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Comparison of Bacterial Presence in Biofilms on Different Materials Commonly Found in Recirculating Aquaculture Systems

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ABSTRACT. Recirculating aquaculture offers an economic potenti for successful fish farming requiring limited resources. However, th form of aquaculture may present a potential unacceptable health risk f the fish. Pathogenic microorganisms may be incorporated into biofilm found in aquaculture systems, causing recurring exposure to diseas agents. The presence of *E. coli*, modified to express a green fluoresce

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protein (GFP *E. coli*) was used to evaluate bacterial incorporation into biofilms in recirculating aquaculture systems.

Disks of Buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, glass, fiberglass, and stainless steel disks, were placed in aquariums stocked with Nile tilapia, *Oreochromis niloticus*. Tanks were inoculated with a known amount of GFP *E. coli* and samples taken on days 1, 3, 7, and 15 post-inoculation. Within 24 hours post-inoculation bacterial presence was observed on all materials. The number of organisms decreased by day three, but remained consistent during the remaining period. There was no significant difference in bacterial presence between the different materials studied. This study demonstrated that bacteria can become incorporated into a biofilm with the potential for pathogen colonization of biofilms in aquaculture systems.

KEYWORDS. Aquaculture, bacteria, biofilms, *E. coli*, green fluorescent protein, pathogen, fish

INTRODUCTION

Recirculating aquaculture systems reuse water, making them environmentally friendly because there is less water used overall. However, 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 fish morbidity and mortality in the system with resultant economic losses for an aquaculture facility.

There are many organisms in an aquatic environment that can affect the health of aquaculture-raised fish. Most are opportunistic pathogens, living freely in the environment and only causing disease if the fish is immunocompromised or if environmental conditions are inappropriate. Obligate pathogens usually do not remain viable in the environment for long periods of time, though it is unknown how many of these pathogens survive in biofilms. It is interesting to note that Margarinos et al. (1994) found dormant, but viable, cells of *Photobacterium damsela*, an obligate pathogen of marine fish, survived in the water and sediment. In another study (King et al. 2004), this same organism was identified in biofilms in saltwater recirculating aquaculture systems.

Biofilms are common in nature and grow at the water/solid interface in most biological systems. They are found on medical implants, on

surfaces in streams, and lead to plaque on teeth (Costerton et al. 198 Wilderer and Characklis 1989; Geesey et al. 1992). Biofilms are respoi sible for the deterioration of ship hulls and underwater building suppor (Blenkinsopp and Costerton 1991; Geesey et al. 1992). Biofilms contai ing *Listeria monocytogenes* have also been found on food preparatic surfaces (Smoot and Pierson 1998a).

Biofilm formation is a response by microorganisms to alterations growth rate due to exposure to sub-inhibitory concentrations of certa antibiotics, or growth on solid surfaces (Brown and Gilbert 199 Sasahara and Zottola 1993; Yu and McFeters 1994; Smoot and Piersc 1998a; 1998b; Kerr et al. 1999). The main advantage of biofilm form tion is protection of the organisms from the effects of an adverse enviroi ment. A multispecies microbial culture can provide and maintain th appropriate physical and chemical environments for growth and su vival. In many cases, the organisms in the biofilm develop resistance t antimicrobials including surfactants, heavy metals, antibiotics, phag cytic predators, and drying (Brown and Gilbert 1993; Ronner and Won 1993; Yu and McFeters 1994; Costerton 1995; Liltved and Landfal 1995; Watnick et al. 1999).

Under certain conditions, planktonic bacterial cells are released fro biofilms (Toutain et al. 2004). With implantable devices the biofilm itse is not always pathogenic for the host, but the presence of biofilms ca trigger planktonic "showering," where free-swimming bacterial cel slough into the host's bloodstream, leading to sepsis (Ehrlich et al. 2004 If fish in a recirculating aquaculture system are in a compromised stressed condition, a disease outbreak could occur from plankton pathogens released into the water from the biofilm.

The purpose of this research was to determine the ability of a specifi bacterium to incorporate into biofilms formed on different substanc under laboratory conditions, using antibiotic-resistant *E. coli* expressin the green-fluorescent protein (GFP). Green- fluorescent protein is a ligh emitting cytoplasmic protein with the cDNA of GFP cloned to develop plasmid used to produce GFP as a marker for detecting bacterial presenc and viability (Prasher, et al. 1992). The GFP fluorescence is stable, inde pendent of bacterial species used, can be monitored non-invasively in liv ing cells with ultraviolet irradiation, requires no cofactors or substrate for activity, and persists in fixed cells (Chalfie et al. 1994; Anonymou 1998).

MATERIALS AND METHODS

Disks, 4 mm × 39 mm, were obtained from rods of Buna-N rubber (which is a soil-resistant copolymer of 1,3-butadiene and acrylonitrile, and can be vulcanized), polyvinyl chloride (PVC), chlorinated PVC (cPVC), and fiberglass, with a 6 mm hole drilled in each material. Glass disks with the same measurements were machine made and stainless steel was represented by the use of 39 mm washers. Twenty-four sets of disks were assembled and autoclaved at 121°C for 30 minutes. Four sets of disks were suspended in six 79.2 L aquaria maintained with an average water temperature of 24.8±0.6°C. Each tank was then stocked with one adult pure Nile tilapia, *Oreochromis niloticus*. Box filters (Jumbo Corner Filter; 10-20 Gallon, Lustar Products Co., Springfield, New Jersey [1]) provided recirculation of aquarium water and removal of nitrogenous wastes.

The challenge strain of *E. coli* (ATCC 25922, American Tissue Type Culture, Rockville, Maryland) represented the model microorganism. The plasmid pGFP (Clontech Laboratories, Inc., Palo Alto, California) was used to transform the microorganism, and a known amount (1×10^{10} CFU/mL) was then placed in each aquarium, giving a final concentration of approximately 1.3×10^5 CFU/tank. One set of disks was removed from each tank and sampled on days 1, 3, 7 and 15 post-inoculation.

Each disk was rinsed with a gentle stream of sterile water from a 500-mL squirt bottle immediately after removal from the tank to ensure only cells incorporated into the biofilm were cultured (Ladd and Costerton 1990; Jass et al. 1994; Karunasagar et al. 1996; Ren and Frank 1993). The disk was then placed in an individual sterile sampling bag with 25 mL of sterile water and stomached (Stomacher 400 Lab Blender, Seward Medical, London, England) for two minutes at medium speed (Gagnon and Slawson 1999). Spread plates, with 0.1 mL of sample on Luria Agar (20 mg Luria broth [Difco, Becton Dickinson Microbiology Systems, Becton Dickinson Company, Sparks, Maryland], 15 mg agar, 1 mL 1 N NaOH/L) were made. Ampicillin (50 mg/mL) and 560 μL/L 420 mM isopropyl-β-D-thiogalactoside (IPTG) were added to the Luria Agar to inhibit the growth of other than GFP containing bacteria and to enhance production of fluorescent green protein. After incubation at 35°C for 24 hours, the plates were examined under long-wave ultraviolet light and green fluorescent colonies counted.

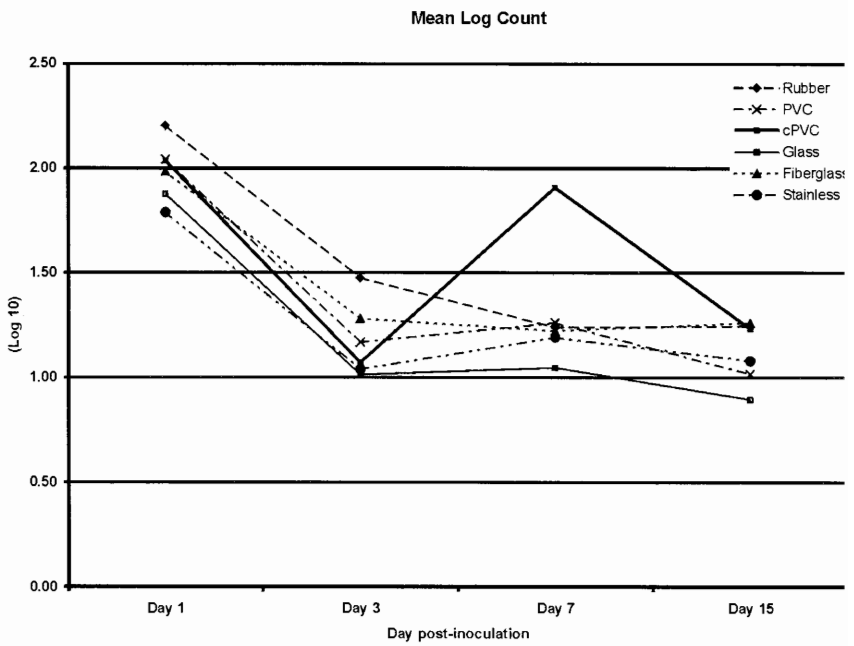
1. Use of trade or manufacturer's name does not imply endorsement.

Statistical analysis was performed using the SAS System (SAS Institute, Inc., Cary, North Carolina). A randomized complete block desig was used, with a two-way factorial treatment structure, at a significanc level of $P = 0.05$. There were six blocks in this study representing eac tank used. The treatments were the day of sampling and the six material used for disks.

RESULTS AND DISCUSSION

The mean log count of fluorescent *E. coli* colonies for each substrat over the 15-day period tested is shown in Figure 1. There was only an ap proximate 0.5 log difference between the substance with the lowest an the highest numbers of GFP *E. coli*. At 24 hours post-inoculation, ther was approximately a 2 log count/cm^2 on each test disk, which was signifi cant when compared to the number of colonies on the other days. On day 3

FIGURE 1. Mean log count (log 10) cfu/cm^2 of GFP *E. coli* on different sub stances used in recirculating aquaculture systems.



all substrates showed decreased numbers to about 1 log count/cm^2, and over the remainder of the test period these numbers remained steady on all materials but the cPVC. The Buna-N rubber consistently had the highest numbers, except on day 7 when the count on both the PVC and cPVC were greater. The microbial count on glass was consistently the lowest. The presence of GFP *E. coli* on each disk was found to be independent of the type of material ($P > 0.05$) used as substrate.

There are conflicting reports concerning the effect of substrate on biofilm growth. In a study by Kerr et al. (1999) comparing the growth of heterotrophic bacteria on different pipe material, pipes composed of cast iron were more likely to have bacterial biofilms present than on plastic pipe materials such as unplasticised polyvinyl chloride or medium density polyethylene. Krysinski et al. (1992) found that the type of surface (stainless steel, polyester, or polyester backed with polyurethane) had little effect on the rate of cell attachment of *Listeria monocytogenes*. Smoot and Pierson (1998a) found *L. monocytogenes* incorporated in biofilms on the Buna-N rubber and stainless steel within short contact times at all temperatures and pH levels tested. However, Ronner and Wong (1993) found there was a lag in the growth rate of a number of microorganisms, including *E. coli* O157:H7, on Buna-N rubber compared to stainless steel, leading these authors to conclude that the rubber exhibited a bacteriostatic effect. After 48 hours however, the recovery of biofilm bacteria from the rubber was equivalent to the number of organisms recovered from the stainless steel.

In this study, there was a substantial amount of variation in the amount of GFP *E. coli* recovered between tanks (Table 1). Though each system was treated similarly, there were slight differences in the environmental parameters. Temperature is considered one of the most important rate controlling parameters for biofilm accumulation (LeChevallier et al. 1990). In this study, the average temperature difference between tanks was not more than 1.2°C, therefore it seems unlikely this difference significantly influenced the incorporation of the GFP *E. coli* in the biofilm.

The amount of organic material present in each aquarium was inconsistent, depending on whether the fish consumed all of its food, and algae and fungal growth were maintained at a minimum concentration. The growth of fungus and algae was not measured in this study. De Franca and Lutterbach (1996) observed that fungal cells in multiorganism biofilms reached a constant number even though bacterial numbers fluctuated. Kerr et al. (1999) also determined that dominant heterotrophs in the biofilms changed over time reflecting continuously changing bacterial populations as a result of changes in the environmental parameters and

TABLE 1. Presence of GFP *E. coli* on six different substances (rubber, PVC cPVC, glass, fiberglass, stainless steel) in six tanks (A1-3 and B1-3) expresse in mean log count (log 10) cfu/cm^2.

Tank	Material	Mean Log Count			
		Day 1	Day 3	Day7	Day 15
A1	Rubber	2.47±0.32	0	0	0
A1	PVC	2.02±0.18	0	0	0
A1	cPVC	2.00±0.21	0	1.95±0.49	0
A1	Glass	1.57±0.81	0	0	0
A1	Fiberglass	2.77±0.13	0	0	0
A1	Stainless	1.74±0.37	0	0	0
A2	Rubber	2.32±0.13	2.53±0.28	1.00±0.00	2.24±0.09
A2	PVC	1.96±0.08	1.50±0.28	1.16±0.28	1.00±0.00
A2	cPVC	2.38±0.69	1.54±0.34	2.13±1.40	2.48±0.35
A2	Glass	2.24±0.46	1.63±0.46	2.71±0.00	0
A2	Fiberglass	1.48±0.67	2.53±0.43	0	1.60±0.43
A2	Stainless	2.30±0.12	2.00±0.00	2.33±1.20	1.77±0.10
A3	Rubber	1.50±0.71	1.3±0.00	0	1.47±0.58
A3	PVC	1.39±0.12	0	0	2.09±0.08
A3	cPVC	1.49±0.50	0	0	2.16±0.22
A3	Glass	2.00±0.00	0	0	2.45±0.13
A3	Fiberglass	1.00±0.00	0	0	3.00±0.49
A3	Stainless	1.00±0.00	0	0	1.00±0.00
B1	Rubber	2.97±0.23	2.14±0.31	2.56±0.00	1.00±0.00
B1	PVC	2.65±0.24	2.40±0.10	1.9±0.00	0
B1	cPVC	2.67±0.16	1.94±0.14	1.80±1.13	0
B1	Glass	2.21±0.21	1.15±0.21	0	0
B1	Fiberglass	2.80±0.34	2.09±0.34	1.30±0.00	0
B1	Stainless	1.93±0.21	1.65±0.07	1.85±0.00	1.30±0.00
B2	Rubber	1.00±0.00	0	0	0
B2	PVC	1.70±1.20	0	1.00±0.00	0
B2	cPVC	1.00±0.00	0	1.98±0.71	0
B2	Glass	0.60±0.00	0	0	0
B2	Fiberglass	1.18±0.31	0	2.30±1.83	0
B2	Stainless	1.00±0.00	0	0	0
B3	Rubber	2.97±0.18	2.88±0.24	3.88±0.00	2.77±0.34
B3	PVC	2.54±0.56	3.11±0.26	3.51±0.24	3.01±0.48
B3	cPVC	2.68±0.17	2.95±0.07	3.59±0.14	2.76±0.15
B3	Glass	2.64±0.68	2.29±0.44	3.57±0.30	2.92±0.23
B3	Fiberglass	2.68±0.01	3.07±0.44	3.74±0.76	2.97±0.71
B3	Stainless	2.77±0.16	2.59±0.35	2.97±0.03	2.41±0.48

also competition with new biofilm members. The presence of the fish in the systems undoubtedly affected the environmental parameters in our study. Thus, the amount of GFP *E. coli* present in the biofilms within each tank was almost certainly dictated by the presence or absence of competitive microorganisms in the organic material as a result of fish being present.

The use of GFP *E. coli* as a model microorganism for monitoring bacterial incorporation into biofilms of recirculating aquaculture systems was demonstrated. Even though the presence of the GFP *E. coli* decreased after 24 hours, the number of bacteria in the biofilm on the test disks remained constant over the remainder of the 15-day time period. It is difficult to know how long the bacteria might remain in the biofilm. The presence of the dominating heterotrophic bacterial colonies changes over time and the amount of the GFP *E. coli* could be affected by these trends. *Escherichia coli* is not a reliable indicator of fecal pollution when testing seafood from cold water because of a rapid decline of the bacteria's presence in low temperatures (Nickelson and Finne 1992). However, in this study, the GFP *E. coli* could be isolated from the biofilms, most likely due to the warmer water temperature required by cultured tilapia, which encouraged the incorporation of the microorganism into the biofilms.

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