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Action of Nitrate on *Pimephales promelus*, (Fathead Minnow) in Stillwater

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ABSTRACT

Many fertilizers contain nitrates that leach out of the soil or enter surface waters through runoff, affecting aquatic life. Fathead minnows, because they are an indicator species, were tested to determine if an increased tolerance to nitrates could be induced. An environmental chamber was used to maintain water conditions at 19 C and to provide 12 hour light/dark cycles. The LD50 was found to be 9 g/L (the average length of minnows was 5.2 cm). Exposing minnows to sublethal concentrations of 75%, 40%, 25%, and 10% of the LD50 was ineffective at yielding an increased nitrate threshold. Chloride, a competitive inhibitor to nitrate, was found to increase nitrate toxicity. These findings indicate that fathead minnows are able to tolerate a fixed amount of nitrate in the stillwater environment. No results were found which supported the hypothesis that chloride reduces nitrates toxicity.

INTRODUCTION

Midwest ponds and reservoirs often contain nitrogen at a concentration of 0.005 mg/ml, typically in the form of ammonia (Lewis & Morris 1986). Runoff from farm ground (containing herbicides and pesticides) and sewage effluents (feedlots) show abnormally high levels of nitrogen compounds (Lewis & Morris 1986, Muller & Lloyd 1994). Many fisheries studies conduct tests in flow tanks or recirculating systems in order to achieve maximum control over variables like pH, dissolved oxygen and excretory products of the fish (Arthur et al. 1987, McCormick 1984). However, these conditions are not representative for fishes inhabiting stillwater environments.

Nitrogen compounds are absorbed through chloride cells in the gill epithelium and Cl^- and other ions may act as competitive inhibitors to nitrogen uptake (Lewis & Morris 1986, Heath 1995). After entering the bloodstream, nitrogen converts hemoglobin to a dysfunctional form, methemoglobin, incapable of binding oxygen (Lewis & Morris 1986, Muller & Lloyd 1994, Heath 1995). When blood volume levels of methemoglobin are greater than 50% (the normal range for methemoglobin is 1-4% of blood volume), hypoxic stress causes disorientation and can result in death (Lewis & Morris 1986). As nitrogen concentrations diminish, methemoglobin is converted back to hemoglobin by the enzyme reductase (in red blood cells). This process is continuous and only exceeds methemoglobin formation at low nitrogen concentrations (Lewis & Morris 1986).

An interesting related study demonstrated that Rainbow trout, *Oncorhynchus mykiss*, are able to tolerate increasing concentrations of aluminum if first acclimated to a sublethal concentration (Reid et al. 1991). This pivotal finding fomented the idea that other species may be capable of adapting to toxic ion concentrations if properly exposed. In another study conducted with chinhook salmon, high concentrations of calcium were found to reduce chloride loss, thereby preventing nitrate uptake from reaching harmful levels (Lewis & Morris 1986). These two hypotheses provide two possible avenues to decrease the toxic effects of nitrogen compounds on fish.

Temperature, pH and dissolved oxygen each contribute to nitrogen compound toxicity indirectly (Lewis & Morris 1986, Thurston 1986). At increasing temperatures, tissue oxygen demand increases as does nitrogen compound toxicity (Lewis & Morris 1986). As external pH decreases, plasma carbonate levels increase and hemoglobin loses its affinity to bind oxygen (i.e. methemoglobin levels increase). Low dissolved oxygen levels in the external medium is unfavorable, and when coupled with nitrogen compounds simply exacerbates low oxygen levels within the fish (Lewis et al. 1986).

Using Fathead minnows, an LD50 value was determined. This nitrate concentration was then used as a measuring point to determine if sublethal nitrate exposures could induce greater tolerance to sodium nitrate. In the final experiment, Fathead minnows were exposed to varying concentrations of a chloride solution and then placed in the LD50 concentration of sodium nitrate to again determine if reduced mortality was possible.

MATERIALS AND METHODS

Fish were obtained from a local bait shop (Bill's Outdoor Sports, McPherson KS.) and placed into a plastic, oxygenated bag for transport back to the lab. The minnows were then transferred to plastic bins with conditioned water (stillwater) and put inside an environmental chamber (Sherer-Cel8VHL). This water was maintained a temperature of 19 C and a pH between 7 and 7.6 (tested with Sargent-Welch pH meter) for twenty-four hours prior to fish being introduced. Before any tests started, all fish were kept under these standard conditions for a period of twenty-four hours to acclimate.

The toxicant chosen was reagent grade sodium nitrate (Na_2NO_3, J.T. Baker Chemicals), 99% pure. Conditioned water with various concentrations of Na_2NO_3 was prepared in order to determine the LD50 for

this species. Acclimated minnows were divided into two groups (usually 6-7 per group); one group received a specified concentration of sodium nitrate, the other group served as a control.

After the LD50 concentration was established, a new battery of tests commenced which exposed minnows to a percentage of this value (10%, 25%, 40%, and 75% of the LD50), the sublethal dose, for twenty-four hours. Following this 24 hour exposure to a sublethal dose, minnows were immediately placed in the LD50 concentration and monitored for the next 96 hours.

In the last series of experiments, sodium chloride (at concentrations of 1, 2, 3, and 4 g/L), potassium chloride (4 g/L) or calcium chloride (2 g/L) was each introduced for twenty-four hours. The purpose of these treatments was to attempt to saturate chloride cells in the gill, then place the fish in the LD50 concentration and monitor them for the next 96 hours. Fish able to survive for 96 hours after being readmitted into the LD50 constituted success.

RESULTS

The LD50 for P. promelas (average length 5.2 cm) was determined to be 9 g/L of sodium nitrate. This value is quite specific for fathead minnows; at 7.5 g/L there was no mortality and at 10.5 g/L there was total mortality over a 24 hour time period.

When minnows exposed to sublethal concentrations of Na_2NO_3 were placed back in the LD50 concentration, mortality was not reduced.

Sodium chloride and calcium chloride each failed to effect the LD50. Potassium chloride decreased the LT50 concentration from 24 hours to 13-15 hours. Figures 2 and 3 both report the minnows alive after 24 hours because all minnows had died before 96 hours. Minnows first placed in any of the three chloride solutions did not show prolonged life in the LD50 concentration (i.e. the LD50 concentration was more potent).

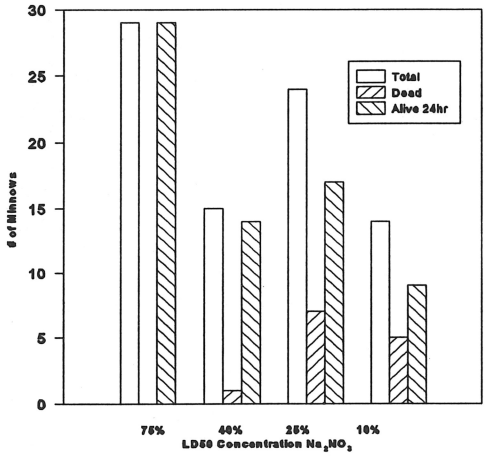

Figure 1. Percentages of the LD50 concentrations used and the coresponding mortality/vitality. (755 is equal to 3.5g/L, 40% is equal to 3.5 g/L, 25% is equal to 2.3 g/L, and 10% is equal to 0.9g/L).

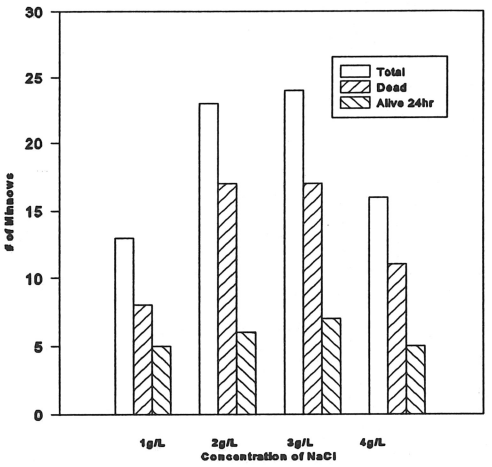

Figure 2. Summary results of minnow mortality exposed to different concentrations of sodium chloride solution. No minnows survived for 96 hours in all treatment groups onced placed back in the LD50 concentration, so minnows alive after 24 hours are reported.

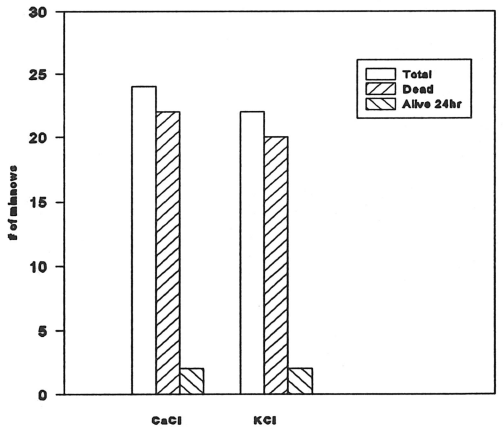

Figure 3. Graph representing calcium chloride and potassium chloride concentration and corresponding 24-hour survival in LD50 concentration. (96-hour values not reported because no fish survived this long.)

DISCUSSION

The 24 and 96 hour effect of nitrate on *P. promelas* (Fathead Minnow) is quite pronounced. Although it has been demonstrated in other fish that acclimation is possible, the method of sublethal concentration exposures using a non-metal toxin was not effective. However, sublethal exposure time was not varied in this experiment. 24 hours was the only length of time used, whereas other experiments exposed fish to sublethal doses for as long as two weeks. Other methods should not be excluded either, such as exposing minnows to a toxin as early as the larval stage. Nevertheless, as the LD50 graph shows, P. promelas is able to tolerate nitrate levels up to 9g/L; concentrations above this value are lethal.

The difference in results between this acclimation experiment and Reid's et al. (1991) is best attributed to the differing actions of each toxicant. Aluminum's effect on fish is due to an impairment in gill ionoregulation (Heath 1995, Reid et al.1991). It's suspected that Ca^{+2} is displaced and therefore unable to initiate cross-binding of intracellular ligands which control trace metal binding (Heath 1995, Reid et al. 1991). By exposing fish to a sublethal concentration, the membrane components are destabilized, but fish are able to respond by producing calmodulin, and other Ca^{+2} specific binding proteins (Heath 1995, Reid et al. 1991). Although the exact gill microenvironment transformation is not known, hypothesized mechanisms include 1) chemical changes in lipid, phospholipid, or glycoproteins of the cell membrane 2) increased rates of membrane turnover, and 3) incorporation or exclusion of membrane components (Reid et al., 1991). Once stabilized, aluminum is no longer able to "clog" cell membranes and interrupt ionoregulation.

Nitrate is able to enter the plasma of fish (unlike aluminum) through the chloride-transporting mechanism found in gill epithelial cells and is also capable of accumulating, even against a concentration gradient in the plasma (Heath, 1995). Once in the plasma, nitrogen compounds are capable of converting hemoglobin into methemoglobin and decreasing its oxygen affinity, causing death due to hypoxia (Heath, 1995). Aluminum, on the other hand, interrupts normal control over essential ions which are constantly being lost in freshwater fish whereas NO_3^- is capable of readily entering the organism and inactivating hemoglobin. This latter process is basically unregulated by ions such as Ca^{+2}, so cell transformations which prohibit nitrate uptake are not possible (Heath, 1995).

The last series of experiments dealt with exposing *P. promelas* to various concentrations of chloride. It was speculated that elevated Cl^- concentrations may slow chloride-transport and in turn slow nitrate uptake. However, as Figures 2 and 3 show, nitrate uptake may have been positively influenced by first exposing the minnows to chloride solutions (LT50 decreased from 24 to 13-15 hours). Although chloride cells may or may not have been saturated, placing them in the LD50 concentration immediately disrupted the surface membrane ion equilibrium, in part due to nitrate's slightly higher affinity for binding. In response to this, chloride cell proliferation is increased to provide more possible sites for Cl^- uptake (Heath, 1995). Unfortunately, because chloride cells in the gill is precisely where nitrate enters fish tissues, and because of nitrate's slightly higher binding affinity, this process is maladaptive (Heath, 1995). Much can still be learned, however, about the exact mechanism which is responsible for Cl^- uptake. Finding Cl^- complexes and testing them in vitro on Cl^- cells may offer further details about the Cl^- receptor/ uptake mechanism.

Fathead minnows (approx. 5.2 cm in length) have little defense against nitrate toxicity at levels greater than 9 g/L. Therefore, rather than remedy fish inhabiting such water, a better solution would be to halt the nitrate entering stillwater environments. Although this suggestion is often economically unfavorable, its clearly the best answer. Herbicides and pesticides containing toxins which easily leach out of soil or enter surface waters via runoff should be replaced with safer alternatives. For example, nitrogen free herbicides and pesticides and feedlots designed to control water runoff are environmentally sound options. Because fish can tolerate relatively high nitrate concentrations (7.5g/L) with very little mortality, water testing should be performed if high concentrations of nitrate are suspected.

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