Far From Superficial: Microbial Diversity Associated With The Skin And Mucus Of Fish

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Abstract

During horizontal or water-borne infection involving an obligate pathogen (e.g. – *Aeromonas salmonicida*, cause of furunculosis), the pathogen interacted with and influenced the microbial diversity of the dermal mucus of fish. Prior to infection, the prevalent bacterial flora cultured from juvenile Atlantic salmon (*Salmo salar*) included *Pseudomonas fluorescens*, *Comomonas terrigenia*, *Acinetobacter* sp., *Moraxella* sp., *Pseudomonas dimunita*, *Alcaligenes denitrificans*, *Pseudomonas pseudoalcaligenes*, and *Pseudomonas alcaligenes*, *Serratia liquefaciens*, *Aeromonas hydrophila*, other motile *Aeromonas* spp., and *Corynebacterium aquaticum*. After *A. salmonicida* was initially detected in this population as an external mucus infection, *Acinetobacter* sp., *Moraxella* sp., *C. terrigenia*, *P. fluorescens*, and *P. dimunita, Staphylococcus* sp., and *A. hydrophila*, were also present in appreciable numbers. Within several weeks, however, the *A. salmonicida* infection amplified and composed 78% of the total flora in the mucus. Only *P. dimunita* (4%). *P. fluorescens* (2%), and *C. terrigenia* (1%) were cultured at that time and more than a third of these fish showed evidence of a systemic *A. salmonicida* infection within their kidneys. Eight weeks after oral oxytetracycline treatments, A. *salmonicida* was no longer isolated from the mucus or kidneys of any fish and glucose inert or other oxidative microbes (e.g., *P. fluorescens*, *C. terrigenia*, *Acinetobacter* sp., *Moraxella* sp.) were beginning to repopulate the external surface of the salmon in increasing frequency. Still present and composing fairly large percentages of the total flora were *A. hydrophila*, as well as *Enterobacter sp.,* and *P. putrefaciens.* A normal microbial diversity was re-established as the fish recovered. In another investigation, reduced biological diversity was noted in the dermal mucus among smallmouth bass that were sampled from the Jackson River (Covington, VA). In these fish, *A. hydrophila* and *P. putrefaciens* were the two predominant microorganisms composing 49.5% and 31.2% of the total bacterial flora, despite the absence of systemic infection or any other clinical signs of disease. In another instance, *P. fluorescens* was the sole bacterium associated with the surface of Atlantic salmon eggs regardless of their viability at the eyed stage of development. Collectively, these results indicate that the kinetics and distributions of the surface bacterial flora on aquatic organisms is affected by numerous factors including pathogen invasion, environmental conditions, and fish culture practices.

Key words: bacterial flora, skin, mucus, fishes, disease, environment

Introduction

Clinicians routinely accomplish the detection of bacterial pathogens in fish by determining whether or not a systemic infection is present, which may involve molecular assay, serodiagnostic detection, or cultural isolation from the kidneys of fish (Noga 2000, AFS-FHS 2004). As an internal organ with an inintegral role in blood circulation, the kidney generally provides for the recovery of pathogens (when present) in monoculture, yielding ample evidence of a putative etiology when bacterial pathogens are suspected of causing disease. Unfortunately, isolation from the kidney is invasive and lethal.

Isolation of *Aeromonas salmonicida*, the cause of furunculosis, from the dermal mucus has been used as a non-lethal

alternative for early, sensitive, and reliable detection of this pathogen within threatened or endangered populations of certain salmonids (Cipriano et al. 1992, 1994, 1996a). By comparison to isolation from the kidney, diverse bacteriological populations are generally present on the dermal surfaces of fish (Cahill 1990). Fish inhabit an aqueous environment and the bacterial flora within their dermal mucus often reflects the microbial population of the surrounding water (Horsley 1973, Austin and Austin 1987, Cahill 1990). Consequently, members of the genera *Aeromonas, Serratia, Flavobacterium, Alacaligenes, Moraxella, Acinetobacter, Enterobacter, Pseudomonas, Cytophaga, Micrococcus, Staphylococcus,* and *Corynebacterium* frequently form a rich, diverse, and normal flora on the dermal surfaces of fresh-water salmonid fishes (Allen et al. 1983, Nieto et al. 1984, Austin and Austin 1987). During the course of studies with salmonids (Cipriano et al. 1992, 1994, 1996b), major shifts were recorded in the bacterial distributions within the dermal mucus that develop before, during, and after the occurrence of overt disease outbreaks caused by *A. salmonicida*. The present study was conducted to highlight the effect of infection by *A. salmonicida* on the normal bacterial flora of Atlantic salmon (*Salmo salar*). In addition, the study attempts to provide further analysis on how these normal bacterial distributions were affected within smallmouth bass and Atlantic salmon eggs as a result of environment and fish culture practices.

Materials and Methods

In order to determine the natural microbial diversity that is inherent to Atlantic salmon cultured at the United States Fish and Wildlife Service (USFWS) White River National Fish Hatchery (Bethel, VT), assays were conducted in a manner sufficient to detect a 10% prevalence of a given bacterial species with 95% confidence (Simon and Schill 1985). This amounted to 27 fish from each of 12 rearing units (containing about 7,500 salmon, apiece) each time the fish were sampled throughout an annual smolt production cycle during which furunculosis was diagnosed. During the cycle in question, fish were initially sampled at 4 months of age (September) prior to any detectable exposure to the fish pathogen, *A. salmonicida*. At 9 months of age (February), *A. salmonicida* was detected only as an external infection within the mucus. Disease and subsequent mortality was reported about two weeks after the bacterium was first detected in the mucus and the fish were sampled once again (March). The salmon were then medicated with oxytetracycline (Terramycin®, Pfizer, Inc.) at the rate of 50-80 mg of drug per kilogram of fish per day (2.5 to 3.75 g per 45.3 kg of fish) for 10 days (Herman 1970). Mortality was controlled by treatment and the salmon were again sampled 8 weeks after the last day of treatment (May).

In each case, bacterial isolations were attempted from the dermal mucus and kidneys of each fish by dilution plate counts (Cipriano et al. 1992) on Coomassie Brilliant Blue (CBB) agar (Cipriano and Bertolini 1988, Cipriano et al. 1992). Fish were euthanized in a lethal aqueous dose (>200 mg/L) of tricaine-methaneosulfonate (MS-222; Argent Laboratories; Redmond, Wash-ington) and a sample of dermal mucus was removed by passing a sterile scalpel along the lateral surface. Each fish was

then necropsied and a portion of the posterior third of the kidney was excised aseptically. Samples were placed in individual, pre-weighed, sterile test tubes. Mucus and kidney sample weights were determined and the samples diluted in a 1:10 (weight/volume) in sterile phosphate buffered saline (pH = 7.2) and emulsified by repeated passage and expulsion through a sterile 1.0 mL pipette. Additional serial $\log_{(10)}$ dilutions were prepared and a 10 µL aliquot of each dilution was pipetted onto CBB agar. Plates were incubated at ambient temperature (15-20^{o}C) for a maximum of 120 hours. The number of colony forming units (cfu) in an appropriate dilution of sample (containing about 10 colonies per 0.01 ml) were recorded and then subcultured onto tryptic soya agar (Difco, Detroit, MI) plates for a maximum of 72 hours at 20^{o}C. These sub-cultures provided inocula for further taxonomic speciation.

Other samples were also processed from twenty smallmouth bass (*Micropterus dolomieu*) electro-shocked from two different sites of the Jackson River near Covington (VA). Fish were euthanized with a lethal aqueous dose (>200 mg/L) of MS-222 and bacterial samples were taken from the mucus and kidneys of each smallmouth bass by quadrant streak (Whitman 2004) on CBB agar.

Dilution counts were also conducted on 32 Atlantic salmon eyed-eggs from each of five lots that had been incubated at the USFWS Craig Brook National Fish Hatchery (East Orland, ME). Eggs were cultured for total bacterial content associated on their external surfaces. Using sterile forceps, individual eggs were aseptically placed into pre-weighed sterile test tubes. Weights of individual eggs were determined and diluted 1:10 in sterile PBS containing 0.5% peptone. All eggs were allowed to sit for 48h at 12-15^{o}C to allow time for bacteria on egg surfaces to become present within the broth diluent. Each egg-containing tube culture was vigorously vortexed to release additional bacteria from the egg surface into the culture medium. Serial $\log_{(10)}$ dilutions were prepared of the broth culture diluent and a 10 µL aliquot of each dilution was pipetted onto agar, as described previously. Tryptic-yeast-glucose (TYG) agar (Bullock et al. 1986) was used as the primary plating medium. Plates were incubated for up to 10 days at 15^{o}C, bacterial colonies were quantified, and individual colonies were subcultured onto additional TYG agar plates for further identification. Bacteria were characterized by classical microbiological and biochemical tube tests and standardized procedures. Media and supplies were prepared and sterilized according to manufacturer's recommendations. Based upon the results from individual tests, bacteria were classified according to referenced flow charts and identification schemes (Carnahan et al. 1991, Murray et al. 1995, MacFaddin 2000, AFS-FHS 2004). Total bacterial distributions were graphically charted in histograms in which the presence of any given species is depicted as percent of the total bacterial isolations made for that particular population of fish or group of eggs.

Results

Collectively, more than 5,000 bacterial identifications were made among the Atlantic salmon cultured at the White River National Fish Hatchery (Bethel, VT). At four months prior to infection (September) by *A. salmonicida*, the bac-

terial flora within the mucus was fairly diverse (Figure 1a). Twelve microbial species were the predominant microorganisms that were identified by culture. Most of these were Gram-negative, glucose inert or oxidative microbes, which included *Pseudomonas fluorescens*, *Comomonas terrigenia*, *Moraxella* sp., *Pseudomonas dimunita*, *Alcaligenes denitrificans*, *Pseudomonas pseudoalcaligenes*, *Acinetobacter* sp., and *Pseudomonas alcaligenes*. Present to a lesser extent were several members of the Enterobacteriaceae including *Serratia liquefaciens*, *Aeromonas hydrophila*, and other motile *Aeromonas* spp. Also identified was one Gram-positive organism, *Corynebacterium aquaticum*. None of these fish displayed any systemic infection within their kidneys. More specifically, *A. salmonicida* was not detected.

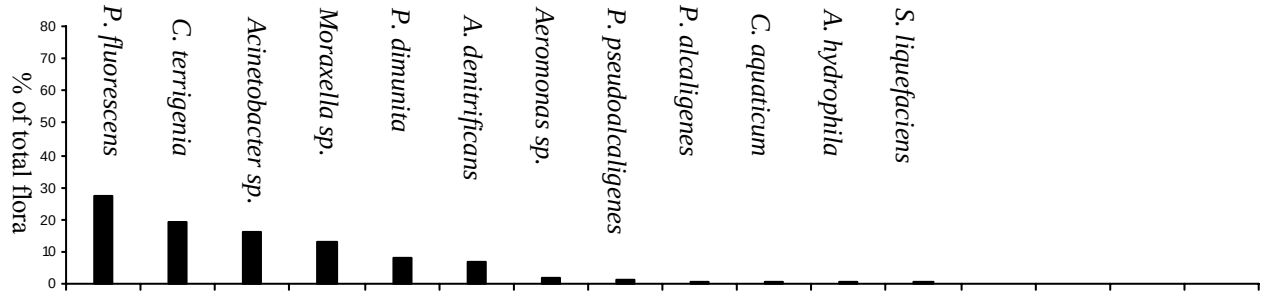
Four months later (February), the salmon within several culture pools became somewhat lethargic and displayed an infrequent but erratic swimming behavior. The fish were sampled once again and revealed that an early, external mucus infection by *A. salmonicida* was developing. At this time, there was no mortality nor was *A. salmonicida* isolated from the kidneys of any of the fish that were sampled. The glucose inert or oxidative microbes, which included *Acinetobacter* sp., *Moraxella* sp., *C. terrigenia*, *P. fluorescens*, and *P. dimunita*, were still present in appreciable numbers and collectively composed the predominant bacterial flora (Figure 1b). Also, present were *Staphylococcus* sp., and *A. hydrophila*, which constituted 7% and 1% of the total bacterial distribution, respectively. *Aeromonas salmonicida*, which collectively constituted approximately 12% of the total bacterial distribution, was now isolated from the mucus of 13 fish. Within several weeks (March), this *A. salmonicida* infection had amplified to the degree that it now established itself as the predominant microorganism and composed approximately 78% of the total bacterial flora in dermal mucus (Figure 1c). *Aeromonas hydrophila* became the second most predominant organism and composed 12% of the total bacterial flora. Also present were *Enterobacter* sp. (2%) and *Pseudomonas putrefaciens* (1%). Of the normal glucose inert and other oxidative microbes that had predominated in healthy fish, only *P. dimunita* (4%), *P. fluorescens* (2%), and *C. terrigenia* (1%) were cultured and identified. More than a third of the cultured fish showed evidence of a systemic *A. salmonicida* infection within their kidneys and accounted for 95.7% of the identifications made from the kidneys of fish at this time.

Eight weeks after oxytetracycline treatments had ended (May), *A. salmonicida* was no longer isolated from the mucus or kidneys of any fish (Figure 1d). Normal glucose inert or oxidative microbes were beginning to repopulate the external surface of the salmon in increasing frequency. This included *P. fluorescens* (31%), *C. terrigenia* (7%), *Acinetobacter* sp. (1%), and *Moraxella* sp. (1%), as well as *C. aquaticum* (9%). Still present and composing fairly large percentages of the total flora were *A. hydrophila* (24%), as well as *Enterobacter* sp. (17%) and *P. putrefaciens* (8%).

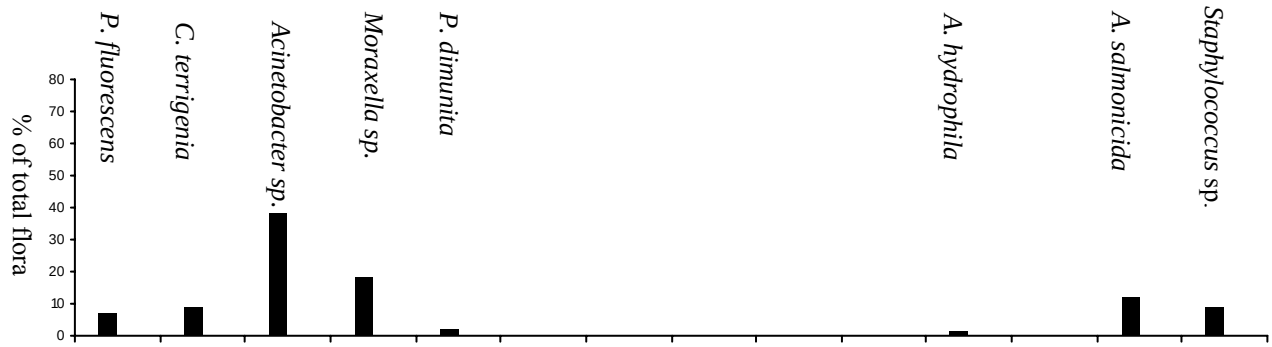
Based on these results, other examples were studied in which significant changes were observed in the biological diversity of the mucus of fish in the

Figure 1: Distributions of microbial species on the dermis of Atlantic salmon (*Salmo salar*) as a result of bacterial invasion by *Aeromonas salmonicida*

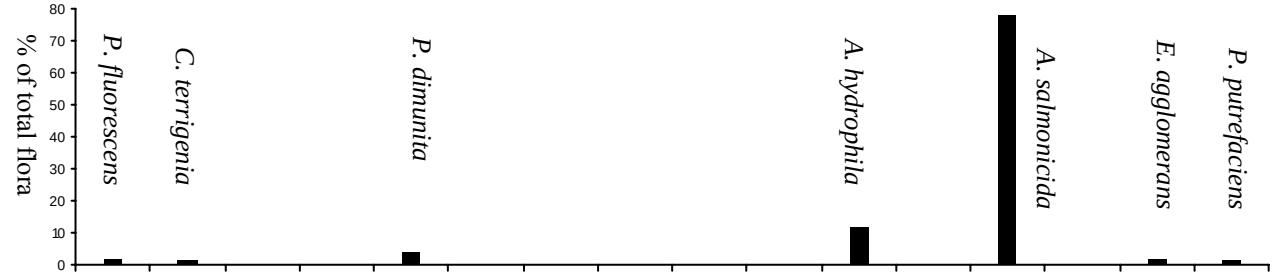
A. Pre-exposure to *Aeromonas salmonicda*



B. Invasion by *Aeromonas salmonicida*



C. Frank disease caused by *Aeromonas salmonicida*



D. Recovery following treatment with oxytetracycline

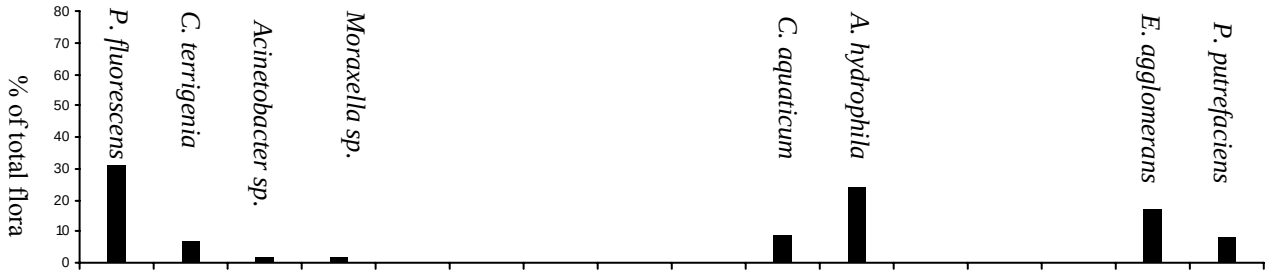
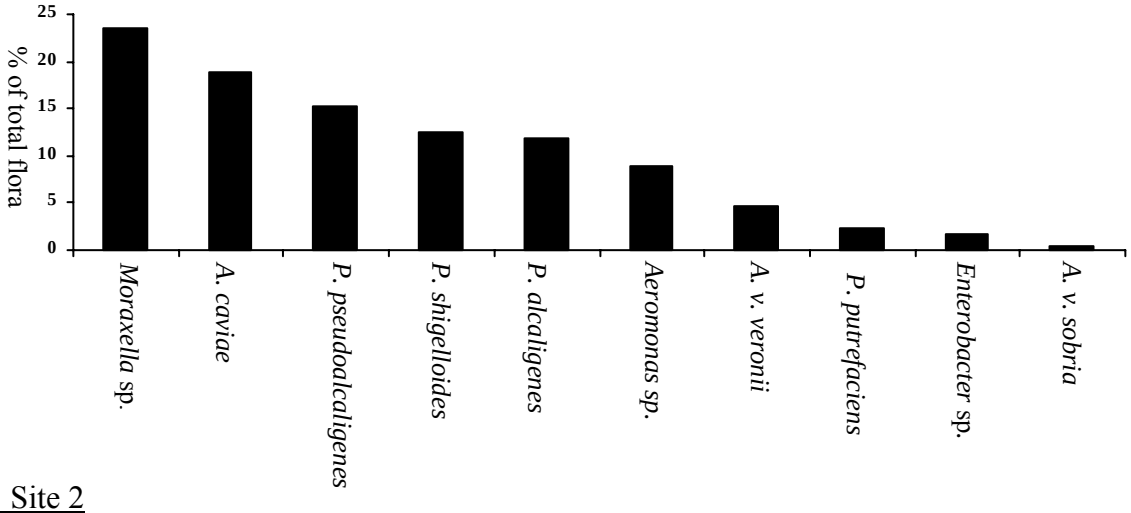
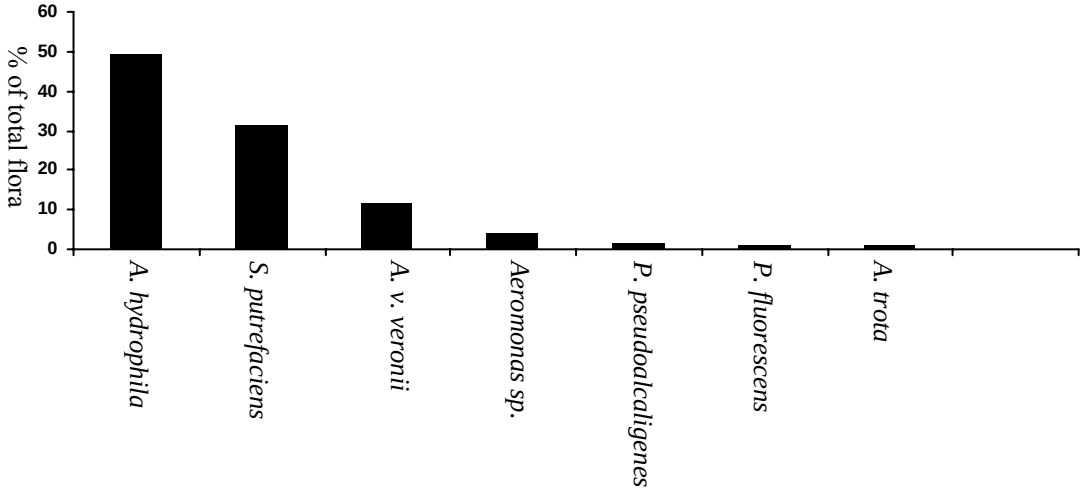


Figure 2: Bacterial distribution within the dermal mucus of smallmouth bass (*Micropterus dolomieui*) at two sites from the Jackson River near Covington, Virginia

A. Site 1:



B. Site 2



absence of clinical signs of disease or apparent infection. For example, the total bacterial flora was evaluated from the mucus of smallmouth bass electroshocked from two different areas of the Jackson River near Covington, Virginia. A total of 2,147 bacterial identifications were made from the dermal mucus of the smallmouth bass at the first site. Four species of Gram-negative, glucose inert bacteria were isolated and comprised significant percentages of the total bacterial distribution of the dermal mucus (Figure 2); - *Moraxella* sp. (23.5), *P. pseudoalcaligenes* (18.8%), *P. alkaligenes* (5%) and *P. putrefaciens* (2.4%). Another five bacterial species readily isolated from this population were members of the Enterobacteriaceae and included *Aeromonas caviae* (19.8%), *Ples-*

iomonas shigelloides (12.4%), *Aeromonas veronii* biovar *veronii* (4.6%), *Enterobacter* sp. (1.8%), *Aeromonas veronii* biovar *sobria* (0.4%) and also present were other non-speciated motile aeromonads (9.0%). At site two, 830 bacterial identifications were made from the mucus of the apparently healthy smallmouth bass. Two organisms, *A. hydrophila* and *P. putrefaciens,* were predominant and composed 49.5% and 31.2% of the total flora, respectively. Furthermore, both of these organisms and as well as the third most prevalent species, *A. veronii* biovar *veronii* (11.7%), produced hydrogen sulfide gas. Collectively, hydrogen sulfide production was produced by just three species composing 92.4% of the bacteria that was isolated from the fish at this site.

Table 1: Bacteria isolated from various lots of eyed-stage Atlantic salmon (*Salmo salar*) eggs from the Craig Brook National Fish Hatchery (East Orland, ME).

Egg lot	% Viability at eye-up	Bacterial species isolated*	# of eggs (% of eggs)	Bacterial range (c.f.u.**/egg)
Penobscot River	84%	*P. fluorescens*	18/18 (100%)	$4.0x10^7 – 1.7x10^8$
Penobscot River	63%	*P. fluorescens*	18/18 (100%)	$2.0x10^7 – 7.5x10^8$
Penobscot River	14%	*P. fluorescens*	36/36 (100%)	$3.0x10^7 – >5.0x10^8$
Penobscot River	14%	*P. fluorescens*	36/36 (100%)	$3.0x10^7 – >5.0x10^8$
Penobscot River	14%	*P. fluorescens*	36/36 (100%)	$6.0x10^7 – >5.0x10^8$

* Bacteria isolated from the outer surface of the egg
** Designates colony forming units per gram of egg; average egg weight = 0.1 g

In another instance, samples were obtained from Atlantic salmon eggs that had displayed varied degrees of survival at the eyed-stage of development (Table 1). Healthy eggs were cultured from the two egg lots that had an 84% and 63% viability at the eyed-stage of egg development. Only blank or dead eggs were available from the other three egg lots that each had a viability of less than 14%. Externally and in all cases, *P. fluorescens* was the sole bacterium associated with the surface of eggs regardless of their viability.

Discussion

Just as biological diversity is often used as an indicator of environmental health, there is a normal microbial diversity on aquatic animals in healthy environments. Because every aquatic organism harbors a normal bacterial flora on their skin, it is logical to presume that the presence and frequency of any given microbial species will indeed affect the overall frequency and total distribution of others species within a mixed microbial community. Such was the case that was observed while evaluating the microbial kinetics of Atlantic salmon before, during, and after a furunculosis outbreak occurring at the White River National Fish Hatchery. On those fish, the normal (pre-infection) flora consisted mostly of gram negative, glucose non-fermenting and other glucose inert bacteria. About a dozen bacterial species within the genera *Pseudomonas, Alcaligenes*, *Moraxella, Acinetobacter, Comomonas,* etc., formed a rich microbial flora on the skin and mucus of healthy fish prior to detection of *A. salmonicida.* These species were consistent with those that had been previously reported by other researchers (Allen et al. 1983, Nieto et al. 1984, Austin and Austin 1987).

During the process of infection, *A. salmonicida* initially established itself as an external infection. During the early stage of this external infection, pathogen numbers remained low for approximately two to three weeks without much influence on total microbial diversity (Figure 1b). As the pathogen developed a stronger foothold, it replicated and displaced other species of the normal flora until it dominated the external flora to the exclusion of almost everything else (Figure 1c). Such an advantage apparently enabled the pathogen to overwhelm host defense mechanisms and initiate systemic infection, which resulted in clinical disease and mortality. The ensuing degradation of tissues and decomposition of carcasses also results in some degree of environmental degradation in fish culture systems. Now and continuing into the recuperative phase of the disease (Figure 1d), a different flora appeared that was generally represented by facultative pathogens of the Enterobacteriaceae (e.g. - motile aeromonads, *Enterobacter* spp., *Serratia* spp., etc.). Horsley (1984) noted that part of the enterobacterial flora of fishes is significantly influenced by water quality. In the current study, the greatest prevalence of the enterobacterial species was observed in the overt disease (Figure 1c) and recuperative (Figure 1d) phases post infection. As the fish and the environment recovered, these species gave way and the fish was eventually repopulated by its normal bacterial flora (Figure 1a; - data not shown). In general, results suggested that healthy fish possess a diverse microbial flora, which is altered and diminished during the process of a horizontal or water-borne infection. During the actual disease outbreak, the pathogen *A. salmonicida* dominated the microbial flora that resulted in significant loss of microbial diversity on the surface of the fish.

In addition to analyzing the kinetics of the microbial flora among Atlantic salmon before, during, and after infection by *A. salmonicida*, several other studies were conducted and pre-sented herein as examples of bacterial shifts in

the dermal flora independent of a bacterial pathogen. Smallmouth bass sampled in this study inhabited a far more eutrophic environment than the Atlantic salmon that were reared in ultraviolet irradiated well-water at the White River National Fish Hatchery. Consequently, members of the Enterobacteriaceae comprised a greater percentage of the microbial flora isolated from fish out of the Jackson River. At site one, four gram-negative, glucose inert bacteria were isolated *Moraxella* sp., *P. pseudoalcaligenes, P. alcaligenes,* and *P. putrefaciens* along with five other representatives Members of the Enterobacteriaceae, which included *A. caviae*, *A. v.* biovar *veronii*, *P. shigelloides*, *Enterobacter* sp., *A. v.* biovar *sobria,* and another group of undifferentiated motile aeromonads. At site two, however, a difference in the dermal flora was observed where A. *hydrophila* and *P. putrefaciens* accounted for 81% of the total bacterial flora isolated from the mucus of smallmouth. Along with *A. v.* biovar *veronii*, 92.4% of the bacteria isolated from the external dermis of these fish produced hydrogen sulfide gas. At site 1, the only isolates that produced hydrogen sulfide were *P. putrefaciens*, which accounted for only 2.4% of the total microbial distribution.

It is well known that bacteria are important decomposers of organic materials in aquatic environments (Chróst et al. 1986). Interestingly, the smallmouth bass at site 2 were collected near the effluent discharge of a large paper mill where the total suspended solids are elevated as compared to other areas of the river. The addition of cellulosic pulp fiber in pulp mill effluents is known to increase the concentration of their metabolic by-product, which is sulphide (Poole et al. 1977), and produce conditions that favor the reduction of sulfates to aqueous H_2S by many bacteria (Rava et al. 2008). Consequently, some of the observations from fish at site 2 may reflect changes in environment. While additional research would be needed to validate this assumption, it is clear that understanding shifts in the biotic flora and also in their metabolic products may provide clues concerning environmental quality. If properly understood, the shifts in bacterial populations could, therefore, be use as a metric to estimate anthropogenic impacts and habitat degradation.

The last example presented in this study involved Atlantic salmon eggs from the Craig Brook NFH. Kubilay et al. (2009) have shown that the bacterial flora of rainbow trout (*Oncorhynchus mykiss*) eggs may consist of members of *Cytophaga*, *Moraxella, Aeromonas, Acinetobacter,* and *Corynebacterium,* plus other species of Enterobacteriaceae. On the other hand, Barker et al. (1989) found that rainbow trout and brown trout (*Salmo trutta*) eggs incubated in flow through systems were predominantly colonized by *A. hydrophila* and various *Pseudomonas* spp. In this study, a diverse microbial flora was not expected because these eggs were first disinfected in iodophor and then maintained in incubators receiving ultraviolet irradiated water. What was surprising was that there was essentially no microbial diversity associated with the egg surfaces. *Pseudomonas fluorescens* produced virtual monocultures on all eggs regardless of whether or not the egg lots were viable at the eyed stage. This suggested that the *P. fluorescens* monocultures were not a cause of poor egg

survival, but was more likely associated with some environmental factor.

Atlantic salmon eggs are often dosed with ant-fungal formalin treatments (Piper et al. 1982). Because of this, the effect of formalin on *P. fluorescens* was evaluated. In data not presented here, *P. fluorescens* isolates from these eggs were standardized and the bacteria were inoculated for 15 minutes in a 1667 ppm formalin solution (Piper et al. 1982). Following such incubations, the bacteria were plated onto tryptic soy agar and those results indicated that *P. fluorescens* resisted the germicidal activity of formalin. Hence, fish culture practices may have profound influences on normal bacterial distributions.

Collectively, these results suggested that the kinetics and distributions of the surface bacterial flora on aquatic organisms (i.e. - distribution of species and the relative proportion of a pathogen) are affected by pathogen invasion, environmental conditions, and fish culture. Bacterial distributions on fish mucus shift away from a natural state of diversity with the onset of infection and bacterial distributions continue to change as the population undergoes frank disease and recovery. During infection and subsequent disease, the changes in microbial community structure may have been a direct result of bacterial infection and replication by *A. salmonicida*, however, it is also possible that *A. salmonicida* is capable of infecting and establishing itself on external surfaces of its host because of changes in microbial community structure that arise from another cause. In this manuscript, an example was presented on how environmental conditions (pulp mill effluent) affected the microbial community structure on the dermis of smallmouth bass and how a fish culture procedure (formalin treatments) affected the flora associated with the surface of Atlantic salmon eggs. It is also possible that subtle changes in environmental parameters may affect the normal microbial structure in a manner that provides an initial foothold for pathogen invasion. Some physiological changes may also be induced in fish, either by the environment or simply as a result of its general condition that affect the composition of the mucus and alters microbial community structure. This could suggest that fish may have evolved in such a manner that allows them to assimilate a diverse dermal flora that enables them to better resist invasion by pathogens. Under this scenario, *A. salmonicida* is less of a primary pathogen initiating disease and becomes more of an opportunist that exploits a compromised animal.

Ecologists have considered biological diversity to function as an indicator on environmental stability. Decreases in a population's biological diversity may increase its vulnerability to perturbations within the environment (Elton 1958). Even if biological diversity does not actually drive ecological structure, it can reflect important ecological mechanisms within ecosystems (McCann 2000). In this study, decreased microbial diversity was noted to occur as a function of pathogen invasion, environmental degradation, and certain fish culture practices. Future investigations should concentrate on determining the role of environment and the host physiological response in relation to its microbial flora. Understanding these shifts are still a work in progress, but monitoring microbial diversity could provide a non-lethal metric to forecast population health,

environmental quality, and habitat degradation.

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