ARTICLE

The Effect of High Total Ammonia Concentration on the Survival of Channel Catfish Experimentally Infected with Flavobacterium columnare

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Abstract
Ammonia concentrations in water can affect the severity of Flavobacterium columnare infections in fish. Two trials lasting 7 d each were conducted to determine the effect of a single immersion flush treatment of total ammonia nitrogen (TAN; 15 mg/L) on the survival of channel catfish Ictalurus punctatus infected with F. columnare; the chemical was added while the water flowed continuously through the tanks. Both trials consisted of four treatments: (1) no ammonia exposure and no bacterial challenge (control), (2) ammonia exposure only, (3) bacterial challenge only, and (4) both ammonia exposure and bacterial challenge. Two hours after exposure to ammonia, the highest un-ionized ammonia level was 0.43 mg/L. The percent un-ionized ammonia is based on TAN, temperature, and pH. Caudal fins from three fish in each treatment were sampled at 24 h posttreatment to be analyzed by quantitative real-time polymerase chain reaction (qPCR). No significant difference in survival (mean ± SE) was noted between the channel catfish in treatment 1 (95.2 ± 1.2%) and those in treatment 2 (95.6 ± 1.0%); however, survival in both treatments 1 and 2 differed significantly from that in treatments 3 (8.5 ± 4.5%) and 4 (41.8 ± 12.7%). Treatment 4 catfish had significantly higher survival than treatment 3 catfish. Quantitative PCR data showed that treatment 4 fish had significantly less F. columnare (7.6 × 10⁵) than did treatment 3 fish (1.2 × 10⁷), and treatment 2 fish (8.5 × 10³) had significantly less bacteria than did treatment 1 fish (6.9 × 10⁴), indicating that ammonia limited the F. columnare infection. The highest mean concentration of the bacteria (3.9 × 10⁷) was found on moribund fish. The ammonia concentrations tested did not negatively influence fish survival but interfered with the infection process. An in vitro assay was also conducted to evaluate the direct effects of ammonia on F. columnare.

Elevated ammonia concentrations in water serve to stress fish. This is especially true for un-ionized ammonia (UI), the toxic form that can cause fish to become more susceptible to infections and, among infected fish, cause higher mortalities (Chen et al. 1982; Noga 1996; Hoole et al. 2001). Ferguson et al. (1992), tested the influence of exposure to UI at 0.4 mg/L (total ammonia nitrogen [TAN] not reported) for 90 d on susceptibility of fish to bacterial infection, found that the mucus layer covering the gills was altered (all types of glycoprotein production were increased) on rainbow trout Oncorhynchus mykiss that were naturally infected with Flavobacterium branchiophila, but not on uninfected fish subjected to the same high ammonia concentration. Ferguson et al. (1992) concluded there is a link between high ammonia concentrations in the water and enhanced susceptibility to F. branchiophila.

The addition of TAN at 1.1 mg/L (UI, 0.1 mg/L) carbon dioxide at 6 mg/L, or a combination of the two resulted in mortality of channel catfish Ictalurus punctatus infected with a sublethal dose of Aeromonas hydrophila (Walters and Plumb 1980). Amin et al. (1988) reported that the severity of columnaris disease increased and the median death time was shortened when infected Nile tilapia Oreochromis nilotica with scarified gills were kept in waters containing NH₄Cl at 168 mg/L (approximately 53 mg/L TAN; UI could not be determined from data given). The mortality in Japanese eel Anguilla japonica associated with columnaris disease increased from 57% to 80%
as TAN (UI concentrations not reported) increased from 0.4 to 1.2 mg/L (Chen et al. 1982).

Ammonia is toxic to both fish and parasites but, because of slight differences in the toxicity of ammonia to host and parasite, it has been used in water-bath control of monogeneans (Chan and Wu 1984). Some research has suggested that elevated ammonia concentrations may actually lessen the impact of bacterial disease. Morris et al. (2006) found that the survival of Lost River suckers Deltistes luxatus exposed to F. columnare significantly increased as UI concentrations increased to 0.4 mg/L (TAN, 0.78 mg/L) from 0.006 mg/L (TAN, 0.01 mg/L). While performing columnaris challenge tests on channel catfish in 2009, the present authors observed that channel catfish in water with the highest TAN concentration (>10 mg/L) had milder infections and greater survival than did similarly challenged fish with lower TAN concentrations (<10 mg/L).

To further investigate the effects of ammonia on the course of a bacterial disease, a study was designed to determine the effect of a single high level exposure of ammonia on channel catfish experimentally infected with F. columnare. The purpose of this study was to determine whether elevated ammonia concentrations would reduce mortality caused by columnaris disease. An in vitro assay was also conducted to ascertain the direct effects of ammonia on the survival of F. columnare.

METHODS

Twenty kilograms of channel catfish (7 ± 0.62 g each [mean ± SD]) from the Harry K. Dupree Stuttgart National Aquaculture Research Center (HKDSNARC), Stuttgart, Arkansas, were placed in 400-L tanks and acclimated for at least 1 week. Fish were then moved to sixteen 18-L tanks containing 10 L of water at a rate of 50 g of fish/L of water (approximately 70 fish/tank); in previous tests, infection did not progress at lower stocking densities. Aeration was provided to each tank with air stones. The tanks received filtered well water at a rate of approximately four water exchanges per day or about 30 mL/min with air stones. The tanks received filtered well water at a rate of 50 g of fish/L of water (approximately 70 fish/tank) in previous tests, F. columnare infections were not produced in higher flows) from an ultra-low-flow water delivery system (Mitchell and Farmer 2010). The water was heated with 50 W electric heaters (HPA-50; Finnex, Westmont, Illinois) that held temperatures between 26.3°C and 27.3°C, and was measured daily with a Wissenschaftlich-Technische Werkstätten (WTW) pH/Oxi 340i/SET meter (Weilheim, Germany) and HOBO Water Temp Pro v2 temperature loggers (Onset Computer Corp., Bourne, Massachusetts).

An Accumet Basic AB15 pH meter (Fisher Scientific, Singapore) was used to measure pH (7.5–8.2) at the beginning of both studies; during the second study, the WTW meter was used to measure pH (7.3–8.2). Dissolved oxygen was measured with the WTW meter during both trials (4.4–8.3 mg/L). Standard titration methods (APHA et al. 2005) were used to measure total alkalinity (217–218 mg/L) and total hardness (119–120 mg/L) in both trials. Chlorides (175.5 mg/L) were measured in both trials by using a mercuric thiocyanate method (Method 8113; Hach Co., Loveland, Colorado).

Experimental design.—Two trials were conducted, each consisting of four treatments: (1) no ammonia exposure and no bacterial challenge (control), (2) ammonia exposure only, (3) bacterial challenge only, and (4) both ammonia exposure and bacterial challenge. Each trial consisted of four replicates for each treatment and lasted for 7 d.

Ammonia exposure.—Ten milliliters of stock solution of ammonium chloride (NH₄Cl, 46 mg/mL; Sigma-Aldrich Co., St. Louis, Missouri) was added to each tank to reach a nominal concentration of 15 mg/L TAN in the water. The NH₄Cl was applied as an immersion flush treatment; the chemical was added while the water continuously flowed through each tank. The TAN concentrations were determined in each tank with a Hach DR/4000 spectrophotometer using Nessler Method 8038 with the following adaptations: assays used 5-mL water samples with 0.2 mL of Nessler’s reagent instead of 25 mL and 1 mL, respectively. The water sampling procedure from each tank was the same for trial 1 and trial 2. In trial 1, TAN samples were taken at 1 min and at 1, 2, 3, 4, and 6 h posttreatment. No pH measurements were taken to correspond to the TAN readings, and therefore no UI concentrations were determined. In trial 2, water samples for TAN determination were taken at 20 min and at 1, 2, 3, 4, 6, 30, and 56 h. Temperature and pH were also measured at each sample time. Un-ionized ammonia was determined from TAN, pH, and temperature by using the spreadsheet of Colt (2002).

Flavobacterium columnare challenge.—Fish were experimentally challenged with F. columnare isolate LV-359-01 within 5 min of the ammonia treatment. The isolate was retrieved from storage in a –80°C freezer and streaked on Ordals medium (Anacker and Ordal 1955); after 48 h the isolate was dislodged from the agar with a sterile cotton swab and inoculated into 5 mL of F. columnare growth medium (FCGM; Farmer 2004). This suspension was incubated at 28°C for 24 h, after which 5 mL of starter culture was used to inoculate 1 L of FCGM. The inoculated 1 L of broth was incubated for up to 24 h at 28°C in an orbital shaker incubator set at 200 revolutions per minute. When the bacterial growth reached an absorbance of 0.70 at 550 nm (approximately 2 × 10¹⁰ bacteria/mL), the flask was removed and placed on a stir plate at room temperature. A 10-mL sample was removed from the broth for serial dilution and determining colony-forming unit (CFU) data. To account for variations in size, age, and fitness of fish used in the two studies, nonreplicated range-finding trials (following methods above) were conducted 1 week before each trial to determine the amount of broth to add to the tanks in each trial to yield acceptable challenge results (target of 90% mortality). In trials 1 and 2, 40 mL and 75 mL of bacterial suspension, respectively, were added to tanks receiving bacterial challenges. Fish were observed daily for clinical signs. Fish unable to maintain neutral buoyancy were considered moribund and removed for sampling.
Identification and quantification of bacteria.—At 24 h after exposure to *F. columnare*, three fish showing no obvious signs of disease were sampled arbitrarily from each tank for all treatment groups. The caudal fins were severed at the base of the peduncle at –8°C. This sample site was selected based on its high surface area for *F. columnare* attachment and low amounts of fish tissue that would clog the DNA filter membranes. Samples were also taken throughout the course of the study from moribund and freshly dead fish. Genomic DNA extraction was performed with a DNaseasy Blood and Tissue Kit (Qiagen GmbH, Düsseldorf, Germany). The extracted template DNA was used for pathogen detection, identification, and quantification, utilizing the procedure of Panangala et al. (2007): forward primer, FcFp, (5′-CCTGTACCTAATTTGGGGAAAAGAGG-3′); reverse primer, FcRp, (5′-CGGTTATGCCCTTTATCATAGA-3′); and FAM-labeled probe (5′-ACAAATGTATTTCGAGGAGA GTATCTGATGGG-3′). This primer and fluorescent probe set targets a region of the chondroitin AC lyase gene of *F. columnare*. Primers and FAM-labeled probe were obtained from Applied Biosystems, Inc. (Foster City, California). All quantitative polymerase chain reaction (qPCR) assays were performed on a Lightcycler 480 Real Time PCR system (Roche, Indianapolis, Indiana). All qPCR assays were run in duplicate for each sample with standards and a no-template control included on each plate. Reactions included 500 nM for both forward (FcFp) and reverse (FcRp) primers, 250 nM labeled probe, 1 μL of template DNA (300–380 ng of genomic catfish and bacterial DNA), Bio-Rad 2× master mix, and molecular-grade water for a 20-μL total reaction volume. The initial DNA denaturation step was 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, and 60°C for 30 s. These data were calculated based on a standard curve generated from bacterial samples that had been previously assayed. The standard curve efficiency was 1.94 and the r² was 0.98; the resulting equation of the line was $y = –3.472x + 47.94$.

In vitro studies.—In vitro minimal inhibitory concentration determination was conducted according to the method of Darwish et al. (2008a). Briefly, an *F. columnare* suspension (LV 359-01) was prepared in sterile saline (0.85% NaCl) and used to inoculate fivefold-diluted Mueller–Hinton broth. This broth was pipetted into a 96-microwell plate; to the initial well was added a stock solution of NH₄Cl, and twofold dilutions were carried out through the tested range of 60, 30, 15, 7.5 mg/L TAN. This was done in triplicate with inclusion of positive and negative control wells. Plates were incubated at 28°C for 24 h and evaluated visually for the presence or absence of growth.

The direct effects of ammonia on the survival of the bacteria were evaluated for the reduction of CFUs (Darwish et al. 2008b). Briefly, *F. columnare* was suspended in sterile well water (same parameters as the water used for the in vivo studies). Ammonium chloride was added to give TAN concentrations of 15 and 30 mg/L. The suspension of water, bacteria, and ammonia were incubated at room temperature for 6 h; samples were removed, serially diluted, and plated on Ordal’s agar medium for CFU counts.

Statistical analysis.—Percent survival in the two trials was compared by using the general linear model (GLM) to perform an analysis of variance (ANOVA) with the statistical software Minitab version 13 (Minitab, Inc., State College, Pennsylvania). Arcsin-transformed data for percent survival and log-transformed qPCR and CFU data were normally distributed with equal variances (Kolmogorov–Smirnov normality test and Bartlett’s test for equal variances; Minitab version 13). A residual model diagnostic method revealed one outlier outside 2σ units and outside the 95% confidence limit for the qPCR data; this outlier was not included in the analysis. A GLM ANOVA was performed on the survival, qPCR data, and CFU data—all transformed as already stated. Differences among treatment means were separated by using the Tukey–Kramer procedure for pair-wise comparisons (Tukey 1953; Kramer 1956). All treatment effects were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Survival of Channel Catfish

Moribund fish in the two bacterially challenged groups displayed signs typical of disease. Infected fish exhibited areas of depigmentation, erosion of the caudal and dorsal fin margins, and typical “saddleback” lesions (Noga 1996); during later stages, infected tissues became necrotic. Clinical signs of the two challenged groups were similar and no signs or lesions were observed in the control or ammonia-only treatment groups. Survival data are shown in Figure 1. There was no significant difference between the survival of the control fish (92.6% and 97.7% for trials 1 and 2, respectively) and the fish exposed only to ammonia (93.3% and 97.9%). This indicated that ammonia alone did not influence survival. The control and ammonia-only groups had significantly higher survivals than the other two groups receiving the *F. columnare* challenge. The two groups challenged with *F. columnare* had significantly different survivals, survival being higher among the fish receiving the ammonia exposure (38.5% and 45.3%); the bacterial challenge-only group had 6.1% and 11.0% survival for trials 1 and 2, respectively. This indicated that the high ammonia concentrations either limited the survival of *F. columnare* or otherwise interfered with the infection process.

Identification and Quantification of Bacteria

In trial 1, identification of *F. columnare* in all treatments was determined. but quantification results were not reported because samples were not properly standardized. In trial 2, each treatment had a significantly different concentration of *F. columnare* as determined by qPCR (Figure 2). Among the two *F. columnare*–challenged groups, the ammonia-treated group had significantly less bacteria (7.6 × 10⁵ compared with 1.2 × 10⁷; 94% reduction); for the nonchallenged groups, the one receiving an ammonia treatment also had significantly less bacteria.
CHANNEL CATFISH INFECTED WITH *FLAVOBACTERIUM COLUMNARE*

165

Z1

Z2

Y1

Y2

X1

X2

0%

10%

20%

30%

40%

50%

60%

70%

80%

90%

100%

Control

Am

Am+Fc

Fc

Trial 1

Trial 2

FIGURE 1. Survival of channel catfish subjected to four different treatments in trials 1 and 2. Treatments included no exposure to ammonia or challenge with *Flavobacterium columnare* (control), exposure to ammonia only (Am), exposure to ammonia and bacterial challenge (Am+Fc), and bacterial challenge only (Fc). Ammonium chloride treatment produced a nominal 15 mg/L TAN. Significant differences are signified by different letters on bars; error bars represent SEs.

(8.5 × 10^3 compared with 6.9 × 10^4; 88% reduction). Although not significantly different, the moribund or recently dead fish from both challenge groups had slightly higher numbers of *F. columnare* than apparently healthy fish from the *F. columnare*-challenge-only group (Figure 2). The moribund or recently dead fish had significantly higher bacterial numbers than the other three groups.

Interestingly, *F. columnare* was isolated from the caudal fins of all channel catfish tested, regardless of treatment. Based on the control fish in our trials, *F. columnare* background concentrations determined by qPCR were 6.9 × 10^4. The background *F. columnare* on nonchallenged fish that received the ammonia treatment was significantly lower than the concentration of *F. columnare* on the control fish (no challenge or exposure), further supporting the idea that ammonia can lower the number of *F. columnare* attached to the fish. At HKDSNARC, no population of channel catfish has tested as being free of *F. columnare* by qPCR, and most have been bacteria between 10^3 and 10^5 (observation by the authors). This indicates that finding this facultative pathogen at fairly high concentrations on channel catfish is normal and does not necessarily signify an impending disease outbreak.

**In Vitro Studies**

The minimal inhibitory concentration of TAN was 60 mg/L; at 30 mg/L, less growth was observed, but growth was not inhibited. Abundant growth was seen at 15 mg/L TAN and at the lower rates. The control, 15 mg/L TAN, and 30 mg/L TAN treatments resulted in mean counts of 1.95 × 10^{10}, 5.18 × 10^9, and 3.4 × 10^9 CFUs, respectively. This represented a 74% reduction in CFUs for the 15 mg/L TAN treatment and 84% for the 30 mg/L treatment counts compared with the CFU numbers of the control group; both were significantly different from the control but were not different from each other. Clearly, ammonia can limit the growth of *F. columnare*, as well as reduce its survival. Even a small reduction in bacterial numbers may have a profound effect on the course of the infection, given that the isolate used in this study was facultative and not a primary
pathogen. A relatively small increase in bacterial numbers may be enough to overwhelm the fish and cause disease. It is also possible that ammonia is interfering with the attachment of bacteria to the fish and thereby reducing mortality; future research should investigate this.

**TAN and UI Determinations**

The measured TAN concentrations for trials 1 and 2 are given in Figure 3a, and the UI concentrations for trial 2 are given in Figure 3b. The TAN concentrations from both trials in the ammonia-treated tanks through 6 h were within 2 mg/L and the UI concentrations in trial 2 were within 0.06 mg/L. The non-ammonia-treated groups also had narrow ranges of TAN and UI concentrations. The highest TAN concentrations (ranging from about 15 to 19 mg/L) were measured from the initial 1- and 20-min samples; UI concentrations peaked at about 0.43 mg/L at 2 h posttreatment. The TAN concentrations in the non-ammonia-treated groups were between 1 and 4 mg/L at 1 h, probably because of the dense stocking of fish (50 g/L of water) and low flow in each tank. At 6 h (one water exchange), both TAN and UI were at about 50% of their peak. By 30 h, the ammonia- and non-ammonia-treated groups had similar TAN or UI concentrations. Thus the effect of ammonia on the course of the *F. columnare* infection was most probably affected within the first 30 h.

The TAN at 1 min in trial 1 exceeded the nominal treatment concentrations of 15 mg/L. Because these readings could be in part due to incomplete mixing of the ammonia in the water within 1 min, the first sample in trial 2 was taken at 20 min. The 20-min samples were within approximately 1 mg/L of the nominal rate.

In preliminary acute TAN/UI toxicity trials, all channel catfish (*n* = 60) survived an immersion flush treatment in the ultra-low-flow system with a measured TAN of 24 mg/L and a calculated UI of 0.49 mg/L. This indicates that TAN and UI concentrations even higher than those used in our trials would not affect survival. For comparison, the 24-h median lethal concentration of TAN and UI at pH 8 was determined to be 38.8 and 1.82 mg/L, respectively, for channel catfish (Tomasso et al. 1980).

In preliminary trials, most fish (88%) survived TAN and UI concentrations up to 49 mg/L and 1.25 mg/L, respectively, and
FIGURE 3. Panel (a) shows the total ammonia nitrogen (TAN) concentration from trials 1 and 2 following the application of 15 mg/L TAN and/or exposure to *Flavobacterium columnare*. Treatments are as described in the previous two figures. The TAN measurements from all ammonia-exposed groups were similar (within about 2 mg/L), decreasing to the measured reading of the non-ammonia-exposed group by 30 h. The error bars represent SEs. Panel (b) shows the un-ionized ammonia from the measured TAN, pH, and temperature following the application of 15 mg/L TAN and/or exposure to *Flavobacterium columnare*. Results are from trial 2. The un-ionized ammonia concentrations from the ammonia-exposed groups were similar (within about 0.06 mg/L), dropping to the measured reading of the non-ammonia-exposed group by 30 h.
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REFERENCES


20% survived 61 mg/L TAN and 2.41 mg/L UI (unpublished data). Fish were held for 4 d and all fish deaths occurred within 24 h of ammonia exposure. A 12-h exposure of channel catfish to 25 mg/L TAN showed no difference in hematocrit, percent total plasma protein, or plasma and muscle chloride (Tomasso et al. 1980). The median lethal time for channel catfish exposed to 2.0 and 1.6 mg/L UI was 25 and 100 h, respectively (Colt and Tchobanoglous 1978). In this study, NH4Cl was added to the water as an acute exposure just before the bacterial challenge. Because this is different from the more chronic ammonia exposures that occur naturally in most pond or tank settings, it may, in part, explain why the ammonia limited rather than enhanced the disease process. Exposing fish with an established columnaris infection to NH4Cl probably would have produced different results. The severity of columnar infections increased when tilapia were exposed to NH4Cl at 168 mg/L (Amin et al. 1988). However, F. columnare rarely affects all fish at the same time in a population; therefore, an acute exposure to ammonia may offer protection to the fish not yet infected and possibly to those that have just been infected. Along with ammonia, NH4Cl added chloride of about 30 mg/L to the water, but this concentration should have little effect on fish or bacteria given that most chloride treatments (NaCl treatments) are more than 30 times the concentration added in this study (Taylor and Bailey 1979). Based on the findings of this study, an immersion flush exposure of NH4Cl at 46.3 mg/L (nominally yielding 15 mg/L TAN) served to lower the fish mortality caused by F. columnare and the number of bacteria recovered from a challenged fish population; moreover, these treatments did not overly affect the health of the fish. Although there may be some contraindications associated with the use of NH4Cl, there are a number of contraindications for other fisheries chemicals commonly used in aquaculture, including copper sulfate and formalin (Noga 1996). Further testing will be necessary to determine the usefulness of NH4Cl or other ammonia compounds as a management tool to control columnaris in controlled laboratory studies to help better understand the mechanisms of infection with F. columnare. Optimal rates, safety margins, and contraindications will have to be determined in further studies.