Influence of N-nitrate pollutant on development of endemic common arboreal frog *Polypedates cruciger* (Amphibia: Rhacophoridae)

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Abstract
Experimentally detected N-nitrate concentration in stagnant water bodies in Mirissa agro-environs (0.1096mg^{-1}) were tested on the development of Polypedates cruciger *under simulated natural conditions provided in the laboratory. Observations were mainly focused on behavioral changes, length of different stages, mortality and growth changes during development and any abnormalities in the pre-adult stage. The detected N-nitrate concentration caused a significant impact on growth in tadpoles, especially in early life stages compared to the later stages of the life cycle. Paired sample t-statistics revealed that mean wet weight of young adults exposed to N-nitrate were significantly lower ($p<0.05$) than the non-exposed controls. In addition, early stage larvae exposed to the detected N-nitrate concentration did not achieve significant growth and may have had unseen impacts during their development. However, impacts of N-nitrate lessened during the later stages of life cycle such as the pre-adult stage. Present study revealed that even a low concentration of N-nitrate in aquatic environments can cause an unseen impact on the amphibian population by altering the growth in highly sensitive developmental stages such as external gill stages.*
Key words: N-nitrate pollutants, aquatic environments, agro-environs, development, *Polypedates cruciger*

Introduction

Amphibians are the highest vertebrate group to retain an essentially "naked" egg, and the only vertebrate group which has an aquatic larval stage and a terrestrial adult phase. Furthermore, the skin of an adult amphibian is a permeable organ used for respiration and maintaining water-balance, whereas the tadpole stage relies predominantly on gills for respiration and water balance. This implies that amphibians may have more modes of exposure to environmental contaminants than other vertebrates, also the notion that amphibians exhibit greater sensitivity to environmental contaminants than other phylogenetic groups (Mann and Bidwell, 1999). A wide array of contaminants affects amphibians including pesticides, herbicides, fungicides, nitrogenous fertilizers and numerous other pollutants (Blaustein *et al.*, 1997; Bonin *et al.*, 1997; Sparling *et al.*, 2000). Among these types of pollutants, nitrogen pollution is becoming a severe problem world wide with unknown consequences on amphibian populations. In the aquatic environment, the most common ionic (reactive) forms of inorganic nitrogen are ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) (Camargo *et al.*, 2004). These ions may be present naturally in aquatic environments as a result of atmospheric deposition, surface and ground water run off, dissolution of nitrogen-rich geological deposits, N_2 fixation by certain prokaryotes and biological degradation of organic matter (Spencer, 1975; Kinne, 1984; Gleick, 1993; Wetzel, 2001; Rabalais, 2002).

Nitrate is the more stable form of oxidized nitrogen but can be reduced by microbial action to nitrite which is moderately reactive chemically. Because of the relative stability of the nitrate ion, most nitrogenous materials in environmental media tend to be converted to nitrates. Therefore, all sources of nitrogen (including organic nitrogen, ammonia and fertilizers) should be considered as potential sources of nitrates. Increasing nitrate levels in surface and ground waters are a potential source of severe environmental stress to aquatic organisms and nitrate is known to be toxic to amphibians (Baker and Weights, 1993, 1994). Inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-) can enter aquatic ecosystems via anthropogenic sources such as animal farming, agricultural application of nitrogen-based mineral fertilizers, manure and their subsequent run-off to surface waters (Bogardi *et al.*, 1991; Oldham *et al.*, 1996), as well as atmospheric deposition of nitric compounds (Stoddard *et al.*, 1999). Moreover, the atmospheric deposition of inorganic nitrogen (mainly in the form of NO_3^-) has dramatically increased because of the extensive use of nitrogen fertilizers and the huge combustion of fossil fuels (Vitousek *et al.*, 1997; Carpenter *et al.*, 1998; Moomaw, 2002, Boumans *et.al*, 2004). As a result, concentrations of nitrate in ground and surface waters are increasing around the world, causing one of the most prevalent environmental problems responsible for degradation of water quality on a world wide scale (Meybeck *et al.*, 1999; Wetzel, 2001; Rabalais, 2002; Smith, 2003).

Amphibians are at the highest risk of exposure and are most sensitive to nitrates when they are in the egg and tadpole life stages. For most amphibian species in Sri Lanka, the egg and tadpole life stages occur in the stagnant water bodies associated with paddy cultivation and during these months, application of fertilizers and other chemicals reach their peak levels. Therefore the main objective of the present study was to investigate the toxicity of nitrate to the developmental stages of the common tree frog (*Polypedates cruciger*) and to assess how nitrate is toxic to the different stages of the frog in stagnant water bodies associated with agricultural landscapes.

Materials and methods

The study was carried out in Mirissa area, Matara, which has stagnant water bodies associated with vegetable and fruit cultivations, *i.e.* papaw cultivated lands. Five study sites were selected with stagnant water bodies associated with adjacent cultivated lands. Five water samples were collected from each water body every 2 weeks for a 3 month period and nitrate concentration of each water sample was measured by reading the absorbance relevant to a particular nitrate concentration given by UV/VIS (Model 6405) spectrophotometer. Maximum and minimum nitrate-nitrogen concentrations of each water sample were calculated during the study period, using reference absorbance values and mathematical formulae. Egg clutches of the common tree frog, *Polypedates cruciger* collected from university premises were transported to the laboratory and allowed to hatch and tadpoles dropped into experimental tanks (fiberglass, 250L) with dechlorinated tap water and aquatic plants, *Hydrilla sp.*

To simulate the effect of environmental nitrate concentration on development of tadpoles, four fiberglass tanks were used (120 L each). Of the four fiberglass tanks filled with de-chlorinated tap water, three tanks were used as test tanks while the other was maintained as the control. Experimentally determined highest concentration of N-nitrate (0.1096mg/l) was added to the water of each test tank. Fifteen newly hatched tadpoles were introduced into each tank separately. All experimental tanks were maintained under almost the same environmental conditions and were observed daily. Experimental nitrate levels were adjusted every other day by checking concentrations with the help of absorbance measured by spectrophotometer and water level was also adjusted. Zooplankton, boiled spinach (Johansson *et al.*, 2001) and commercially available fish food were (Helfrich *et al.*, 2001) provided to the tadpoles in each tank at a similar schedule. Floating objects like stout branches / cork bark were provided for each tank and tanks were maintained close to the natural environment of tadpoles.

Impacts of recorded environmental N-nitrate concentrations in water bodies of Mirissa area were observed ex-situ and evaluated on behavioral changes, time duration for development, mortality during development, growth changes during development and any abnormalities or deformations in pre-adult stage.

That is, daily observations were recorded under following categories; behavioral changes, time duration for completion of the life cycle, mortality, growth changes and abnormalities shown by growing tadpoles. Wet weight of each individual was measured every other day by an electronic balance (accuracy, 0.001). Data recorded for tadpoles exposed to nitrate and control group were compared by parametric one-way ANOVA and Student's t-test using SPSS (version 10) statistical packages.

Results

Environmental nitrate concentration in Mirissa

Different water bodies in Mirissa agri-environs showed different concentrations of N-nitrates. Stagnant water bodies associated with vegetable and papaw cultivated land in the Mirissa area had the highest nitrate concentration. Measured nitrate concentrations in different study sites at Mirissa agro environments are summarized in Table 1.

Table 1. Nitrate concentration of five selected study sites at stagnant water bodies with adjacent vegetable and fruit cultivated lands in Mirissa area.

Site	Minimum (NO^3-N) mg/l	Maximum (NO^3-N) mg/l	Average (NO^3-N) mg/l
1	0.0008	0.0041	0.0038
2	0.0153	0.0771	0.0767
3	0.0096	0.0482	0.0480
4	0.0219	0.1098*	0.1096
5	0.0081	0.0409	0.0406

* Highest N-nitrogen concentration

Behavioral changes and mortality

There were no significant behavioral changes observed in any stage of tadpoles between the control group and nitrate induced group, during the experiment, i.e., tadpoles in the two groups exhibited similar behavioral patterns. Moreover, significant changes were not observed on the time duration for completion of life cycle, abnormalities in pre-adult stage, and mortality during development (No mortality was recorded during the development of tadpoles in control and nitrate exposed experimental groups).

Time duration

Time taken for the completion of the life cycle was not statistically significant ($p \leq 0.05$). That is, length of the life cycle was not significantly affected by the N-nitrate concentration prevailing in Mirissa area. Detailed morphological and behavioral changes during development and the time taken for the development of anatomical structures or to show specific behavior are summarized in Table 2.

Table 2. Prominent morphological and behavioral changes exhibited by tadpoles and the time taken for each major change

Morphological changes	Time taken (days)	
	Control group	Nitrate exposed group
Emergence of larvae from egg mass	7(5-8)	7(5-8)
Feed on remaining yolk (larvae do not feed)	10(10-11)	10(10-11)
Start to swim	11(10-11)	11(10-11)
Start to feed on algae	11(11-12)	11(11-12)
Disappearance of external gills	11(11-12)	11(11-12)
Appearance of internal gills	11(11-12)	11(11-12)
Begin to feed on zooplankton	21(21)	21(21)
Begin to feed on pellets provided externally	24(24)	24(24)
Begin to feed on boiled spinach provided	26(26)	26(26)
Start air breathing	35(34-36)	35(34-36)
Appearance of hind limb bud	47(43-47)	46(43-46)
Appearance of' joints of toes	50(48-50)	49(47-49)
Appearance of real long tail	50(48-50)	49(47-49)
Begin to feed on large plants	51(51-52)	50(49-50)
Appearance of distinct head & elongated body	51(51-52)	50(49-50)
Start to degenerate lower horny jaw	53(52-53)	52(50-52)
Limbs become longer and stouter	53(52-53)	52(51-52)
Start formation of pigmented patches on limbs	53(52-53)	52(51-52)
Toes become longer	54(52-54)	53(51-53)
Hind limbs become gradually stouter and wider	56(54-56)	56(54-56)
Appearance of distinct head region	59(56-59)	60(56-60)
Usage of floating substances	59(59)	60(60)
Visibility of fore limbs	61(59-61)	67(64-67)
Widening of mouth gape	61(60-62)	67(65-67)
Appearance of transparent tail	61(60-62)	67(65-67)
Start the resorption of tail fin	61(61-62)	67(66-67)
Eyes are bulging out	62(62-63)	68(65-68)
Hind limbs become longer and stouter	62(62-63)	68(66-68)
Coloration of tail fin with black	62(62-63)	68(67-68)
Start to reduce the tail fin	64(62-64)	69(67-69)
Stop feeding completely	65(62-63)	70(67-71)
Reduced half of tail fin	65(63-64)	70(68-72)
Appearance of tail stub	66(63-65)	71(68-71)
Become adult frog	68(65-68)	72(70-72)

Growth changes

During the development of *P. cruciger*, tested N-nitrate concentration prevailing in environment at Mirissa area caused a significant impact on growth of tadpoles into adults, especially in early life stages (up to 25 days). That is, showing the effect of experimental environmental nitrate in Mirissa area on the development of the tadpoles, mean wet weight was significantly higher in N-nitrate exposed animals compared to that of controls (Figure 1). Paired samples t-test revealed that mean wet weight was lower in nitrate exposed young adults when compared with that of controls (Figure 1).

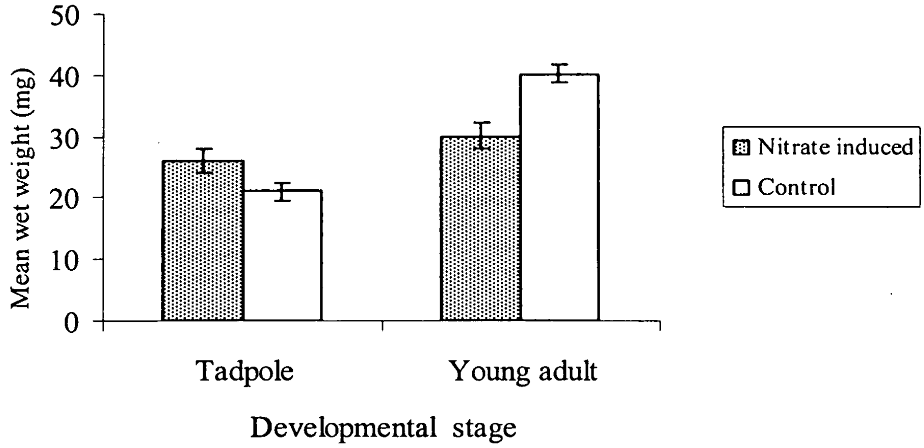

Figure 1. Mean wet weights of tadpoles (25 days old) and young adults of *P. cruciger* exposed to in N-nitrate compared to the control groups during their development

In contrast, a growth increase was detected in control animals after 25 days of the development compared to the N-nitrate exposed animals. These growth increases in control animals were prominent from 25 to 45 days of their development. After 45 days there was no significant difference in growth among the two groups of animals (Figure 2).

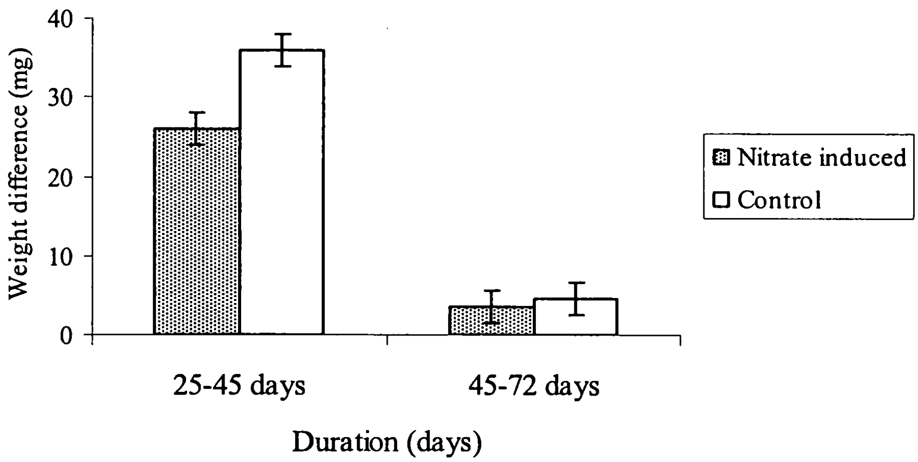

Figure 2. Mean wet-weight increase of N-nitrate exposed and control animals of *P. cruciger* from 25 to 45 days and after 45 days of development.

Discussion

Today many amphibian populations are in decline throughout the world due to industrial chemicals, pesticides, heavy metals, salts, and nitrogen fertilizers which are considered as possible anthropogenic causes for the decline (Blaustein and Wake, 1995). The nitrogen contamination that occurs in stagnant water bodies is known to cause significant effects on amphibians globally (Bogardi *et. al.*, 1991). Therefore evaluation of the concentration of nitrate in the aquatic ecosystem is very important. Stagnant water bodies associated with agricultural landscapes are the preferred breeding sites of most amphibians. However these water bodies may contain elevated concentrations of various agrochemicals mainly due to agricultural activities. The relative concentrations of these substances are constantly changing due to human activities such as use of fertilizers, and climatic factors such as rain fall. In the present study the nitrate ion concentration of the water body was measured every two weeks only, because of the relative stability of the nitrate ion. Most nitrogenous materials in environmental media tend to be converted to nitrates (Bogardi *et al.*, 1991). Therefore, all sources of nitrogen (including organic nitrogen, ammonia and fertilizers) should be considered in long term studies as potential sources of nitrates which may cause a synergistic effect on the target organisms. Since both groups, experimental and control animals, exhibited similar behavioral patterns, it indicates that N-nitrogen concentration did not significantly affect the behavior of tadpoles.

However, the present study indicates that environmental N-nitrate concentration tested ex-situ has significant effects on the growth changes during the development of the tadpoles into adults. The growth of the early stages of tadpoles (up to 25 days) was induced by the N-nitrate in the environment, as N- nitrate exposed animals had a higher growth rate compared to that of the control. However, during 25 to 45 days of their life cycle, growth was retarded in test groups indicating some kind of adverse effect due to the early exposure to toxic substances on their internal developments. Baker and Waights (1993 and 1994) have observed similar effects on the exposure of two species of tadpoles to nitrate pollutants under laboratory conditions. After 45 days, tadpoles normally reach the transition period of their amphibious life. During these days prominent changes occur in the life cycl, making them morphologically adapted to terrestrial life. Usually, tadpoles stop feeding during this period. As there was no prominent weight increment, growth rate of both groups were not significantly different. In addition, there were no abnormalities in the adults of both groups.

The present results are in line with the findings of Blaustein and Wake (1995) Brown, *et al* (1997) and Rouse *et al* (1999), i.e., nitrate pollutants in aquatic environments significantly affect the development and survival of different amphibian species throughout the world. *P. cruciger* is an endemic amphibian species widely distributed in Sri Lanka. The present study indicates that even N-nitrates alone could significantly affect the early development of *P. cruciger*. In addition, there is a record of Gramoxone toxicity for this species (Wijesinghe *et al.*, 2006). It is well known that pollutants in aquatic environments may cause synergistic effects on susceptible organisms such as amphibians (Mann and Bidwell, 1999). It indicates that in their natural habitats *P. cruciger* larvae may face a severe threat due to the synergistic effects of various pollutants in the water bodies. Hence, it could be concluded that, although *P. cruciger* adults live in terrestrial or arboreal habitats, exposure to pollutants during their early aquatic life may adversely affects the survival of this endemic frog species.

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