. Biofilms form on all aquaculture system components, incorporating microflora present in the water. Pathogenic microorganisms are found in this biofilm, causing recurring exposure to disease and the presence of asymptomatic carriers.

The project objective is to increase the understanding of pathogen incorporation into biofilms in recirculating aquaculture systems by determining the relationship of the various materials used in construction of those systems and the bacteria’s ability to attach.

Six freshwater, two mariculture, and one hydroponics facilities were sampled, with eight different types of material tested. Pathogenic bacteria were identified using BAM methods and the Becton-Dickinson BBL Crystal ID Kit.

Various pathogenic bacteria were identified, including Aeromonas hydrophila, other Aeromonads, Vibrios, Yersinias, and Bacillus cereus. Some of the microorganisms identified are pathogens for both fish and humans and can be significant in further processed seafood. Bacillus cereus is a spore-forming bacteria which is thermally resistant, making it difficult to eliminate with normal food processing techniques. Vibrio species were found in the biofilms in both freshwater and saltwater systems. Some Yersinia species, such as Yersinia enterocolitica, are becoming more recognized as food or water-borne pathogens.

Whether the presence of these organisms in biofilms could lead to food-borne illness is unclear, but the potential is there. More research on pathogenic organisms in biofilms is required. The most significant variation in biofilm pathogens was observed in facility type and not construction material indicating increased biosecurity measures leading to pathogen elimination should also be investigated.

Aquaculture is one of the most rapidly growing areas of agriculture. With the decreasing numbers of wild fisheries, (Pauly et al., 2000) the demand for seafood will
need to be filled, and aquaculture will help to meet that demand. There are many methods of aquaculture including net pen farming, raceway systems, pond systems and recirculating systems. Recirculating aquaculture systems reuse water, making them environmentally friendly because there is less waste produced and the systems use less space. They also use less water than raceway systems, and there is less danger of contamination of the food source when compared to some pond systems. A disadvantage of recirculating systems is that if a pathogenic organism is introduced into the system, it may survive in the system indefinitely. This can lead to tremendous economic losses for the facility, as well as the possibility of asymptomatic fish being ingested by humans.

One component of the recirculating systems that may harbor pathogenic organisms is the biofilm that forms the water/solid interface on tanks and equipment. Biofilms are common in nature and grow at the water/solid interface in most all biological systems. They are found on medical implants, on surfaces in streams, and lead to plaque on teeth. (Costerton et al., 1987) Biofilms are responsible for the deterioration of ship hulls and underwater building supports. (Geesey et al., 1992) Research has also been done investigating the presence of biofilms formed by Listeria monocytogenes on food preparation surfaces. It was found that exposing L. monocytogenes in a biofilm to sublethal treatments of antimicrobials or stresses would result in a unique adaptive response and the stress of starvation was often accompanied by an increase in cell surface hydrophobicity and adhesiveness, with a decrease in cell size. (Smoot and Pierson, 1998a; Smoot and Pierson, 1998b; Wong, 1998). These cellular changes could lead to food contamination if inadequate amounts of cleaners and sanitizers are used on food contact surfaces.

Biofilms are often multispecies cultures attached to surfaces. The bacteria live in glycocalyx-enclosed microcolonies whose location, size and shape are determined by nonrandom species-specific factors. Primary colonizers may not all produce an exopolymer, but those that do may attract other bacteria which have no propensity for attachment from the planktonic phase. The multispecies biofilms form highly complex structures with cells arranged in clusters or layers with anastomosing water channels which bring nutrients to the lower layers and remove waste products. As the biofilm develops some cells will slough and become planktonic.

Biofilm formation is a response by microorganisms to alterations in growth rate, exposure to subinhibitory concentrations of certain antibiotics, and growth on solid surfaces (Brown and Gilbert, 1993; Kerr et al., 1999; Sasahara and Zottola, 1993; Smoot and Pierson, 1998a; Smoot and Pierson, 1998b; Stoodley et al., 1999; Yu and McFeters, 1994). The sessile cells of biofilms are very different from planktonic cells. There are differences noted in cellular enzymatic activity, cell wall composition, and surface structures between bacterial cells adherent to surfaces and planktonic cells of the same organism. The molecular composition of bacterial cell walls is essentially plastic and is very responsive to the cell’s growth environment. Environmental signals and cellular structures required for adhesion to intestinal epithelium, nonnutritive abiotic surfaces and nutritive, abiotic surfaces are distinct. (de Franca and Lutterbach, 1996; Watnick et al., 1999).
The main advantage for microorganisms to form biofilms is that the organisms are protected from the effects of an adverse environment and host immune defenses. The multispecies culture can provide and maintain the appropriate physical and chemical environments for growth and survival. In many cases the organisms in the biofilm develop a resistance to antimicrobials including surfactants, heavy metals, antibiotics, phagocytic predators and drying. (Brown and Gilbert, 1993; Lilved and Landfeld, 1995; Ronner and Wong, 1993; Yu and McFeters, 1994)

There are many organisms in the aquatic environment affecting the health of aquaculture raised fish that can lead to disease in humans. Some are opportunistic pathogens, living freely in the environment and only causing disease if the individual is immunocompromised or if environmental conditions are sufficient. Some of these organisms are obligate pathogens. Obligate pathogens usually do not remain viable in the environment for long periods, though it is unknown if pathogens survive longer in biofilms.

Most of the bacteria are ubiquitous in an aquatic environment and humans and fish are exposed to them regularly. Examples of this are the *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* and *Proteus* species. These bacteria are found in the normal intestinal flora of most animals. Coliform infections in man are most commonly caused by *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia marcescens*, and *Proteus mirabilis*. *Citrobacter* species can also cause disease, though they are not as significant and are usually pathogenic in individuals with predisposing conditions.

Shigellosis and vibriosis are caused by organisms that are associated with poor sanitation. *Shigella* species are related to *E. coli*, with *S. dysenteriae* and *S. flexneri* the most virulent, though in some parts of the world, *Shigella sonnei* is most virulent. (Hale and Keusch, 1996) The disease in humans is dysentery, a severe gastroenteritis. *Vibrio cholerae*, which causes cholera, is found in water and will persist in shellfish and plankton in beds that have been contaminated with polluted effluent. *V. parahemolyticus* causes enteric disease in humans who eat raw or improperly cooked seafood. *Vibrio vulnificus* can cause wound infections and septicemia in humans, though it is most commonly seen in immunocompromised patients. Vibriosis in fish is most common in marine species, though it has been isolated from freshwater fish. (Roberts, 1985) Disease outbreak varies with the temperature, the virulence of the strain present, and the amount of environmental stress present. *V. anguillarum* is the most common causative agent in vibriosis of fish, though *V. vulnificus* can also cause disease.

*Bacillus* species are spore producing bacteria. The spores are resistant to host physical and chemical environments and can survive in harsh environments, such as deserts, hot springs, frozen ground, fresh water and marine environments. (Turnbull, 1996) The most significant species is *B. cereus*. This bacterium is implicated in food-borne illnesses and is emerging as a very important pathogen due to its hardiness. It
produces two hemolysins that are necrotic and toxemic in nature. Other *Bacillus* species may cause food poisoning, but their significance is unknown.

*Pseudomonas* species are also ubiquitous in the environment. *P. aeruginosa* is the most common etiological agent of pseudomonas infections in man. *P. maltophilia* causes opportunistic infection in man. In fish, *P. anguilliseptica* causes red spot disease. *P. fluorescens*, *P. putida* and *P. putrefaciens* are often isolated from the soil and water and may cause either primary or secondary disease in fish.

*Yersinia enterocolitica* and *Y. pseudotuberculosis* have been implicated in food borne illness. They both cause an acute gastroenteritis that mimics appendicitis. *Y. enterocolitica* can survive refrigeration temperatures and low oxygen environments, so it has been isolated from refrigerated foods. *Yersinia ruckerii* causes enteric redmouth disease in salmonid species of fish. It has been isolated from other fish species as well, indicating a possible reservoir. (Furones et al., 1993a; Roberts, 1985a) *Y. ruckerii* is considered to be an obligate pathogen, though it can survive in the aquatic environment for several months. It is felt that farmed fish may be more prone to clinical disease caused by this organism because of the environmental stress involved. (Plumb, 1999a; Plumb, 1999a)

*Paseturella multocida* and *P. hemolytica* are bacteria that can cause disease in humans, though most often they are related to cat and dog bites. *Photobacterium damsela*, previously known as *Paseturella piscicida*, causes a pseudotuberculosis type disease in marine fish. It has been found that *P. damsela* does not live in estuarine water for more than 4 – 5 days so transmission is most likely horizontal from carrier fish. (Toranzo et al., 1982)

*Aeromonas* species are commonly found in the aquatic environment. Most aeromonads are opportunistic pathogens. *A. hydrophila* is found in the normal flora of fish. It has also been implicated in diseases in man, though it is not common. (Goncalves et al., 1992a) *Aeromonas salmonicida* is an obligate pathogen of fish. It causes furunculosis primarily in freshwater salmonid species such as trout.

Streptococci are considered part of the normal flora of animals and man. There are many species of streptococci and many of them are virulent. These bacteria cause multiple forms of human disease including scarlet fever, rheumatic heart disease, glomerulonephritis, and pneumococcal pneumonia. (Patterson, 1996) Group A streptococci are most commonly associated with human disease though other species can be pathogenic. Streptococcciosis can be seen in fresh water and saltwater fish. The susceptibility of fish to streptococcus infection is most probably due to environmental stress. It is believed that the bacteria are found in the water and sediment around fish cages. (Kitao et al., 1979) Seven species of *Streptococcus* which have been isolated from diseased fish are *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. equisimilis*, *S. faecium*, *S. pyogenes* and *S. zooepidemicus*. *Streptococcus iniae* is another *Streptococcus* species which is becoming more prevalent as a fish pathogen. There is some concern that this
organism causes disease in humans, though there is little conclusive evidence to indicate this. (Plumb, 1999)

Other aquatic organisms associated with disease outbreaks in fish and humans are *Plesiomonas shigelloides, Edwardsiella tarda* and *E. ictaluri*. *Plesiomonas shigelloides* is considered to be an opportunistic pathogen mostly seen in young, overcrowded tilapia and in immunocompromised people. *Edwardsiella* species are primarily piscine pathogens causing non-specific lethargy, internal abcessation, and skin inflammation. Edwardsielliosis is not confined to fish, having been isolated from birds, humans and other animals. (Plumb, 1999)

Other organisms found in aquatic environments that are of public health significance are *Clostridium botulinum, Listeria monocytogenes*, and *Campylobacter* species. These are all considered food-borne pathogens which cause gastrointestinal disease or neurological symptoms.

In recirculating aquaculture systems, it is possible that the pathogens causing a disease outbreak have become incorporated into the normal biofilm. When the conditions are right, planktonic cells are released. If the fish are in a stressed condition, another outbreak may occur. The purpose of this research was to determine the presence of piscine and human pathogenic bacteria in biofilms of recirculating aquaculture systems. If pathogens were isolated, the correlation between the number of pathogens recovered, the history of disease at the facility and the surface material was determined.

**METHODS:**

Biofilms were sampled by swabbing the surface with sterile culturettes. When pipes were sampled water flow was temporarily discontinued. Tank walls were sampled just above the water surface. Rotating biological contactors (RBC) and other filter types (e.g. fluidized beds) were sampled on surfaces that were out of the water.

The culturettes were then streaked onto McConkey’s Agar, Trypticase Soy Agar (TSA), 5% Blood Agar and Marine Agar if the sample was from a mariculture facility. The plates were then incubated at 35°C for 24 hours. Colonies were removed from plates by differing morphology and streaked on TSA plates. The TSA plates were then incubated another 24 hours at 35°C. Using growth from the TSA plates the organism was Gram stained. Oxidase and indole tests were performed on Gram negative organisms, and the growth media for the non-fermenting enteric BBL Crystal™ ID Kit was inoculated. The morphology of Gram positive organisms was noted and the growth media for the Gram-Positive BBL Crystal ID kit was inoculated. The kits were incubated for 18 – 24 hours at 35°C.

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*Becton Dickinson Microbiology Systems, Becton Dickinson and Company, Cockeysville, MD 21030*
RESULTS AND DISCUSSION:

Nine different commercial or research recirculating aquaculture facilities were sampled. Two were mariculture facilities, growing flounder. The six freshwater facilities were all producing tilapia. There was one hydroponics research facility that was circulating filtered fish wastewater through plant beds before being sent back to the fish tanks.

The nine facilities tested used different materials. Almost all of them used polyvinyl chloride (PVC) for water pipes. Many of the systems used fiberglass and/or plastic tanks. Other substances used in the aquaculture systems tested were glass, stainless steel, rubber, aluminum, foam and cement. An unbalanced random block statistical model was used to test whether pathogen growth was influenced by the type of material, the type of facility, and the history of disease, or whether all of them were significant.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Facilities</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1 Fiberglass/Plastic</td>
<td>1</td>
<td>17</td>
<td>9</td>
<td>1</td>
<td>12</td>
<td>4</td>
<td>7</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2 PVC</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>11</td>
<td>2</td>
<td>1</td>
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<td>6</td>
</tr>
<tr>
<td>3 Other</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>5</td>
<td>2</td>
<td>16</td>
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</tr>
</tbody>
</table>

| History of Disease | N | N | N | Y | N | N | Y | Y | Y |

Figure 1.

Figure 1 shows the data used to determine the statistical analysis. The SAS System for Windows V7™ program was used to determine the relationship between the growth of pathogens and the type of material. The p value was well above the 5% significance level, indicating the type of material was not a significant factor in determining the presence of pathogenic bacteria. A similar response was obtained when comparing the history of disease. Running a comparison of the least squares means and doing Tukey’s test supports this. However, when comparing facilities the p-value = 0.0027. This indicates that at least one facility showed a significant difference in pathogen growth.

SAS Institute, Inc., Cary, NC 27513
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>No. of Facilities</th>
<th>No. of Different Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter spp.</td>
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<td>3</td>
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<tr>
<td>Aeromonas hydrophila</td>
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<td>7</td>
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<tr>
<td>Aeromonas spp.</td>
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<td>7</td>
</tr>
<tr>
<td>Bacillus cereus</td>
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<td>4</td>
</tr>
<tr>
<td>Bacillus spp.</td>
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</tr>
<tr>
<td>Citrobacter freundii</td>
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<td>4</td>
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<td>Enterobacter cloaceae</td>
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<td>4</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
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<td>3</td>
</tr>
<tr>
<td>Escherichia coli</td>
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<td>3</td>
</tr>
<tr>
<td>Photobacterium damsela</td>
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</tr>
<tr>
<td>Plesiomonas shigelloides</td>
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</tr>
<tr>
<td>Serratia marcescens</td>
<td>2</td>
<td>4</td>
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<tr>
<td>Shigella spp.</td>
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</tr>
<tr>
<td>Sphingobacterium multivorum</td>
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<tr>
<td>Sphingomonas spp.</td>
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<td>4</td>
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<tr>
<td>Vibrio cholerae</td>
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</tr>
<tr>
<td>Vibrio spp.</td>
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<td>3</td>
</tr>
<tr>
<td>Vibrio-like spp.</td>
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<td>4</td>
</tr>
</tbody>
</table>

* Organisms were listed in this category if the test kit response was a Vibrio spp. or an Aeromonas spp. but not one or the other specifically.

The isolated and identified organisms are listed in figure 2. All of the bacteria listed are potential human pathogens. Some of them, such as *A. hydrophila*, other *Aeromonas* species, *P. damsela*, *P. shigelloides* and the *Vibrio* species, are also fish pathogens. Many of the organisms isolated could not be definitively identified. This is a shortcoming of any of the rapid test kits, as they are designed to identify human pathogens. If the bacteria isolated are strictly fish pathogens or if they are normal, non-pathogenic flora, the test kits will probably not identify them. An example in this instance is that one organism isolated was identified as *Yersinia pestis*. *Y. pestis* is not considered a water borne bacteria. In this case the organism was probably misidentified, though a positive identity was not determined. It may have been *Y. ruckerii* or a normal, non-pathogenic organism that produced test results similar to *Y. pestis*. Misidentification with the test kits could occur if biofilm bacteria react differently to the biochemical tests than the planktonic counterparts. This raises the possibility of the presence of other pathogens which are unidentifiable with this method.
CONCLUSIONS:

Bacterial pathogens, both fish and human, are present in the biofilms of recirculating aquaculture systems. Most of them are ubiquitous in an aquatic environment and are opportunistic pathogens. The bacteria can grow on any surface tested in this case, and environmental conditions such as salinity and water temperature are the determinant factors for species of bacteria present. The numbers and types of organisms present may be underrepresented. If bacteria alter their chemical functions to survive as sessile organisms, they may not respond to the chemical tests in a predictable manner.

The potential for food-borne illness as a result of consuming fish raised in recirculating systems is unclear, but with the increased consumption of rare fish and the increased survival of immunocompromised persons, there is a possible risk. Also, cross contamination could occur in a processing facility between incoming and processed product. Raising fish in recirculating aquaculture systems exposes them to high density populations and other stressors. The presence of pathogenic bacteria in biofilms in these systems increases the possibility of recurring disease, with resultant economic losses to the aquaculturist.

The next step in research would be to determine the most effective methods to decrease pathogen presence. The aquaculture industry is heavily regulated with regards to the use of antibiotics and chemicals in the water. The proliferation of species of antibiotic resistant organisms is a great concern because the antibiotics in use today are becoming increasingly ineffective. The environmental effect of antimicrobial treatment of wastewater must also be considered. Developing methods of elimination will require a better understanding of the viability of pathogens in biofilms, as well as a better knowledge of the sensitivity of sessile versus planktonic organisms to antimicrobials.

Another area of investigation would be in developing biosecurity measures in the aquaculture industry similar to those in the poultry industry. In the poultry industry hatchlings are introduced into one building and they never leave that building until time for slaughter. Then all the birds are removed, and the building is cleaned and sanitized. It is then left empty for a short period of time to aid in the further elimination of pathogenic bacteria which could affect the new flock. Keeping facilities pathogen-free is an impossible task, but if levels of pathogens can be kept below infective doses, the chance of fish becoming infected would be very low. For economic and public health reasons, the aquaculturist should strive for this goal.

References


Transmission of Furunculosis and Enteric Redmouth by Means of Fluidized Sand Biofilters

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Abstract

Two bacterial pathogens of salmonids, *Aeromonas salmonicida* cause of furunculosis, or *Yersinia ruckeri*, cause of enteric redmouth, were added for five days to the pump inlet of a recirculating culture system, without fish, in order to determine if the pathogens would become established in the fluidized sand biofilters and infect newly added salmonids. Before addition of the cultures, the biofilters were sampled and found free of both pathogens. However, both pathogens could be cultured from biofilters after cultures were added. When culture addition was completed the recycle system, except for biofilters, was disinfected with 200 ppm chlorine for two hours. Biofilters were continuously washed for 24 hr with springwater. Rainbow trout (*Oncorhynchus mykiss*) to detect *Y. ruckeri*, or Arctic char (*Salvelinus alpinus*), to detect *A. salmonicida*, were then added to the recycle system. Three weeks after addition of rainbow trout, *Y. ruckeri* was cultured from feces and trout began dying from enteric redmouth. Two weeks after char were added clinical furunculosis was diagnosed and *A. salmonicida* was isolated from survivors. After enteric redmouth or furunculosis had been transmitted from biofilters, fish were removed, and the entire system was treated with 10 ppm chloramine-T in an attempt to remove the pathogens from the biofilters. The system was then restocked with either char or rainbow trout. After six weeks neither furunculosis nor redmouth occurred and neither pathogen could be isolated from salmonids or biofilters. Biofilter transmission experiments were repeated with both pathogens, and an additional procedure for disease transmission tested.

Introduction

Infectious diseases are a major cause of mortality in intensive aquaculture. Precautions such as use of specific pathogen frees stocks of fish, clean water supply, proper sanitation, and biosecurity are necessary to reduce disease outbreaks. In a recirculating culture system another possible source of pathogens is biofilters. If a disease outbreak occurs there is a possibility the pathogen may become established in biofilters and infect newly stocked fish. Several reviews have described disease problems in recirculating...
systems (Egusa, 1981; Mellergaard and Dalsgaard, 1987). Recently, Noble and Summerfelt (1996) described diseases that occurred in a rainbow trout (Oncorhynchus mykiss) recirculating system and mentioned that biofilters were cultured for \textit{A. salmonicida} during a furunculosis outbreak, but the pathogen could not be isolated (Cipriano et al., 1996). The present work was undertaken to determine if either \textit{A. salmonicida} or \textit{Y. ruckeri} could be established in fluidized sand biofilters and could infect newly stocked salmonids.

**Materials and Methods**

All infectivity trials were conducted in two recycle systems, each having the configuration shown in Figure 1. The culture tank was 1.5 m in diameter and stocked with 60-80 kg of rainbow trout or arctic char (\textit{Salvelinus alpinus}). Water flowed from the culture tank into a drum filter containing 100 µm screen and was then pumped at 8 L/min per filter to six fluidized sand biofilters, each 2.5 m tall and 0.17 m in diameter. Water leaving the biofilters fell by gravity through two columns containing 5-cm plastic Norpac media (NWS Corp., Roanoke, VA) to remove carbon dioxide. Water discharged from the two packed columns was piped into the culture tank (Figure 1). Water temperature in the system ranged from 14-17 °C and oxygen levels were maintained between 9-12 ppm. Samples of biofilm-coated sand from all biofilters were cultured before beginning infectivity trials to insure \textit{A. salmonicida} or \textit{Y. ruckeri} were not present. Bioparticle samples were taken, processed and quantified as previously described (Bullock et al., 1993) and cultured onto Coomassie brilliant blue (CBB) agar plates (Udey, 1982) to detect \textit{A. salmonicida} and Shotts Waltman (SW) plates (Waltmann and Shotts, 1984) to detect \textit{Y. ruckeri}. Suspect \textit{A. salmonicida} colonies were dark blue and 1-2 mm in diameter after 48 hr growth on CBB. Blue colonies were transferred to tryptic soy agar plates and confirmed as \textit{A. salmonicida} if they produced a brown water soluble pigment on TSA and were cytochrome oxidase positive, fermentative in O/F glucose, and nonmotile in a hanging drop. After 48 hr growth on SW, suspect \textit{Y. ruckeri} colonies were 1-2 mm in diameter and surrounded by a zone of precipitation caused by degraded Tween 20 and calcium chloride. Suspect colonies were confirmed as \textit{Y. ruckeri} if they showed an acid slant in triple iron agar slants and a positive slide agglutination test with type I anti \textit{Y. ruckeri} antiserum. Stocks of rainbow trout were obtained from the National Fish Health Research Laboratory and the Freshwater Institute, while arctic char (Labrador strain) were obtained from the Freshwater Institute. Although neither rainbow trout nor char had been exposed to furunculosis or enteric redmouth, feces from 25 rainbow trout and mucus from 25 char were cultured onto CBB or SW plates to insure these pathogens were absent. Two types of infectivity procedures were conducted in which all fish were removed from the system and broth cultures of \textit{A. salmonicida} or \textit{Y. ruckeri} were continuously added to the system at the pump intake, for five days using a peristaltic pump. On the first and fifth day, bacterial cells in the broth cultures were quantified using serial log_{10} dilutions and the drop plate technique of Miles and Misra (1938). Fish tank water and biofilters were then cultured for the pathogen under study. In the first infectivity procedure the entire culture system, except for biofilters, was disinfected for 2 hr with 200 ppm chlorine and the biofilters continuously washed with spring water for 24 hr after which fish were added. Fish were observed for clinical
disease and posterior kidney of mortalities streaked onto CBB or SW. If no mortality occurred in three to four weeks, mucus or feces from 25 fish and all biofilters were cultured on CBB or SW. In the second procedure, the entire system was treated with 10 ppm chloramine-T for 1 hr and the system was then rinsed with spring water for 1 hr. The system was stocked and fish observed for clinical disease. If no disease occurred in six weeks mucus or feces and all biofilters were cultured for the pathogen under study. In trials where chlorine was used to disinfect the system and spring water used to wash biofilters and disease occurred, fish were removed and the entire system disinfected with 10 ppm chloramine-T and new fish added. These fish were observed for disease for six weeks and then cultured if disease did not occur. The experimental protocol and the methods described were in compliance with the Animal Welfare Act (9CFR) requirements and were approved by the Freshwater Institute Institutional Animal Care and Use Committee.

Figure 1. Recirculating system used in bacterial transmission experiments.

Results and Discussion

Results of trials using the chlorine disinfection and biofilter washing procedure are given in Table 1. In all 3 trials, the pathogens were easily cultured from biofilters after the five day addition of culture and in 2 of the 3 trials they could be cultured after the 24 hr washing of biofilters. Clinical furunculosis or enteric redmouth occurred within 11-21 d after the fish were stocked. Prevalence of the pathogens in mucus, feces or biofilters varied regardless of whether fish and biofilters were sampled before or after mortality occurred. In trial 1 with *Y. ruckeri*, where trout were sampled before mortality occurred, the pathogen occurred in 6 of 25 fecal samples and one biofilter. However, in trial 2, fish were sampled after mortality occurred and *Y. ruckeri* was present in only 2 of 25 fecal samples and in none of the biofilters. In the *A. salmonicida* trial, char were sampled after mortality occurred and only 2 of 25 mucus samples were positive while all biofilters were positive for the pathogen. When rainbow trout and char were removed from the
recirculating system after mortality occurred and systems, including biofilters, disinfected with 10 ppm chloramine-T and new fish added neither enteric redmouth nor furunculosis occurred within six weeks after restocking. Culturing feces, mucus or biofilters also failed to show the pathogens.

Transmission of *Y. ruckeri* and *A. salmonicida* by disinfecting the entire system with 10 ppm chloramine-T immediately after the five day culture addition, procedure 2, failed to produce clinical disease in rainbow trout or char. Culture of feces from 25 rainbow trout 3 wk after chloramine-T disinfection failed to show *Y. ruckeri*, while 1 of six biofilters was positive for the pathogen. Five wks after chloramine-T disinfection none of the 25 mucus samples from char was positive for *A. salmonicida*, while one of six biofilters was positive. Monitoring of both rainbow and char is being continued.

Our results to date show that when *Y. ruckeri* or *A. salmonicida* are continuously added to our recycle system for five days these bacteria remain in the biofilters, even after 24 hr of continuous washing, and can produce overt disease in rainbow trout or char. However, if infected fish are removed and the entire system disinfected with 10 ppm chloramine-T and then restocked no disease occurs and the pathogens cannot be cultured from feces, mucus, or biofilters. When the entire culture system is disinfected with 10 ppm chloramine-T and fish stocked no disease occurs and bacteria cannot be isolated from feces or mucus, but can be isolated from biofilters. It is possible that chloramine-T disinfection reduces the pathogens to a level where months may be required before overt disease is produced. However the presence of pathogens in the biofilters is a threat for newly stocked fish. We will continue to monitors fish and biofilters over several months to determine if disease will occur if pathogens are not added again and new fish stocked into the systems.

Table 1. Transmission of *Yersinia ruckeri* and *Aeromonas salmonicida* after rinsing biofilters and disinfection of the rest of the system with chlorine.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1 <em>Y. ruckeri</em></th>
<th>Trial 2 <em>Y. ruckeri</em></th>
<th>Trial 1 <em>A. salmonicida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 day culture addition</td>
<td>6.0 x 10⁹/ml</td>
<td>6.0 x 10⁹/ml</td>
<td>2.5 x 10⁹/ml</td>
</tr>
<tr>
<td>Pathogen in system after culture addition</td>
<td>1/6 biofilters + tank water +</td>
<td>3/6 biofilters + tank water +</td>
<td>6/6 biofilters + tank water +</td>
</tr>
<tr>
<td>Pathogen in system After disinfection</td>
<td>not done</td>
<td>1/6 biofilters + tank water -</td>
<td>1/6 biofilters + tank water -</td>
</tr>
<tr>
<td>Mortality after restocking</td>
<td>21 days</td>
<td>11 days</td>
<td>15 days</td>
</tr>
<tr>
<td>Prevalence before or after mortality</td>
<td>6/25 feces + 1/6 biofilters + before mortality</td>
<td>2/25 feces + 6/6 biofilters + after mortality</td>
<td>2/25 mucus + 6/6 biofilters + after mortality</td>
</tr>
<tr>
<td>Presence of pathogen after Chloramine-T</td>
<td>0/6 biofilters + tank water -</td>
<td>not done</td>
<td>0/6 biofilters + tank water -</td>
</tr>
<tr>
<td>Mortality after restocking Or presence of pathogen</td>
<td>no mortality 6 wk biofilters and feces -</td>
<td>no mortality 6 wk biofilters and Feces -</td>
<td>no mortality 6 wk biofilters and Mucus -</td>
</tr>
</tbody>
</table>
Acknowledgments

This work was supported by the United States Department of Agriculture, Agricultural Research Service under agreement number 59-1930-8-038. Any opinions findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the USDA.

References


Fish Health Monitoring and Maintenance of Small Scale Recirculating Systems in Virginia

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The strong interest in aquaculture in the United States and especially in Virginia is resulting in many individuals considering aquaculture as a business opportunity. The Virginia Fish Farmers Association has taken the approach that small recirculating system growing tilapia integrated with hydroponics may be the best option. Many of these systems, but not all, are self-designed and unproven to generate adequate and profitable production. Nevertheless, these individuals are committed to learning and growing fish in small systems. Many of these systems are designed to generate between 25,000 to 100,000 lbs. per year.

As with every production system, there are inherent problems. Fish health concerns with recirculating systems are a topmost priority with the tilapia industry. The Virginia State University Aquaculture Diagnostic Laboratory has responded to many of this small scale recirculating system producer requests for assistance with fish health problems. The disease laboratory has assisted producers using small scale recirculating systems since 1993. Fish health cases from recirculating systems ranges from about 25% to 50% per year of the total casework for the VSU Fish Disease Laboratory. The goal of keeping fish healthy means that a small-scale producer will have a marketable fish to sell. This paper summarizes specific fish health problems associated with recirculating systems in Virginia. This paper also provides brief guidelines for fish health monitoring and maintenance of these using small-scale systems in Virginia based on recent diagnostic casework.

Inspections/Routine Checks

Some producers have requested their fish be examined for fish health problems as a preventive measure or for fish health inspection for specific pathogens. This categorization of inspections/routine checks represents one third to one-half of the casework from Virginia producers using recirculating systems. VSU laboratory receives on the average of 15 cases from recirculating systems per year. VSU laboratory has done numerous inspections for specific pathogens (using AFS Fish Health Section bluebook standards for detecting specific pathogen) and routine checks (necropsy of 5 to 20 fish for external parasites and gross pathology) for producers that are primarily raising tilapia. From 1996 to 1999 laboratory has done 39 inspections/routine checks.
Parasites

Clinical signs showed that in many cases tilapia had high numbers of external parasites but showed no signs of a disease outbreak. *Ichthyobodo (Costia)* constituted the most commonly found the external parasite. *Ichthyobodo* was found about 50% of the time during these inspections/routine checks. Numbers found on the fish were considered to be of significant threat to cause a possible disease outbreak. Under these circumstances, it was recommended to the producers to use formalin at 15 to 25 ppm as an indefinite treatment. Routine check follow-ups to treatments showed that treatment with formalin was effective in controlling these parasites without upsetting the balance of the biological filter.

Other parasites found, but not as frequently, in significant number were *Trichodina* and *Gyrodactylus* (skin flukes). Formalin also was effective in controlling these parasites.

Bacteria

No bacteria were recovered from any inspections/routine checks. *Streptococcus* was the specific pathogen targeted by these checks. No *Streptococcus* was recovered at any time from checks. Only time that this VSU laboratory recovered *Streptococcus* was in 1995 from tilapia during an epizootic in progress. *Streptococcus* inspection were done using AFS Fish Health Section Blue Book Standards for sample size (sixty fish) for detecting specific pathogens. CNA agar, which is designed for recovering gram positive bacteria, was used as the media for *Streptococcus* detection. Fish brain was used as the inoculum onto the plate.

Outline of Fish Health Maintenance

The first and most important step in maintaining healthy fish is to implement best management practices. A facility operating under poor cultural management will consistently have fish diseases. Facilities that have best management practices will have healthy fish.

The second step in maintaining healthy fish is to identify possible stressors. Fish stress is the primary initiator of diseases at a production facility. Management practices should avoid stressing fish during production.

Quarantine Facility

Regardless of facility size, it should have a quarantine facility that is separate or isolated from the rest of the facility. The quarantine facility is the most important management practice that can be done towards preventing the introduction of diseases into the facility. The quarantine room should be physically separation either by rooms, walls or even by building. Quarantined fish should be held for at least two weeks, before introducing new fish into production facility. After two weeks, fish should be checked for potential
disease problems. For tilapia, a specific pathogen examination should be done for *Streptococcus*. The *Streptococcus* check should come about 1½ to 2 weeks before moving fish. Before introduction of new fish into quarantine room, it should be clean from last use.

**Floors**

Try to avoid the use of dirt or gravel floors at a facility. These types of floors are not suitable or conducive for keeping facility clean. Debris and fish material works itself down in the gravel setting up possible contamination to tanks. Concrete floors can be power spray down to wash away debris and other materials out of building. An operation should have the ability to wash down everything with a high-pressure system. Keeping things clean and washing away all fish debris are critical for a good operation.

**Prophylactic Treatments**

Fish should be treated prophylactically each time when moved to the next stage of production. Formalin can be used either as an indefinite or as a short-term bath treatment. The range for treatment is 15 to 25 ppms. Using 20 ppm has been used to treat systems with success. For bath treatment, formalin can be used at 125 to 250 for up to one hour. Only use a bath treatment, if a tank can be isolated and is capable of being flushed out.

**Medicine Cabinet**

Preventing disease outbreaks in tilapia is critical due to the lack of FDA approved chemicals and antibiotics. At this time, the only approved chemotherapeutic treatment for tilapia is formalin. Efforts are underway to obtain approval for other chemotherapeutic treatments such as Terramycin and Romet 30. These antibiotics are presently available and approved for use in the catfish and trout industry to control bacterial infections.

**Water Quality**

The vital key in the prevention of disease outbreaks at a facility is water quality. It is well known that recirculating systems water quality can go from good to bad very quickly. When this happens, formidable disease problems often appear and seem to never go away. Poor water quality episodes can be the cause of fish diseases weeks later. Monitoring and managing of water quality parameters on a daily schedule are necessary and should be a mandatory management practice for recirculating facilities.

**Broodstock and Fingerlings**

In order to keep the risk of introducing diseases to your facility from stock coming from an outside production facility, one should think about developing their own brood stock and fingerlings facility to produce the needed fingerlings needed for the operation. This will allow for control over fish health issues at all stages of production. Clean broodstock
should produce clean fingerlings. When buying fingerlings from outside sources you never know what the fish have been exposed to (esoteric and unusual pathogens) or what they are carrying.

System Setup

Having all production on one or two systems can lead to disaster. If disease outbreaks occur, the entire production facility will become infected with the disease. When developing a production facility, a fish health management plan should be used to guide construction. A producer should have a production system that is easily treatable and manageable for diseases. A facility that uses a few large units would most likely have difficulty in treating for a disease outbreak. This would result in shutting down the system for disinfecting and having no production capabilities for months. If a disease gets into a facility with many small units, individual systems can be isolated and disinfected without total facility shut down.

Summary of Fish health Maintenance and Monitoring Recommendations

2. Quarantine system that can be physical separated from the main system.
3. Prophylactic treatments for external parasites.
4. Water quality monitoring program.
5. Development of on site broodstock and fingerling production.
6. Fish health training for the producer.
7. Multiple systems for controlling disease outbreaks.
The Role of Fish Density in Infectious Disease Outbreaks

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Introduction

In food animal production, crowding of animals in the necessarily intensive culture environment is well-known to increase the risk of infectious disease outbreaks. In aquaculture, estimation of carrying capacity on a loading rate (kg/L/min) basis accounts for consumption of oxygen and feed and production of carbon dioxide and ammonia (Westers, 1981; Colt and Watten, 1988). The effect of density (kg/m³) on fish behavior and product quality (e.g., fin erosion and skin abrasion) are also considered (Bosakowski and Wagner, 1994; Wagner et al., 1996; Wagner et al., 1997). Acute and chronic losses due to infectious disease outbreaks in aquaculture can impose a significant cost to productivity and, therefore, to profit. A few field studies have demonstrated that mortality during infectious disease outbreaks is higher at higher fish densities (Fagerlund et al., 1984; Mazur et al., 1993; Banks, 1994; LaPatra et al., 1996). Also, some general “rules of thumb” for suggested densities to avoid infectious disease outbreaks have been published for salmonid culture (Wedemeyer and Wood, 1974; Piper et al., 1982). However, details about the relationship between fish density/loading rates, pathogen concentration and the risk of infectious disease outbreaks have not been studied. This relationship should be quantified so that it can be included in estimates of system carrying capacity and in the development of risk analyses for aquaculture production.

We used a disease model to explore three questions about the relationship between fish density, pathogen concentration and characteristics of infectious disease outbreaks. 1) As fish density and pathogen concentration increase, how does this change affect the probability of survival for an individual fish? 2) How does the maximum population death rate change as pathogen load changes? 3) As pathogen load changes, what is the effect on the time at which the maximum death rate is reached?

The model system

The design requirements for studies that will answer these questions make it practically impossible for them to be done in a commercial aquaculture facility. Large numbers of fish and tanks are needed. The requirements can be restrictive even for a well-equipped...
aquaculture research laboratory. After considering these requirements, we chose infectious pancreatic necrosis (IPN) as our disease model. IPN, which is a viral disease, is an appropriate model because it affects small salmonid fish. Therefore, small tanks containing large numbers of fish can be used in an investigation.

Young rainbow trout (34 days old) were used for the study. Conditions were set up to mimic rainbow trout fry culture in flow-through conditions. The study consisted of two experiments. The first lasted 59 days and the second lasted 53 days. The day before each experiment began, all of the fish in a single tank were infected with the IPN virus. These fish will be referred to as infectious, or INF, fish. The next day, these INF fish were added to tanks containing various densities of uninfected, susceptible fish (Table 1). Either 1, 2 or 3 of the infectious fish were added. Additional tanks were set up that contained no INF fish or all INF fish. Tank densities ranged from about 23 to 162 kg/m$^3$. The total densities were chosen to be near to, or greater than, the density of 0.5 lbs/ft$^3$ recommended for culturing one inch rainbow trout (Piper et al., 1982) without any outbreaks of infectious disease. Tank volume was 1 liter of spring water at 54$^\circ$F. Tank turnover rate was 15 times per hour. This high flow rate was chosen to maintain good water quality at high fish densities. Each day dead fish were removed and counted. All remaining fish were counted at the end of each experiment.

Table 1. INF = Number of fish that were exposed to IPN virus (INF) and added to the tanks. Total density = total density of fish in the tank. X is a tank that was lost when water flow to the tank was disrupted.

<table>
<thead>
<tr>
<th>INF</th>
<th>Total density</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>15,15,25,25,26,30,30,34,37,45,48,50,59,60,74,75,75,75,76,87,90,90,98,100,104,105</td>
</tr>
<tr>
<td>2</td>
<td>14,15,27,28,45,45,60,60,75,75,89,92,105,105</td>
</tr>
<tr>
<td>3</td>
<td>14,15,25,25,25,30,30,45,49,50,52,X,57,60,64,75,75,75,76,81,90,90,95,100,105,105</td>
</tr>
<tr>
<td>All</td>
<td>15,15,30,30,45,45,60,60,69,75,75,86,90,105,105</td>
</tr>
<tr>
<td>0</td>
<td>14,15,25,26,44,45,48,49,72,73,74,75,96,97,103,104</td>
</tr>
</tbody>
</table>

Results

Effect on probability of survival for individual fish

The probability of survival to the end of the experiment was >0.98 for tanks where no INF fish were added (Fig 1A). As the number of INF fish added to the tanks increases, the probability of survival to the end of the experiment (S(t)) decreases (Fig. 1A). When each group of infectious fish is considered separately, the effect of density can be seen (Fig. 1b – Fig. 1d). For the tanks where INF = 1, the probability of survival is higher at densities <40 fish/L than it is at densities ≥40 fish/L (Fig. 1b). As the number of INF fish increases (Fig. 1c and Fig. 1d), the advantage of lower densities disappears until the probability of survival at given time point is about the same regardless of the culture density (Fig. 1d).
This apparent interaction between total density and number of INF fish has been confirmed with regression models.

**Figure 1a – 1d.** Survival curves. Fig. 1A: Each curve represents number of infectious (INF) fish added (l = 1 INF fish; h = 2 INF fish; c = 3 INF fish; y = All INF fish; ~ = 0 INF fish). Figure 1B: Survival curves for tanks where 1 INF fish added. Figure 1C: Survival curves for tanks where 2 INF fish. Figure 1D: Survival curves for 3 infectious fish added. For figures 1B – 1D, l = density < 40; g = 40 ≤ density < 80; c = density ≥ 80).

![Graphs showing survival curves](image)

**Effect on the maximum death rate and time to the maximum death rate**

When one infectious fish was added to tanks of susceptible fish, the maximum death rate was 0.18 fish/(fish⋅day) (Fig. 2a). As the number of infectious fish increased, the maximum death rate increased, until it reached 0.60 fish/(fish⋅day) in tanks where all fish were challenged by the IPN virus.

When one infectious fish was added to tanks of susceptible fish, the time taken to reach the maximum death rate was 26 days (Fig. 2b). As the number of infectious fish added
increased, the time to the maximum death rate decreased, until it reached 13.5 days for tanks where all fish were infectious.

**Figure 2a - 2b.** Fig 2a. Maximum death rate (fish/(fish·day)) vs. number of infectious fish added. Fig. 2b. Time to maximum death rate (fish/(fish·day)) vs. number of infectious fish added.

![Graphs showing maximum death rate and time to maximum death rate vs. number of infectious fish added](image)

**Discussion**

More quantification is needed before fish density/loading, pathogen concentration and the risk of infectious disease outbreaks can be used in carrying capacity and risk analysis estimates. However, this work contributes new information that describes how fish density and pathogen load will interact to affect characteristics of disease outbreaks in an aquaculture system. It also emphasizes the importance of effective biosecurity measures. The effect of fish density on the probability of survival for an individual fish will depend on the pathogen load. For example, when the pathogen concentration is lower (e.g., INF=1), fish in tanks containing lower densities have a greater probability of survival than fish in tanks containing higher densities. However, as the pathogen load increases (e.g., INF=2, INF=3), the advantage of lower density disappears, that is, at a given point in time, the probability of survival is similar regardless of density. Pathogen load also affects the maximum death rate and the time to the maximum death rate. The lower the pathogen concentration, the lower the maximum death rate and the longer the time taken to reach the maximum death rate.
References


