SURVEY OF CULTURABLE HETEROTROPHIC BACTERIA ON THE GILLS OF JUVENILE RAINBOW TROUT DURING HATCHERY REARING

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ABSTRACT

Bacterial colonization of the gills of juvenile rainbow trout (*Oncorhynchus mykiss*) was surveyed during hatchery rearing in normal (high water flow) and potentially pathogenic conditions (low water flow). The changes in bacterial colony forming units (CFUs) per gram of gills were monitored, and the most common bacterial colony morphotypes by biochemical tests and sequencing of 16S rRNA genes identified. Total bacteria CFUs were considerably greater in the low water flow tanks than in the high flow tanks. Both an increase in the rate of water flow or treatment with chloramine-T greatly reduced bacterial CFUs in the low water flow tanks. *Flavobacterium* sp. exhibited a twofold increase in CFUs after chloramine T treatment, while *Deinococcus* sp. was more abundant in low water flow tanks. *Flavobacterium* sp. may opportunistically recolonize the gills after treatment with chloramine-T.

Keywords

Rainbow trout, *Oncorhynchus mykiss*, gills, *Flavobacterium*, chloramine-T, bacteria

INTRODUCTION

Bacterial colonization of the gills of salmonid fish in hatcheries is of importance, particularly because of occasional outbreaks of bacterial gill disease (BGD). BGD is caused by the bacterium *Flavobacterium brachiophillum* and often leads to a fatal infection (Lumsden et al. 1994; Wabayshi et al. 1989; MacPhee et al.. 1995; Speare et al. 1991). Few studies have examined the composition of the communities of heterotrophic bacteria on the gills of salmonids. Pigmented, aerobic, gram-negative rods, including species of *Cytophaga* and *Flavobacterium*, are abundant on the gills of adult salmonids in fresh water, along

with species of *Pseudomonas*, *Aeromonas*, and *coryneform* bacteria (Trust 1975; Nieto et al. 1984). Total numbers of culturable bacteria on the gills of adult, hatchery-reared salmonids are reported to be about 2 X 10^5 colony forming units per g gill tissue (Trust 1975), which suggests that at least some species of *Flavobacterium* may normally be abundant on the gills of healthy fish.

Scanning electron micrographs of the gills of turbot indicated that much of surface of gill lamellae is free of bacterial cells (Mudharris and Austin 1988), probably because of the constant flow of mucus over the gills (Dersen et al. 1998). Physiological conditions that inhibit the flow of mucus over gills, such as low oxygen content in water, may greatly increase the number of particles and bacteria (Dersen et al. 1998). Other factors, such as the induction of an immune response due to previous bacterial exposure, may be important in reducing bacterial numbers on the gills of adult fish, and protecting them from BGD (Lumsden et al. 1994). One recent study suggests that growth of the pathogen *Flavobacterium columnare* may be inhibited due to the production of compounds, such as siderophores by *Pseudomonas* species (Tiirola et al. 2002).

In a brief survey of heterotrophic bacteria on the gills of 20 juvenile (six month old) rainbow trout (*Oncorhynchus mykiss*), colony forming units (CFU) were highly variable but averaged about 5 X 10^4 CFU/g of gill tissue (D.J. Bergmann and G. Crabtree, unpublished, 2002). Most of the bacterial colonies recovered from gills had morphological and biochemical characteristics of the genus *Flavobacterium* (Holt et al. 1994).

Although the normal bacterial flora of the gills of adult salmonids has been studied by traditional cultivation-based methods, the bacterial flora of the gills of juvenile trout, which is the stage most susceptible to BGD, is not known. The etiology of BGD and mode of infection of the gills by *F. brachiophilum* have been extensively studied, but there have not been any studies of whether any other species of bacteria normally present on gills might either enhance or inhibit infection of gills by *F. brachiophilum*. Yet another question concerns the use of the bactericide chloramine-T, which has been used experimentally to treat BGD (Bullock et al. 1991). It might be possible that, following chloramine-T treatment, the gills of fish are re-colonized by different assemblages of bacterial species than were initially present. In this study, we describe how bacterial density on the gills of juvenile rainbow trout is affected by the rate of water flow in rearing tanks and by treatment with chloramine T, and identify the common bacterial genera on the gills of juvenile trout.

METHODS

A total of about 400 juvenile (Cleghorn strain) rainbow trout, approximately 4 cm long, were placed into each of six 100L tanks. The study was conducted from 4-12-03 until 5-6-03 at McNenny State Fish Hatchery, Spearfish South Dakota. Two control tanks were designed to simulate good hatchery rearing conditions with high water flow (16 L/min) and daily cleaning. Another two tanks were subjected to low water flow (0.5 L/min) and poor sanitation (no cleaning of tanks) in an attempt to foster BGD. In these two low-flow tanks, flow was

increased to 16 L/min after mortality of 10% of individuals had been observed. A third set of two tanks was also subjected to low water flow (0.5 L/min) and poor sanitation. After mortality rates of 10% were observed, chloramine T treatments occurred (8 mg/L for 60 min), followed by increasing water flow to 16 L/min. Mortalities were removed from each tank daily. Following major mortality events, approximately 10 dead fish from the low flow tanks were frozen and the gills were examined by light microscopy to detect signs of BGD, such as clubbing of lamellae. Fish in all tanks were fed Silver Cup fish feed for trout fry (Nelson and Sons, Murray, UT) at a rate of 45 g per day for two days, then 60 g per day for two days, and then 100 g per day for the rest of the experiment. Ten live fish from each tank were removed for bacterial samples every three to five days.

The gills were removed with sterile forceps. Half of the gills from each tank and sample date were combined and frozen at -70 ºC for later sampling of bacterial DNA. The other half of the gill samples of 10 fish were ground up in 2.5 ml of sterile tap water with a glass homogenizer and serially diluted 1/10. 100 uL of the undiluted and 1/10 diluted extract of ground fish gills was plated onto three Cytophaga agar (CTA) (Anacker and Ordal 1959) and three Tryptic Soy agar (TSA) plates, and incubated 3-5 days at 22 ºC. The numbers and types of colonies on plates with colonies were recorded, and the plates stored at 5ºC in plastic boxes.

Ten to 20 colonies of each morphological type were transferred to either CTA or TSA slants and cultured three to seven days at 22 ºC before storage at 5 ºC. Each isolate was gram-stained, tested for production of acid in glucose phenol red and lactose phenol red broth, tested for catalase activity with hydrogen peroxide, and tested for the presence of cytochrome oxidase with tetramethyl-*p*-phenylenediamine.

Genomic DNA from each bacterial isolate was obtained from a loop-full of culture (about 200 mg) using the Dneasy kit, using the protocol for gram-negative bacteria supplied by the manufacturer (Qiagen, Inc., Valencia, CA), with the final volume of genomic DNA solution (5-20 ng/uL) being 50 uL. The genes for 16S rRNA were amplified from genomic DNA of most isolates using the eubacterial primers C and D developed by Lane (1991). The primers were diluted to 0.1 nMoles/ uL before use, and stock solutions of dNTPs were 10 mM. The buffers and Taq DNA polymerase used for PCR were provided in the MasterTaq PCR kit by Eppendorf (Westbury, NY). Each PCR reaction consisted of 1.0 uL of bacterial genomic DNA, 0.5 uL of each diluted primer, 5 uL of 5X PCR extender (Eppendorf), 2.5 uL of 10X buffer (Eppendorf), 0.5 ul of a 25 mM stock of magnesium acetate, 14.5 uL water, and 0.5 uL of Taq DNA polymerase (Eppendorf). PCR was performed in MJ Minicycler thermal cyclers (MJ Research, Watertown, MA) using the program: 94ºC for 4 min; then 35 cycles of (94ºC for 30 s, 59 ºC for 1 min, and 72 ºC for 2 min); 72ºC for 10 min and hold at 5ºC.

For a few bacterial isolates, the primers C and D would not amplify the 16s rRNA genes from genomic DNA. In these cases, the forward primer LRNNF1, 5'-CGG-CCC-AGA-CTC-CTA-CGG-GAG-GCA-GCA- 3', (bp 310 – 340 of the *E. coli* 16S rRNA gene) and reverse primer LRNNR1, 5'-GCG-TGG-ACT-ACC-AGG-GTA-TCT-AAT-CC- 3' (bp 770 – 740 of the E. coli 16S rRNA

gene) (developed by Jeffery D. Newman, http://lyco.lycoming.edu/~newman) were used for PCR using the conditions for PCR described previously but with the following program: 94°C for 4 min; then 35 cycles of (94°C for 45 s, 59 °C for 45 s, and 72 °C for 1 min); 72°C for 10 min and hold at 5°C

16S rRNA gene PCR products (200 ng) from each bacterial isolate were digested separately with 5U of restriction endonucleases *Taq*I and *Hae*III, and the restriction fragments (RFLPs) separated on a 2% agarose gel. Bacterial isolates were grouped together based on sharing a common pattern of RFLPs. At least one isolate from each RFLP group had the 16S rRNA PCR product purified using the Qiagen (Valencia, CA) PCR cleanup kit in preparation for dideoxy-chain termination sequencing using the primers RNNF1 and RNNR1 and the Big Dye 3.1 kit (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed on Applied Biosystems Avanti 3100 Genetic Analyzer at Black Hills State University.

For each PCR product, which was sequenced, the nucleotide sequence obtained using primer RNNF1 were aligned with the reverse complement of the sequence from primer RNNR1 to edit the final sequence of the 16S rRNA gene of the isolate prior to conducting database searches at the University of California San Diego supercomputing center (http://workbench.sdsc.edu/). The final edited 16S rRNA sequences were matched against 16S rRNA sequences in the Ribosomal Database project at Michigan State University (http://rdp.cme.msu.edu/cgis/seq_align.cgi?su=SSU).

RESULTS

Bacterial Colony Morphotypes

Colonies of bacteria on CTA or TSA agar were divided into seven classes based on their visible morphology (Table 1). The most abundant colony morphotypes were cream-colored colonies of moderate size (CRM), small, yellow pigmented colonies (YS), and small, reddish colonies (PR) . Some colonies, which did not fit readily into the seven categories above were classified as "other." In some cases, colony morphology differed on CTA or TSA media.

Differences in CFU from Trout Gills vs. Treatment and Time

Despite over 10% mortality of fish in low water flow tanks, BGD was not observed based on the absence of gill filament clubbing of or visible bacterial growths on gills. When the numbers of colony forming units (CFU) of all morphological types from the fish in each tank are examined throughout the sampling period, it is apparent that there is considerable day-to-day variation in CFU (Figures 1-3). This variation was especially evident in the two high-flow tanks. In all tanks, CFU were fairly low during the first two sampling periods, increased during subsequent sampling periods, and (with the exception of one of the control tanks) declined to low CFU by the end of the experiment. As expected, bacterial CFU declined following treatment with chloramine-T (after

Table 1. Classification of bacterial isolates from gills of juvenile rainbow trout based on colony morphology.

Agar	Bacterial Colony			
	Acronym	Color	Description	Diameter (mm)
TSA	CRM	creamy-tan	round, low-convex, smooth	5-12
	SID	creamy-tan	secrete yellow siderophore, low-convex, smooth	8-12
CTA	IW	whitish	slight irregular, flattened, translucent border, erose edge	4-8
	IL	whitish	very irregular, large, flat, thin	10-50
TSA and CTA	YS	bright yellow	round, convex, smooth	2-3
	PR	pink-reddish	round, smooth, convex	1-2
	WS	whitish	round, smooth, convex	1-2

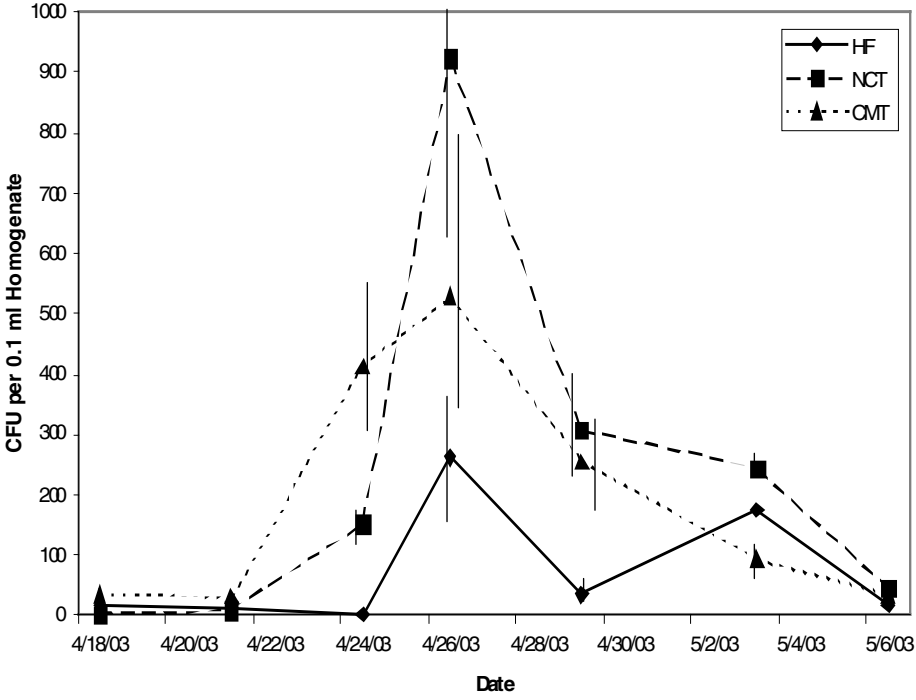

Figure 1. Mean bacterial colony forming units (CFU) on TSA plates per 0.1 mL of gill homogenate vs. sampling date for fingerlings of rainbow trout reared in high water flow tanks (HF, diamonds), low water flow tanks without chloramine T treatment (NCT, squares), and low water flow tanks treated with chloramine T (CMT, triangeles). For each treatment, mean CFU for two tanks are shown, with a vertical line indicating standard error of the mean.

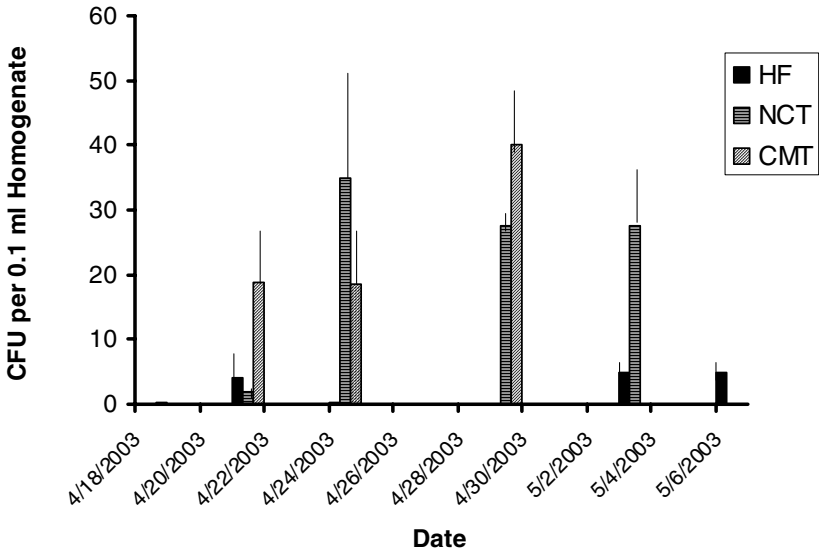

Figure 2. Mean CFU of YS colonies on TSA plates from 0.1 mL of gill homogenate vs sampling date for fingerlings in high water flow tanks (HF), low water flow tanks with no chloramine T treatment (NCT), and low water flow tanks treated with chloramine T (CMT). For each treatment, mean CFU for two tanks are shown, with a vertical line indicating standard error of the mean.

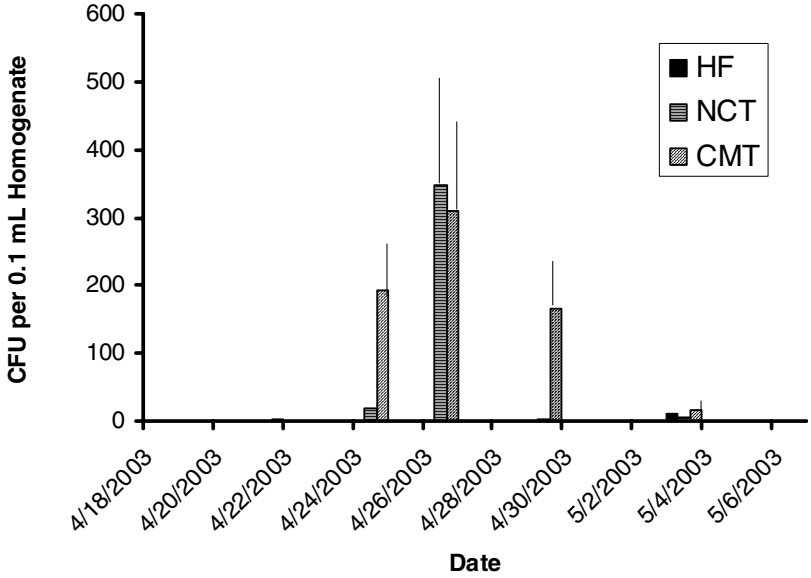

Figure 3. Mean CFU of PR colonies on TSA plates from 0.1 mL of gill homogenate vs. sampling date for fingerlings in high water flow tanks (HF), low water flow tanks with no chloramine T treatment (NCT), and low water flow tanks treated with chloramine T (CMT). For each treatment, mean CFU for two tanks are shown, with a vertical line indicating standard error of the mean.

4/29/03) in the two low flow tanks, but abrupt declines in CFU were observed in tanks not treated with chloramine-T as well. This decline in bacterial CFUs in the two low-flow tanks not treated with chloramine-T appeared to coincide with an increase in water flow from 0.5 L/ min to 16 L/ min following increased mortality in these tanks. However, large differences in total CFUs and types of colonies were observed between tanks having the same experimental treatments, for example the two high-flow tanks.

The PR colony morphotype was most abundant in the four low water flow tanks and was uncommon in the two high water flow tanks (Figure 4). The YS colony morphotype appeared to increase in relative abundance in the two tanks treated with chloramine-T.

Characterization of Bacterial Isolates From Each Colony Morphology Type

Colonies of the CRM morphotype were abundant from fish gill bacteria plated on TSA. Twenty CRM colonies were transferred to TSA agar slants for further characterization. Using primers C and D, 16S rRNA PCR products were obtained from 18 of these isolates. When 16S rRNA PCR products were digested with *Hae*III and *Taq*I, four groups of RFLPs were noted. Ten of the 18 PCR products had the same RFLP pattern. The PCR product of one of these, CRM5, was partially sequenced and closely matched (98% identity over a 171 nt region) *Acinetobacter* lwoffii (ascession number U10875). Four of the 18 PCR products had another RFLP pattern- one of these, from isolate CRM4, was partially sequenced, and most closely matched (100% identity over 365 nt) *Pseudomonas* strain PsI (ascession number AF105387). Two isolates constituted another RFLP group; the PCR product of one,CRM10, was partially sequenced and closely matched (100% over 379 nt) *Aeromonas salmonocida* subspecies *salmonicida* (accession number X60405) a serious pathogen of fish. The PCR product of one isolate, CRM2, constituted another RFLP group; its partial sequenced closely matched that of Pseudomonas strain DhA91 (accession number AF177916), which had been isolated from hydrocarbon-contaminated soils in the arctic.

Colonies of the YS morphotype were frequently recovered from low-flow experimental tanks. Ten of these had 16S rRNA genes analyzed by PCR and they constituted two RFLP types. One of each RFLP type was sequenced, and both were similar (100% identical over 184 nt) to *Flavobacterium aquatile* ATCC 11947 (ascession number M2797).

Colonies of the PR morphotype were also frequently recovered from low-flow tanks. The 16S rRNA gene PCR products analyzed all belonged to a single RFLP group, whose sequence was similar (100% identical over 583 nt) to *Deinococcus* sp. Strain MBIC3950 (ascession number AB022911).

Colonies of the IW type were recovered from cytophaga agar plates. The RFLP patterns of the PCR products of the 16S rRNA genes of ten colonies were analyzed. Three RFLP groups were present. Seven of the ten had same RFLP pattern as isolate IW6, whose sequence closely matched that of *Acinetobacter* strain

LY1 (ascession number AJ007008). Another RFLP group, shown by isolate IW1, was similar to *Pseudomonas* strain P51 (ascession number AF015487).

DISCUSSION

Numbers of culturable heterotrophic bacteria on the gills of juvenile trout could not be estimated with precision due to high variability between different samples in the same experimental treatment, although estimates of CFUs/g of gill tissue are the same magnitude as those found in literature. Perhaps the high variability in estimated CFU per fish in a tank might be due to high variability in CFU on the gills of individual fish, coupled with the low sample sizes used in the study. To improve the rather poor accuracy of CFU estimates, one would ideally wish to increase the number of fish sampled in each treatment group. However, because about five min were required to process the gills of each individual fish, sampling large numbers of fish would be very labor-intensive.

In general, bacterial numbers on gills were higher in tanks with low water flow. Increasing water flow in low-flow tanks appeared to reduce bacterial numbers as much as addition of chloramine-T (combined with increased water flow). *Flavobacterium* species, but not *F. branchiophilum*, were common, especially in low-flow tanks. However, PR colonies were more abundant in low-flow tanks. Other common bacteria included species of *Pseudomonas*, *Acinetobacter*, and *Deinococcus*. *Aeromonas salmonocida* may also be present. While some colony morphotypes, such as YS (*Flavobacterium*) and PR (*Deinococcus*) appeared to represent a single species, others, such as CRM, contained multiple genera.

Our methods have provided estimates of CFU on the gills of juvenile trout comparable in magnitude to those obtained by others (Austin 2002). A few tentative conclusions can be drawn from numbers of CFU from the gills of hatchery trout. Maximum numbers of CFU from plates of CTA 2753 +- 2831 (4.6×10^5 CFU/g gill tissue) were higher than those from TSA plates (645+- 507 (1.1×10^5 CFU/g gill tissue).

We did not observe outbreaks of BGD in the low water flow tanks, despite mortality of over 10% of the individuals in the tanks. The rate of water flow in hatchery tanks has a strong influence on the numbers of bacteria on the gills of juvenile trout, with bacterial numbers much higher on the gills of fish reared under low water flow conditions than under high water flow conditions. Possibly, poor oxygenation in tanks with low water flow might inhibit the flow of mucus over the gills of fish, as was reported by Derksen et al.. (1998). It is possible that increasing the rate of water flow in rearing tanks might reduce the risk of infection of juveniles from *F. branchiophilum*, or other bacteria, simply by increasing the rate at which bacteria and other particles are removed from the gills.

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LITERATURE CITED

Anacker, R. L., and E. J. Ordal. 1959. Studies on the myxobacterium *Chondrococcus columnaris*. I. Serological typing. Journal of Bacteriolology 78:25-32.

Austin, B. 2002. The bacterial microflora of fish. The ScientificWorld Journal 2:558-572.

Bullock, G. L., and R. L. Herman. 1991. Hatchery efficiency trails with chloramine-T for control of bacterial gill disease. Journal of Aquatic Animal Health 3:48-50.

Derksen, J. A.,V. E. Ostland, and F. W. Ferguson. 1998. Particle clearance from the gills of rainbow trout (*Oncorhynchus mykiss*). Journal of Comparative Pathology 118:245-256.

Ferguson, H. W., V. E. Ostland, P. Byrne, and J. S. Lumsden. 1991. Experimental production of bacterial gill disease by horizontal transmission and by bath challenge. Journal of Aquatic Animal Health 3:118-123.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. Bergey's Manual of Determinative Bacteriology, 9th Ed. Williams and Wilkin, Baltimore, Maryland.

Lane, D. J. 1991. 16S/23S rRNA sequencing. Pages 115-175 *in* E. Stackebrandt and M. Goodfellow, editors. Nucleic acid techniques in bacterial systematics. John Wiley and Sons, Chichester, United Kingdom.

Lumsden, J. S., V. E. Ostland, D. D. MacPhee, J. Derksen, and H. W. Ferguson. 1994. Protection of rainbow trout from experimentally produced bacterial gill disease caused by *Flavobacterium brachiophilum*. Journal of Aquatic Animal Health 6:292-302.

MacPhee D. D., V. E. Ostland, J. S. Lumsden, and H. W. Ferguson. 1995. The influence of Feeding on the development of bacterial gill disease of rainbow trout (*Oncorhynchus mykiss*). Diseases of Aquatic Organisms 7:500-505.

Mudharris, M., and B. Austin. 1988. Quantitative and qualitative studies of the bacterial microflora of turbot, *Scophthalmus maximus* L., gills. Journal of Fish Biology 32:223-229.

Nieto, T., A. E. Toranzo, and J. L. Barja. 1984. Comparison between the bacterial flora associated with fingerling rainbow trout cultured in two different hatcheries in the north-west of Spain. Aquaculture 42:193-206.

Speare, D. J., H. W. Ferguson, and F. W. M. Beamish. 1991. Pathology of bacterial gill disease: sequential development of lesions during natural outbreaks of the disease. Journal of Fish Diseases 14:21-23.

Tiirola M., E. T. Valtonen., P. Rintamaki-Kinnunen, M. S. Kulomaa. 2002. Diagnosis of flavobacteriosis by direct amplification of rRNA genes. Diseases of Aquatic Organisms 51(2):93-100.

Trust, T. J., 1975. Bacteria associated with the gills of salmonid fishes in freshwater. Journal of Applied Bacteriolology 38:225-233.

Wabayashi, H. G., J. Huh, and N. Kimura. 1989. *Flavobacterium brachiophila* sp. nov., a causative agent of bacterial gill disease of freshwater fishes. International Journal of Systematic Bacteriology 39:213-216.