

Nitrate Toxicity: A Potential Problem of Recirculating Systems

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Introduction

Rearing fish successfully depends on a number of factors, including the maintenance of good water quality. Adverse water quality can decrease production, reduce growth, and decrease a fish's resistance to disease (Nicholson et al., 1990). Ammonia is the major nitrogenous excretory product of fishes and is also formed from the breakdown of uneaten feed. The conversion of ammonia by bacterial oxidation to nitrate (NO_3^-) is termed nitrification. Bacteria of the Nitrosomonas species convert NH_3 to nitrite (NO_2^-) which is acted upon Nitrobacter species to form NO_3^- . Nitrates are taken up by plants, converted anaerobically to nitrogen gas, or removed by regular water changes.

Ammonia and nitrite are both toxic to fishes. Ammonia toxicity is thought to occur from osmoregulatory imbalance causing renal failure and gill epithelial damage resulting in suffocation, decreased excretion of endogenous ammonia, and general neurological and cytological failure (Meade, 1985). Elevated nitrite levels cause methemoglobinemia (brown blood disease). Nitrate is generally considered nontoxic to fishes (Bromage et al. 1988). In most aquaculture systems, nitrate levels are below 50 mg/L, but in intensive culture systems, nitrate levels often exceed 100 mg/L. Nitrate levels, in recirculating systems that have limited fresh water input, can be 200 mg/L or greater.

The fact that fish are often raised for extended periods of time in water with elevated nitrate levels prompted research into the effects of prolonged nitrate exposure.

Results

Presented here are summaries of studies conducted to investigate the effects of prolonged exposure

to elevated nitrate concentrations. The first study examined the effect of elevated nitrate on the humoral immune response (antibody production) of hybrid striped bass to a formalin killed *Aeromonas salmonicida* bacterin (Hrubec et al. 1996a). Hybrid striped bass were acclimated to the experimental conditions over a two week period by slow addition of sodium nitrate to the tank until levels reached 200 mg/L NO₃-N. An identical tank with untreated water was used as a control. Fish were maintained at the elevated nitrate level for 4 weeks prior to immunization. Nitrate treated and control fish were immunized and the antibody response monitored for three months. Antibody response was determined with an ELISA and expressed as a percent of the positive ELISA control. The maximum antibody response in control and nitrate treated fish was 72% and 48% of the positive control respectively. Randomized control block analysis of variance (ANOVA) showed significant ($p < 0.0001$) differences in the antibody response between the nitrate exposed and control groups.

Hematological and serum biochemical parameters were also monitored in nitrate treated fish. Hybrid striped bass were acclimated to elevated nitrate levels as described above. After five weeks at elevated nitrate levels fish were bled for hematologic and biochemical determinations following procedure determined previously (Hrubec et al. 1996b). There were notable changes in the blood of nitrate exposed fish as compared to control fish. Numbers of reticulocytes (immature red blood cells) were increased in the nitrate exposed fish. In control fish, there were one to two reticulocytes per microscope field under oil immersion, while in the elevated nitrate treated fish there were five to six reticulocytes per field. Nitrate exposed fish had higher monocyte and neutrophil counts and lower thrombocyte-like cell (TLC) counts. The TLC is an unidentified cell type that superficially resembles a thrombocyte, and is probably an immature form of leukocyte. Serum biochemical changes included higher creatinine, and calcium values, and lower chloride concentration than controls. All of the changes seen in the blood parameters were statistically significant.

In addition to the changes in humoral immune function and blood parameters, the nitrate treated fish appeared to become blind three weeks into the experiment, one week after the nitrate concentration reached 200 mg/L. The fish would often swim into objects and the walls of the tank. The skin color became darker, but there were no obvious gross lesions, or changes to the eyes. After seven weeks at the elevated nitrate level, the fish began to die. The fish were found moribund, swimming weakly upside down or lying on the bottom. About 50% of the moribund fish were icteric (jaundiced) around the operculum, gills, ventral abdomen, and in the viscera and fat. The other moribund fish showed no clinical signs. The icteric fish were anemic with a packed cell volume (PCV) of 20 - 25%, while the other moribund fish had a PCV of 30 - 45%. The serum biochemical profile of icteric fish demonstrated elevated creatinine (1.8 mg/dL), total bilirubin (2.5 mg/dL), and AST (585 mU/mL), and decreased sodium (114 mEq/L) concentrations. This hematological profile is consistent with an intravascular lysis of red cells. Histopathological changes included inflammation, hyperplasia and fusion of the gills. The spleen was hypocellular with erythrocytic and lymphoid depletion, and multifocal necrosis. The liver was vacuolated with necrotic foci and the head kidney was depleted of lymphoid tissue. The posterior kidney was edematous with dilated tubules; and there was mineralization and necrosis of the tubules.

These results demonstrate hematological and immunological changes in fish exposed to nitrate, but do not preclude that the changes could be due to other factors. A possible explanation for the

responses seen in the nitrate exposed fish was contamination of the sodium nitrate with some other toxicant. However, analytical examination of the sodium nitrate used in the experiment, revealed no evidence of contamination. Another possible explanation was that the changes observed were due to increased sodium and not nitrate since the addition of sodium nitrate increases sodium levels as well as nitrate. To determine whether the changes in blood parameters were produced by nitrate we examined the hematology and blood chemistry of hybrid striped bass in recirculating systems with elevated nitrate levels.

Hybrid striped bass, raised in recirculating systems with nitrate concentrations above 200 mg/L, were bled. Changes in blood parameters from these fish were compared to hybrid striped bass raised in low density tanks with a nitrate level less than 10 mg/L (Hrubec et al. 1996c, 1996d). Fish from the recirculating system had higher plasma protein values higher leukocyte, lymphocyte, neutrophil, monocyte and reticulocyte counts, and lower TLC counts than fish in tanks. Serum chemistry changes included higher concentrations of total protein, albumin, globulin, creatinine, phosphorus and calcium, and lower chloride values. Many of these changes were the same as seen in the sodium nitrate treated fish. Although these data suggested that the blood changes were caused by elevated nitrate levels, they were not conclusive. Many high density recirculating systems have pH fluctuations that are controlled by addition of sodium bicarbonate to increase alkalinity. This was the case with the systems used in our study. The amount of sodium, added as sodium bicarbonate to the recirculating system, was approximately equivalent to the amount added as sodium nitrate in the original study. Although this study showed similar changes in sodium nitrate treated fish and fish exposed to nitrate produced by nitrification in the tank, the question of whether these changes were due to nitrate or sodium was still not resolved.

To address the nitrate versus sodium issue, a study was conducted where hybrid striped bass were exposed to 200 mg/L nitrate produced by three different salts of nitrate and to elevated bicarbonate levels. Nitrate levels were increased in three separate tanks over a two week period by addition of sodium, calcium and potassium salts of nitrate into the respective tanks. Sodium bicarbonate was added to a fourth tank at the same rate of sodium addition as in the sodium nitrate tank. Fish in a tank with untreated water were used as controls. Differences between the water treatments for each blood value were determined by ANOVA. When a significant difference was detected ($P < 0.05$), the means were compared with a Tukey's means comparison test. The results are shown in Table 1. In most instances, there was little difference between the control and sodium bicarbonate treated fish.

Consistent differences were seen in all nitrate fish for many of the blood parameters. All nitrate treated fish had significantly higher plasma protein, monocyte and reticulocyte counts, and lower TLC counts than control fish. Additionally, total protein, albumin, globulin, and calcium levels were elevated and chloride values depressed when compared to control or bicarbonate treated fish. Fish exposed to the sodium nitrate and potassium nitrate began to die after six weeks at the elevated nitrate levels.

Table 1. Hematologic and serum biochemistry values for fish maintained at different salts of nitrate (200 mg/L N-Nitrate) elevated sodium bicarbonate (NaHCO₃) and control water.

Analyte	Control		NaHCO ₃		NaNO ₃		CaNO ₃		KNO ₃	
Plasma Protein (g/dL)	4.9	a1	4.5	b	5.4	c	5.4	c	5.6	c
Leukocytes (10 ³ /mL)	52.73	a	54.40	a	48.63	a	50.41	a	51.56	a
Lymphocytes (10 ³ /mL)	40.96	a	40.60	a	26.55	a	40.18	a	40.44	a
Neutrophils (10 ³ /mL)	1.32	a	1.37	a	2.16	a	2.35	a	1.79	a
Monocytes (10 ³ /mL)	2.55	a	2.66	a	4.84	a,b	3.79	a,b	5.87	b
TLC ² (10 ³ /mL)	2.74	a	2.22	a	0.95	b	0.97	b	1.11	b
Thrombocytes (10 ³ /mL)	54.77	a	47.60	a	48.32	a	44.33	a	48.06	a
Total Protein (g/dL)	2.7	a	2.6	a	3.73	b	3.2	c	3.3	c
Albumin (g/dL)	1.0	a	1.1	a	1.6	b	1.3	c	1.3	c
Globulin (g/dL)	1.6	a	1.5	a	2.0	b	1.9	b	2.0	b
Creatinine (mg/dL)	0.2	a	0.2	a	1.0	b	0.3	a	0.3	a
T. Bilirubin ³ (mg/dL)	0.0	a	0.0	a	0.4	b	0.0	a	0.0	a
Sodium (mEq/L)	152	a,b	148	c	147	c	149	a,c	154	b
Potassium (mEq/L)	3.5	a	3.4	a	3.5	a	3.5	a	6.0	b
Chloride (mEq/L)	150	a	143	b	52	c	103	d	113	e
Calcium (mg/dL)	9.71	a	9.41	a	11.49	b,c	10.44	b	12.12	c
Phosphorus (mg/dL)	8.1	a,b	8.1	a,b	7.7	a	8.7	b	9.1	c

1) values with different letters are statistically different; 2) Thrombocyte-Like cell; 3) Total Bilirubin

Discussion

Fishes are exposed to environmental nitrate from nitrification of excretory waste, runoff from agricultural lands, and waste water effluent from sewage treatment plants. Nitrate concentrations in aquaculture are not usually elevated due to high water flow through a system or removal of nitrate by plants. In recirculating systems, however, fresh water exchange is often limited. Bonn (1976) reported that adult striped bass tolerated nitrate levels up to 800 mg/L, while fry showed signs of stress at 200 mg/L, however, both adult and fry fed and grew better at levels below 38 mg/L. Unfortunately, the original study was not referenced; and the duration of exposure to the nitrate and clinical signs are not known. There is no information available on the nitrate tolerance of hybrid striped bass. The fish in our study were maintained at elevated levels for extended time periods, which may have enhanced any pathophysiology associated with nitrates. Water quality conditions for rearing striped and hybrid bass have been proposed (Warren et al., 1990). With the exception of nitrate concentrations purposefully outside these ranges, our water quality was within the proposed ranges for all water quality parameters except for nitrite. When nitrate levels were elevated due to addition of a nitrate salt or from nitrification, increased nitrite levels were observed. This increase in nitrite was most likely due to enhanced reduction of nitrate to nitrite caused by the elevated nitrate concentrations.

The effects of ammonia and nitrate on serum enzymes have been investigated using domestic waste water which has high concentrations of these two compounds (Weiser and Hinterleitner, 1980, Bucher and Hofer 1990). Domestic waste water did not increase serum enzymes, but did cause histopathological changes characterized by heavy hyaline droplet degeneration of the kidney tubular cells and progressing to necrosis of the kidney tubules and hepatocytes (Bucher and Hofer 1990). These histopathologic lesions are similar to our observations of nitrate treated fish. The elevated creatinine levels seen in nitrate exposed fish may be an indication of compromised renal function as is observed in mammals. In fishes, creatinine is excreted by the kidneys, but it is not known if blood levels become elevated with impaired renal function (McDonald and Milligan 1992). The results of this study indicate that creatinine may reflect renal function.

The cause of the severe hypochloremia observed in all nitrate treated fish is unknown. Severe hypochloremia has been demonstrated in response to handling stress, (Tomasso et al. 1980) and a mild hypochloremia occurs with gill injury (Byrne et al. 1989) The hypochloremia is due to increased loss of chloride across the gills. Whether the hypochloremia demonstrated in the nitrate exposed fish is in response to stress, gill damage or some other mechanism is unclear. A possible explanation is that chloride moved passively out of the fish to balance the sodium added to the water as sodium nitrate. However the hypochloremia was also observed in calcium nitrate and potassium nitrate treated fish and no hypochloremia was noted in the sodium bicarbonate treated fish.

Electrolyte concentrations are indicative of a fish's ability to osmoregulate. This ability is often compromised with stress, disease, or gill lesions that increase gill permeability to ions (McDonald and Milligan 1992). Blood electrolytes appear to be influenced to a greater extent than other biochemical parameters in the nitrate exposed fish. This may be because nitrate damages the gills and kidneys affecting osmoregulatory ability. Alternatively, the pathology is caused by an electrolyte imbalance

in the water (from the added salts of nitrate) affecting the physiology of the fish. The fact that similar biochemical changes were produced in fish from all three salts of nitrate but not elevated sodium bicarbonate provides evidence that the biochemical changes seen are due to elevated nitrate and not the added cation. At the same time, however, the added cation does affect electrolyte values as the potassium concentration is elevated in fish from the potassium nitrate tank.

The changes observed in the nitrate treated fish most likely represent a pathological response as apposed to a generalized stress response. In mammals the stress response is well characterized. There are three stages; an initial alarm phase, a stage of resistance and a final stage of exhaustion. Exhaustion is reached if the stress is sufficiently prolonged or severe, and is characterized by decreased levels of cortisol, depletion of liver glycogen, immunosuppression and other changes reducing the survivorship of the organism. This progression has been demonstrated in fishes with long term exposure to environmental pollutants (Hontela et al. 1992). Fish from polluted sites had decreased cortisol and atrophied pituitaries, indicating an exhaustion of the cortisol producing system. Hematologic changes associated with stress include a leukopenia characterized by a lymphopenia, and a neutrophilia (Ellsaesser and Clem 1986, 1987). Serum biochemical changes with stress include a hyperproteinemia, hypercortisolemia, hyperglycemia, hyponatremia, hypochloremia, and a hypocalcemia from increased permeability of the gills (McDonald and Milligan 1992). The hematologic and biochemical profiles of the nitrate treated fish were not indicative of a stress response as no lymphopenia, hyperglycemia, hypercortisolemia, hyponatremia nor hypocalcemia was noted (Hrubec et al. 1996b, 1986c, 1986d). It is possible that the nitrate treated fish had reached a state of exhaustion and were unable to elevate glucose and cortisol levels, however, both values were well within the reference interval (Hrubec et al. 1986a, 1996c). Thus it is unlikely that the changes seen in the nitrate treated fish were solely stress induced.

The cause of the clinical signs and mortality in the nitrate treated fish is unclear. Some fish suffered from a hemolytic crisis, becoming anemic and icteric prior to death, but whether this was a direct result of exposure to nitrate is not known. There is a report of jaundice in cultured eels associated with a hemolytic crisis, although, excessive destruction of erythrocytes was not the primary cause of jaundice as the bilirubin was mainly conjugated (Endo et al. 1992). The cause of the cholestasis in eels was not determined, but based on histologic changes, the authors felt there was an intrahepatic disorder. The water quality in this report was not mentioned, so it is not known if the nitrate concentrations were elevated.

Conclusion

The data presented here support the theory that prolonged exposure to elevated levels of nitrate may decrease the immune response, induce hematological and biochemical changes indicative of a pathologic response, and may increase mortality. If elevated nitrate levels are responsible for the pathologic changes seen in these fish, then management of recirculating systems must change to lower nitrate levels. The pathologic changes are sufficient to affect the normal physiology of the fish and will probably result in decreased growth and increased susceptibility to disease. These results however do not conclusively show that elevated nitrate levels are responsible for the pathology seen. Further studies demonstrating a dose response to nitrate levels should be conducted prior to making

major management changes in a recirculating system.

References

Bonn, E.W., Bailey, W.M., Bayless, J.D., Erickson, K.E., and Stevens, R.E., 1976. Guidelines for striped bass culture. Striped Bass Committee, Southern Division, American Fisheries Society, Bethesda, Maryland, p. 69.

Bromage NR, Shepherd CJ, Roberts J. 1988. Farming systems and husbandry practice. In: Shepherd CJ Bromage NR, eds. *Intensive Fish Farming*. Oxford: BSP Professional, pp. 94-95.

Byrne P, Speare D, and Ferguson HW. 1989. Effects of a cationic detergent on the gills and blood chemistry of rainbow trout Salmo gairdneri. *Dis Aquat Org* 6:185-196.

Bucher F, and Hofer R. 1990. Effects of domestic waste water on the serum enzyme activities of brown trout (Salmo trutta). *Comp Biochem Physiol* 97C:381-385.

Ellsaesser CF, and Clem LW. 1986. Hematological and immunological changes in channel catfish stressed by handling and transport. *J Fish Biol* 28:511-521.

Ellsaesser CF, and Clem LW. 1987. Cortisol-induced hematologic and immunologic changes in channel catfish (Ictalurus punctatus). *Comp Biochem Physiol* 87A:405-408.

Endo M, Sakai T, Yamaguchi T, et al. 1992. Pathology of jaundice in the cultured eel Anguilla japonica. *Aquacult* 103:1-7.

Hontela A, Rasmussen JB, Audet C, et al. 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Arch Environ Contam Toxicol* 22:278-283.

Hrubec, TC, Robertson JL, Smith SA, and Tinker MK. 1996a. The effect of temperature and water quality on antibody response to *Aeromonas salmonicida* in sunshine bass (*Morone chrysops* X *Morone saxatilis*). *Veterinary Immunology and Immunopathology*, in press.

Hrubec TH, Smith SA, Robertson JL, et al. 1996b. Comparisons of hematological reference intervals between culture system and type of hybrid striped bass. *American Journal of Veterinary Research* 57:618-623.

Hrubec TH, Smith SA, Robertson JL et al. 1996c. Blood biochemical reference intervals for sunshine bass (Morone chasgps x M saxatilis) in three culture systems. *American Journal of Veterinary Research* 57:624-627.

Hrubec TH, Smith SA, and Robertson JL 1996d. The effects of water quality on the hematologic and serum biochemical profiles of hybrid striped bass (*Morone chrysops* X *Morone saxatilis*). Submitted to *American Journal of Veterinary Research*.

Meade, J.W., 1985. Allowable ammonia for fish culture. *Prog. Fish. Cult.* 47:135-145.

McDonald DG, and Milligan CL. 1992. Chemical Properties of Blood. In: Hoar WS, Randall DJ, Farrell AP, eds. *Fish Physiology Vol. 12 Part B, The Cardiovascular System*. New York: Academic Press, 56-135.

Nicholson, L.C., Woods, L.C., and Woiwode, J.G., 1990. Intensive culture techniques for the striped bass and its hybrids. In: R.M. Harrell, J.H. Kerby, and R.V. Minton (Editors), *Culture and Propagation of Striped Bass and its Hybrids*, American Fisheries Society, Bethesda, MD, pp. 141-158.

Tomasso JR, Davis KB, and Parker NC. 1980. Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass X striped bass) during netting and hauling. *Proc World Maricult Soc* 11:303-310.

Warren, H.J., Harrell, R.M., Geiger, J.G., and Rees, R.A., 1990. Design of rearing facilities for striped bass and hybrid striped bass. In: R.M. Harrell, J.H. Kerby, and R.V. Minton (Editors), *Culture and Propagation of Striped Bass and its Hybrids*, American Fisheries Society, Bethesda, MD, pp. 17-28.

Weiser W, Hinterleitner S. 1980. Serum enzymes in rainbow trout as tools in the diagnosis of water quality. *Bull Environ Contam Toxicol* 25:188-193.