Vol. 65: 115–120, 2005 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published June 30

Treatment of columnaris disease of rainbow trout: low pH and salt as possible tools?

L.-R. Suomalainen*, M. Tiirola, E. T. Valtonen

Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland

ABSTRACT: The impact of salt and low pH on columnaris disease of fish was studied. Survival of *Flavobacterium columnare* after exposure to either 4% NaCl (pH 7.2) or pH 5.0, pH 4.86 or pH 4.6 for 15 min or 1 h was studied *in vitro*. All conditions significantly reduced the numbers of viable bacterial cells. The effects of salt (4 and 2%) and acidic baths (pH 4.6) were studied in 2 experiments *in vivo* with rainbow trout *Oncorhynchus mykiss* infected with *F. columnare*. Both salt and acidic baths failed to prevent fish mortality; the overall mortality reached 100% in all groups. However, according to survival analysis, the mortality rate was lower in fish treated with 4% salt baths compared to a control group. The buffering capacity of fish skin mucus against low water pH was also studied. Fish skin mucus was an efficient buffer against decreased water pH and the pH of the skin could be remarkably higher than that of the mucus. This may explain the failure of bath treatments to prevent mortality providing that attached *F. columnare* are located below the mucus surface. We suggest, however, that salt and acidic bath treatments can be used to disinfect water containing *F. columnare* cells shed by infected fish and thus prevent the transmission of the disease.

KEY WORDS: *Flavobacterium columnare* · Treatment · Bathing · pH · NaCl

INTRODUCTION

Flavobacterium columnare causes columnaris disease of freshwater fish worldwide. The disease emerged in Finland in the 1990s (Koski et al. 1993), and has been one of the most harmful bacterial pathogens of cultured freshwater fish over the last few years (annual statistics of National Veterinary and Food Research Institute of Finland). Columnaris disease is treated with antimicrobial drugs, and other methods of treatment and prevention as effective against the disease are not available. Bath treatments can be used against columnaris disease, as it is generally restricted to the skin, gills and fins of fish. Chloramine-T, potassium permanganate, benzalkonium chloride, hydrogen peroxide and copper sulphate have all been tested experimentally and shown to be effective (Jee & Plumb 1981, Wakabayashi 1991, Speare & Arsenault 1997, Altinok 2004). In a study by Thomas-Jinu & Goodwin (2004), Diquat® (herbicide) was shown to be very effective against columnaris disease, and no fish mortality occurred after challenge with *F. columnare*. However, the chemicals mentioned above can be harmful to the fish and the user (e.g. Nemcsok & Hughes 1988, ChemDat® 1999, Straus 2004). In ecological disease management terms, the chemicals used should be as environmentally friendly as possible. An increasing effort should, therefore, be directed towards cost-effective and environmentally friendly prevention methods of bacterial diseases.

Water quality factors such as ion concentration, organic matter and temperature play a role in the infection of fish with *Flavobacterium columnare* and survival of the bacterium in the aquatic environment (Wakabayashi & Egusa 1972, Wakabayashi 1991, Decostere et al. 1999a, Altinok & Grizzle 2001). In a previous study, we detected that Finnish *F. columnare* strains are highly sensitive even to low salt concentrations *in vitro*. *F. columnare* cells were not able to multiply in salt concentrations above 0.1% and pH values below 6.2 (authors' unpubl. data). However, *F. columnare* strain NCIMB 2248^T grows in media with

*Email: lottari@bytl.jyu.fi

0.5% NaCl (Bernardet & Grimont 1989). Soltani & Burke (1994) have also shown that *F. columnare* is sensitive to salt concentrations above 1% and a pH below 5.0. In the study by Altinok & Grizzle (2001), rearing of fish in salinities of 3 and 9‰ after challenging with *F. columnare* prevented mortality associated with *F. columnare* infection. In Finland, columnaris disease has been detected in inland fish farms but not in coastal brackish water areas (salinity between 2 and 7‰). Previous results and the distribution of the disease in Finland suggest that low pH and increased salt concentration may offer a way to treat columnaris disease.

In this study, the survival of *Flavobacterium columnare in vitro* at pH values of 4.68, 4.8 and 5.0 or in 4% NaCl solution was investigated. We also studied how bathing fish in low pH (4.6) and high salt concentrations (2 or 4%) affects the progression of columnaris disease under experimental conditions. The buffering capacity of fish skin was also examined in order to explain the contradictory results obtained from the *in vitro* and *in vivo* experiments of this study.

MATERIALS AND METHODS

***In vitro* experiment.** Seven *Flavobacterium columnare* strains were previously isolated from disease outbreaks at Finnish fish farms and were chosen to represent different genotypes based on length differences in their intergenic spacer regions (Table 1, authors' unpubl. data). Ten ml of Shieh medium pH 7.2 (Shieh 1980) was inoculated with 500 µl of fresh bacterial culture and cultivated at 22°C with constant agitation (200 rpm) for 16 h for the culture to reach the late-exponential growth phase. Bacterial cultures were diluted 1:10 in Shieh medium supplemented with 4% NaCl, or their pH was adjusted to 5.0, 4.86 or 4.6 with hydrochloric acid. Three replicates were performed for each treatment on a microtiter plate in a volume of 300 µl. Sampling of viable cells was carried out after 15 min and after 1 h using plate counting on Shieh agar. The plates were incubated at room temperature for 3 d. The number of bacterial colonies was counted and compared to untreated control dilutions. Arcs transformation was used to analyse percentage data using repeated measures ANOVA and SPSS 11.0 software.

Table 1. *Flavobacterium columnare*. Strains used in *in vitro* and *in vivo* experiments. In the *in vivo* experiment with rainbow trout *Oncorhynchus mykiss*, a mixture of strains was used

Strain	Original code	Fish species
A	3294/95	Trout *Salmo trutta*
B	8128/97	Arctic charr *Salvelinus alpinus*
C	8239/97	Rainbow trout *Oncorhynchus mykiss*
D	1397/00	Rainbow trout *Oncorhynchus mykiss*
E	Ra/03	Atlantic salmon *Salmo salar*
G	Os/03	Atlantic salmon *Salmo salar*
H	Htan6/03	Rainbow trout *Oncorhynchus mykiss*

***In vivo* experiments.** Rainbow trout *Oncorhynchus mykiss* fingerlings (average weight = 1.7 g) were exposed to *Flavobacterium columnare* by bathing in 2 experiments. Seven strains representing different genotypes of *F. columnare* were inoculated in Shieh medium for 48 h and the cultures were mixed thereafter. Mixed strain exposure was chosen to equalise the slight differences in tolerance to salt treatments between strains observed in our *in vitro* experiment and also because several genetically different *F. columnare* strains can co-occur in outbreaks. The water temperature was 25°C. During the experiment, acclimation from 20°C was allowed for 2 d. Fish (n = 270) were challenged under aeration in 15 l of water containing 4.3×10^6 cfu ml^{-1} *F. columnare* cells for 30 min. After the challenge, fish were transferred to 9 experimental tanks (water volume = 6 l) and received a continuous flow of aerated well water with an original pH of 6.9. The flow rate was adjusted to between 300 and 330 ml min^{-1}, and the water turnover time was 18 to 20 min. Tanks were randomised into 3 treatments each with 3 replicates: (1) control with no changes in water conditions, (2) bathing with 4% NaCl and (3) decrease in water pH to between 4.5 and 4.6. In both treatments, the first bath was given 8 h after bacterial challenge to allow *F. columnare* to invade fish (Day 1). The first bath lasted for 15 min and subsequent baths were 5 min (given on Days 3 and 5, see Fig. 1a). The reason for the reduction of bath duration was the strong reaction of infected fish to the fast change in water quality. Water circulation was stopped during the treatments. In the NaCl treatment, 1 l of 20% NaCl solution was added to experimental tanks with 4 l of water. In the pH treatment, the first bath was given by adding 3 ml of 20% acetic acid to each tank to decrease the pH to 4.5. For the second and third baths (Days 3 and 5) 2.5 ml of acetic acid was added decreasing the pH to 4.6. Fish were not fed during the experiment. Mortality was monitored twice a day for 6 d. Mortality data were analysed with Kaplan-Meier log rank survival analysis and SPSS 11.0 software.

In the second experiment, the exposure was performed similarly, but the NaCl bath was given only once; the NaCl concentration was lower and the duration of the bath longer. The bath was given 1 h after bacterial challenge to study whether a single long bath

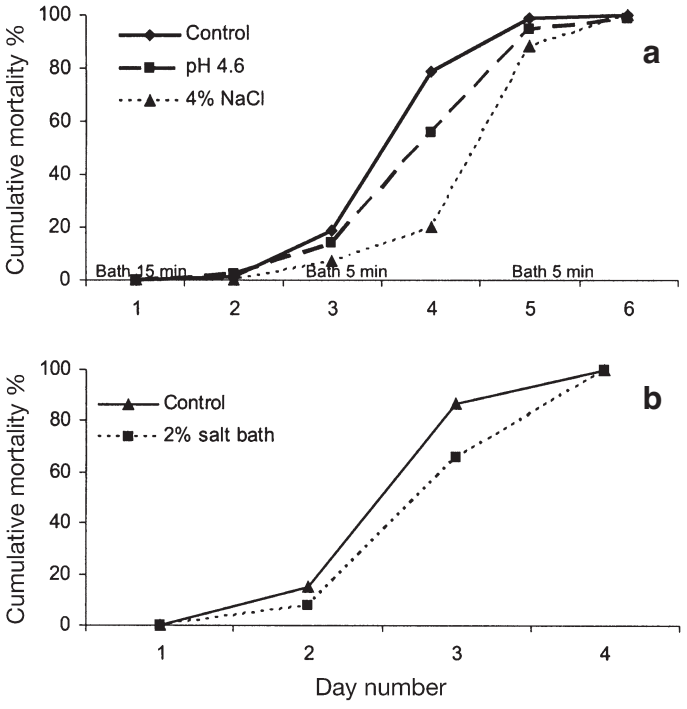

Fig. 1. *Oncorhynchus mykiss* infected with *Flavobacterium columnare*. Cumulative mortality (%) of rainbow trout *O. mykiss* suffering from columnaris disease. (a) In the first experiment, fish were exposed to *F. columnare* on Day 1 as well as to salt (4% NaCl) or acidic baths (water pH 4.6) on Days 1 (duration = 15 min), 3 and 5 (duration = 5 min). (b) In the second experiment, fish were exposed to *F. columnare* on Day 1 as well as to a 2% salt bath (duration = 30 min) given 1 h after bacterial exposure

could prevent columnaris infection when no signs of the disease occur. Fish (n = 106) were exposed to a mixture of inoculated *F. columnare* strains in a bath containing 2.8×10^6 cfu ml^{-1} *F. columnare* cells. Exposed fish were randomly placed in 6 experimental tanks (water volume = 6 l) and a 2% NaCl bath lasting for 30 min was introduced to 3 tanks as described previously. The water temperature was 25°C. In both experiments, an additional control group of fish with no challenge was kept under rearing conditions similar to the experimental fish. This control group was not replicated.

Buffering capacity of fish skin mucus. The buffering capacity of fish skin mucus against fluctuations in water pH was studied using 105 rainbow trout fingerlings (average weight = 1.7 g). Fish were placed in 3 aquaria with aeration (water volume = 15 l). The pH was adjusted to 4.6 by adding 35 ml of NaOH-buffered 20% acetic acid (buffered to pH 4.5) to each aquarium. Samples (3 fish from each aquarium) were taken prior to the decrease of water pH and 15 min and 1 h after the decrease in pH level. pH from fish skin mucus, mucus rinsed with distilled water and fish skin (mucus removed) was measured directly from the skin of freshly killed fish using an HD 8705 pH meter (Delta OHM) with a BlueLine 27 pH electrode (Schott), which is designed for measuring the pH of surfaces. The fish were killed by breaking the spinal cord with a pair of scissors. Data were analysed with SPSS 11.0 software using ANOVA.

RESULTS

In the *in vitro* experiments, all the treatments significantly reduced the number of viable *Flavobacterium columnare* (repeated measures ANOVA, $F = 67.9$, df = 3, $p < 0.001$) and the effect of incubation time within treatments was significant ($F = 4.5$, df = 1.93, $p = 0.013$). An interaction between treatment and bacterial strain was also found ($F = 4.5$, df = 9, $p = 0.002$), as well as a 3-way interaction between all 3 variables (treatment, time and bacterial strain, $F = 3.6$, df = 21, $p = 0.008$), reflecting the differences between strains in each treatment. For the 15 min exposure, a pH of 4.6 was the most effective disinfectant, reducing the number of viable bacterial cells by 99.9%. Fifteen minutes exposure in 4% NaCl, pH 5 or pH 4.86 were not as effective, though the efficacy of these treatments increased with an exposure time of 1 h. After a 1 h treatment, practically all cells (99.9%) lost their viability in pH values of 4.6 and 4.86. Over 99% of cells were eliminated in 4% NaCl, and pH 5.0 eliminated 98 to 100% of the bacterial cells following a 1 h exposure (Table 2).

In the experimental challenge of fish with *Flavobacterium columnare*, the bathing of fish in 4% NaCl or in an environment of pH 4.6 every other day (once for 15 min and twice for 5 min) significantly affected the survival of fish, keeping the daily mortality rate lower when compared to an untreated control group (Kaplan-Meier survival analysis, log rank 8.78, $p = 0.003$ and log rank 51.32, $p < 0.001$, respectively). However, bath treatments did not affect the overall mortality of fish; after 6 d, all the fish in the experiment had died (Fig. 1a).

In the second experiment, in which a 2% NaCl bath was given only once for 30 min, the mortality in the salt-bathed group was approximately 10% lower than in the control group during the first 3 d. This difference was, however, not statistically significant. During the experiments, 3 kinds of symptoms of columnaris disease were seen in the majority of the fish: (1) a greyish area around the dorsal fin, (2) erosion of the tail and (3) gill necrosis. In both experiments, the additional control group without bacterial challenge or treatments did not have these signs.

Fish skin mucus was shown to buffer the variation of water pH (Fig. 2). At the beginning, mucus pH was

Table 2. *Flavobacterium columnare*. Average percentage (±SE) of viable cells after exposure to 4% NaCl and different pH values in culture (Shieh broth) after 15 min and 1 h (3 replicates for each treatment). nd = not determined

Strain	pH 5.0		pH 4.86		pH 4.6		4% NaCl	
	15 min	1 h	15 min	1 h	15 min	1 h	15 min	1 h
A	nd	1.1 ± 0.25	157.6 ± 11.4	0 ± 0	0.1 ± 0.07	0 ± 0	4.2 ± 0.3	0.4 ± 0
B	nd	0.7 ± 0.05	13.0 ± 13.0	0 ± 0	0.02 ± 0.009	0 ± 0	0.25 ± 0.25	0.8 ± 0.8
C	nd	0 ± 0	16.1 ± 2.0	0 ± 0	0.002 ± 0	0 ± 0	1.6 ± 1.2	0.1 ± 0.1
D	3.4 ± 1.05	2.2 ± 0.7	12.2 ± 3.0	0 ± 0	0.1 ± 0.01	0 ± 0	1.0 ± 0.1	0 ± 0
E	1.8 ± 0.6	0 ± 0	22.3 ± 4.0	0 ± 0	0 ± 0	0 ± 0	1.3 ± 0.2	0.6 ± 0.5
G	0.6 ± 0.3	0.6 ± 0.4	3.7 ± 0.4	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.3	0.001 ± 0.001
H	7.0 ± 4.4	0 ± 0	16.5 ± 3.2	0.01 ± 0.01	0 ± 0	0 ± 0	2.9 ± 0.3	0 ± 0

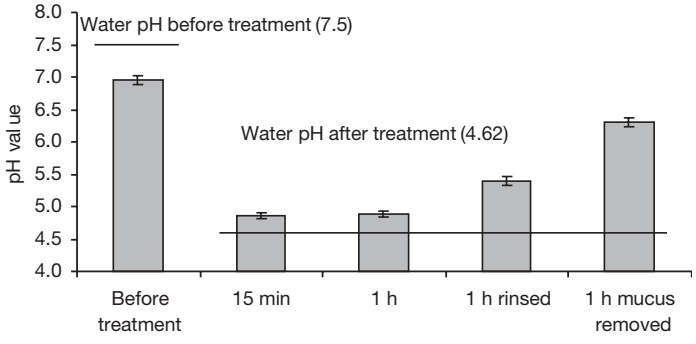

Fig. 2. *Oncorhynchus mykiss*. Buffering capacity of rainbow trout skin mucus in acidic water (pH 4.62). Mean pH (±SE) before and after addition of acetic acid are presented as bars and the ambient water pH as lines

significantly lower than ambient pH (6.96 and 7.5, respectively, $t = -8.13$, df = 8, $p < 0.001$). Fifteen minutes after the decrease of water pH to 4.62, the pH of skin mucus also decreased, but only to pH 4.86; the difference between mucus and water pH was significant ($t = 5.72$, df = 8, $p < 0.001$). After 1 h of exposure to low pH, the difference between pH values of skin mucus and water still remained significant. Fish skin mucus pH remained at 4.89 but when the ambient water was rinsed from the mucus with distilled water, the pH of the mucus was 5.4. Differences between these pH values and water acidicity were both significant ($t = 3.55$, df = 8, $p < 0.001$, $t = 11.03$, df = 8, $p < 0.001$, respectively). When mucus was removed from the fish, the pH of the skin was found to be significantly higher (6.3) than that of the water ($t = 24.3$, df = 8, $p < 0.001$).

DISCUSSION

Our *in vitro* results strongly suggest that salt or acidic bathing could be used to treat columnaris disease, because 95 to 100% of *Flavobacterium columnare* cells did not even tolerate exposure to 15 min pH 4.6 or 4% salt treatments. In addition, we had previously found that the doubling time of *F. columnare* cells is strongly reduced by addition of NaCl, and growth ceases completely when salinity reaches 0.1% (authors' unpubl. data). These Finnish *F. columnare* strains are, thus, highly sensitive to salinity. However, sensitivity to salinity seems to vary between isolates of different geographical origins. In this study, it was also shown that Finnish *F. columnare* strains exhibit differences in tolerance to low pH and 4% salinity *in vitro*. According to Bernardet & Grimont (1989) and Bernardet (1989), 9 *F. columnare* strains grew in media with 0.5% NaCl, but not with 1.0% NaCl. Some strains isolated from fathead minnow and catfish did not grow in 0.5% NaCl (Shamsudin & Plumb 1996). The low tolerance of *F. columnare* to elevated salt concentrations has led to suggestions that salt could be used as a treatment for columnaris disease (Soltani & Burke 1994, Altinok & Grizzle 2001). Inland waters in Finland are usually acidic, with pH ranging from 6.6 to 6.9. In many fish farms, the pH of incoming water is raised slightly above 7 using lime. Together with a temperature rise, however, this apparently favours the occurrence of *F. columnare* in summer. The optimum pH for the growth of Finnish *F. columnare* isolates is between 7.2 and 7.4 (authors' unpubl. data).

Although *Flavobacterium columnare* was sensitive to increased salt concentration and water pH below 4.8 *in vitro*, acid and salt baths had no effect on overall mortality *in vivo*. Mortality reached 100% in all groups within 6 d in the first experiment and within 4 d in the second experiment. However, during the first 4 d of the first experiment, bath treatments significantly reduced mortality, perhaps by killing the bacteria shed from the fish and thus hampering the transmission of the disease. In the second experiment, with prolonged bathing time, a single 2% salt bath reduced the mortality rate during the first 2 d of the experiment, but the difference was not statistically significant.

The failure of acidic baths to prevent columnaris disease can probably be explained by the buffering

capacity of fish skin against decreased water pH. In the beginning of the buffering capacity experiment, the mucus pH was lower than the ambient water pH. When acid was added, the pH of the mucus decreased, but remained higher than the pH of water. When mucus was removed, the pH of the skin remained considerably higher than that of the mucus. This demonstrates that the mucus layer provides a strong shield against environmental changes. During columnaris infection, *Flavobacterium columnare* cells may be located underneath the mucus layer and may, therefore, be protected from pH changes created by experimental bathing. In conclusion, this study shows that results obtained from *in vitro* experiments must be considered with caution when applied to *in vivo* conditions, especially in the case of fish farming systems.

Adhesion of the pathogen to host surface is a critical point in the colonisation process. Adhesion of *Flavobacterium columnare* cells has been shown to be temperature-dependent (Decostere et al. 1999a,b) and the adhesion of *F. columnare* cells is reduced by 83% in 0.9% salinity (Altinok & Grizzle 2001). Salinity and pH have also been reported to influence the adhesion of fish pathogenic *Vibrio* strains (Balebona et al. 1995, Bordas et al. 1996). In fish farming, salt baths could be used to prevent the adhesion of freeswimming pathogens. This is, however, only a preventative method and should be carried out before disease signs occur. For example, salt is already used in fish farming to treat ectoparasitic *Chilodonella* spp. infections. The use of salt could also be beneficial in cases whe fish are subjected to facilitated bacterial invasion due to ectoparasitic infection and damage of the skin.

The use of 4% NaCl or low pH to kill *Flavobacterium columnare* seems to be promising *in vitro*. However, the use of these treatments *in vivo* is complicated and uncertain. Fish skin mucus can resist the osmotic pressure due to strong salt concentration and also buffer the ambient low pH, as was shown in this study; thus, the pathogenic cells, presumably located inside and under the mucus layer, are protected. Furthermore, the exposure time of fish which can be used for such extreme treatments is limited (Alabaster & Lloyd 1980); this was also noticed in the first experiment. Infected fish did not tolerate the fast change of water salinity or pH, thus the duration of 4% salt and pH 4.6 baths had to be shortened to 5 min. This is likely to be a result of difficulties in osmoregulation of fish due to columnaris infection of the skin and gills.

In the light of this study, both of the treatments (salt and low pH) seem to be ineffective against acute columnaris disease, but their efficacy as preventive methods should be studied further in normal fish farming environments. However, we suggest that salt baths could be used to reduce the number of *Flavobacterium columnare* shed from fish into the water, when heavy mortality has not yet occurred. Even though the use of acidic baths in fish farming may be limited due to the poor tolerance of salmonid fishes to low pH, the use of salt baths could be more practical from the fish farmers point of view since salt is being used in treatment of other disease-causing agents such as *Chilodonella* protozoans.

Acknowledgements. This work was supported by the University of Jyväskylä (Rector's grant for L.-R.S.), Academy of Finland (program on Sustainable Use of Natural Resources) and Graduate School of Biological Interactions, Turku, Finland. The authors would like to thank D. Benesh for helpful comments and A. Vainikka for help with statistical analysis. The bacterial strains were kindly donated by Dr. P. Rintamäki-Kinnunen (University of Oulu) and National Veterinary and Food Research Institute (Oulu regional unit).

LITERATURE CITED

Alabaster JS, Lloyd R (1980) Water quality criteria for freshwater fish. Butterworths, London

Altinok I (2004) Toxicity and therapeutic effects of chloramine-T for treating *Flavobacterium columnare* infection of goldfish. Aquaculture 239:47–56

Altinok I, Grizzle JM (2001) Effects of low salinities on *Flavobacterium columnare* infection of euryhaline and freshwater stenohaline fish. J Fish Dis 24:361–367

Balebona M, Moriñigo M, Faris A, Krovacek K, Månsson I, Bordas M, Borrego J (1995) Influence of salinity and pH on the adhesion of pathogenic *Vibrio strains* to *Sparus aurata* skin mucus. Aquaculture 132:113–120

Bernardet JF (1989) '*Flexibacter columnaris*': first description in France and comparison with bacterial strains from other origins. Dis Aquat Org 6:37–44

Bernardet JF, Grimont PAD (1989) Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter pschrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida and Masumura 1996. Int J Syst Bacteriol 39:346–354

Bordas MA, Balebona MC, Zorrilla I, Borrego JJ, Morinigo MA (1996) Kinetics of adhesion of seleceted Fish-Pathogenic *Vibrio* strains to skin mucus of Gilt-head sea bream (*Sparus aurata* L.). Appl Environ Microbiol 62: 3650–3654

ChemDat® (1999) The Merck chemical database. Merck, Darmstadt

Decostere A, Haesebrouck F, Turnbull JF, Charlier G (1999a) Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. J Fish Dis 22:1–11

Decostere A, Haesebrouck F, Van Driessche E, Charlier G, Ducatelle R (1999b) Characterization of the adhesion of *Flavobacterium columnare* (*Flexibacter columnaris*) to gill tissue. J Fish Dis 22:465–474

Jee LK, Plumb JA (1981) Effects of organic load on potassium permanganate as a treatment for *Flexibacter columnaris*. Trans Am Fish Soc 110:86–89

Koski P, Hirvelä-Koski V, Bernardet JF (1993) *Flexibacter columnaris* infection in arctic charr (*Salvelinus alpinus* L.): first isolation in Finland. Bull Eur Assoc Fish Pathol 13:66–69

Nemcsok JG, Hughes GM (1988) The effect of copper sulphate on some biochemical parameters of rainbow trout. Environ Pollut 49:77–85

Shamsudin MN, Plumb JA (1996) Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from 4 species of fish. J Aquat Anim Health 8:335–339

Shieh HS (1980) Studies on the nutrition of a fish pathogen, *Flexibacter columnaris*. Microbios Lett 13:129–133

Soltani M, Burke CM (1994) Responses of fish-pathogenic *Cytophaga/Flexibacter*-like bacteria (CFLB) to environmental conditions. Bull Eur Assoc Fish Pathol 14: 185–187

Speare DJ, Arsenault GJ (1997) Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 54:2653–2658

Straus (2004) Comparison of the acute toxicity of potassium permanganate to hybrid striped bass in well water. J World Aquat Sci 35:55–60

Thomas-Jinu S, Goodwin AE (2004) Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds. J Fish Dis 27:23–28

Wakabayashi H (1991) Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish. J Fish Dis 14:279–290

Wakabayashi H, Egusa S (1972) Premilinary experiments on environmental factors influencing the prevalence of columnaris disease. Fish Pathol 7:58–63

Editorial responsibility: David W. Bruno, Aberdeen, UK

Submitted: October 6, 2004; Accepted: January 27, 2005
Proofs received from author(s): June 10, 2005