Methemoglobin in *Chironomus* Larvae as Potential Biomarker of Nitrate Contamination in Water

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Abstract

Nitrate contamination of groundwater and surface water has been found to be high in various areas. Nitrates are known to have health impacts if consumed in different concentrations. The study set out to investigate the potential of using methemoglobin within chironomid larva e as a biomarker for nitrates in water. The ubiquitous and hemoglobin – containing chironomid larvae from Lake Victoria basin were identified using morphological characteristics and Chironomuswas found to bethe most common genera. The larvae can also withstand polluted waters. The Chironomus was therefore chosen for the study and was exposed to different concentrations of nitrate in water in the laboratory. The Chironomus larvae were analyzed for methemoglobin using a spectrophotometer and the levels compar ed to the exposure nitrate concentration of the test solution. Acute toxicity test was carried out by exposing the larvae to different concentrations of nitrate and determining the LC_{50}. Results showed a positive correlation between nitrate concentration and hemoglobin absorbance in the tested cases. The LC_{50} after 48hrs was found to be 34.2 (30-39; 95% confidence limit) mgL^{-1} NO_3-N for third instar larvae and for first instar larvae after 96hrs was 41.3 (35.9-50.0; 95% confidence limit) mgL^{-1} NO_3-N. From the results it can be seen that chironomid larvae have the potential to be used to indicate differences in nitrate concentration in water containing nitrate concentrations of upto 40 mgL^{-1} NO_3-N. The study can help in the development of a bioassessment tool for nitrates in water. However, further work needs to be carried out on effect of age of the larvae on methemoglobin formation.

Keywords *: Chironomus, Biomarker, Methemoglobin, Nitrate*

Introduction

Chironomids, one of the widespread insect groups in the world, belong to the dipteran family hironomidae (Marziali, Armanini, Cazzola, Erba, & Toppi, 2010; McGavin, 1992). Its first three life stages are aquatic; with the larval stage consisting of four instars, with a complete molt between each instar, while the adult stage is aerial (McGavin, 1992; Péry, Mons, & Garric, 2005). Some chironomid‘s contain hemoglobin, synthesized in the larval fat body and then secreted into the hemolymph (Weber & Vinogradov, 2001), making it easy to identify due to the red color of the hemolymph(Hankeln *et al.*, 2002). Due to the hemoglobin, chironomid larvae can tolerate polluted waters and have been known as potential indicators of water quality (Eggermont, Verschuren, & Dumont, 2005) being used for different studies, as well as biomarkers for different substances (Park & Kwak, 2010). Biomarkers are biochemical or physiological indicators of either exposure to, or effects of, environmental contaminants at the suborganism or organism level (Hyne & Maher, 2003). Researchers have recently been using chironomids as a tool of environmental impact assessment, toxicity testing and evaluation of aquatic ecosystem health (Carew, Pettigrove, & Hoffmann, 2003).

This study need arises because of the widespread contamination of n itrate in both surface and groundwater here as well as worldwide. Maximum contaminant level (MCL) for nitrate in drinking water is 10 mgL^{-1}Nitrate-Nitrogen (NO_3-N), given by various authorities (Almasri, 2007) as well as the Government of Kenya (Republic of Kenya, 2006). Elevated nitrate concentrations in drinking water are linked to health problems such as methemoglobinemia in infants (Almasri, 2007) among others. Methemoglobin has been detected in bullfrog (*Ranacatesbeiana*) tadpoles exposed to nitrites (Rouse, Bishop, & Struger, 1999) and even in cattle (Al-Qudaha, Rousan, & Ereifej, 2009), reptiles and fish (Aime *et al.*, 1992). Methemoglobin expected to be produced in chironomids through the oxidation of hemoglobin by nitrate, was to be us ed as a biomarker in this study. No literature on use of methemoglobin in chironomid larvae as a biomarker for nitrates in water was found as well as information on the most common genera in the Lake Victoria Basin. The study intends to make a contribution towards knowledge in this direction.

Nitrates are analysed using different chemical methods (APHA, 1992), but in the third world countries well-equipped laboratories are scarce and costly. The study can also help in the development of a bioassessment tool which, if availed, will result in value addition. Bioassessment is a cost effective means, which makes it possible to involve the local community in the monitoring of river health and would therefore be beneficial to third world countries.

Materials and Methods

Experimental Design

The experimental design involved exposing the *Chironomus* larvae, which was found to be abundant in the Lake Victoria basin from an earlier study, to different concentrations of nitrate in water for a period of time. Methemoglobin levels of these larvae were then determined.

Organisms

The larvae used in the study were obtained from two sites (Huruma and KCC) on River Soisani in Eldoret. A D-frame net sampler of mesh size 0.5mm was used to scoop sediment from the river as described in (Khazenzi, Osano, Wakhisi, & Raburu, 2011). The live larvae were placed in cleaned open - mouthed 1-L plastic bottles containing river water and taken to the laboratory within 6 h where they were left overnight to acclimatize under room temperature with natural lighting conditions, before exposing them to water containing different concentrations of nitrate.

A proportion of these larvae were cultured in a 3- L aquarium containing the river water and sterilized sand that provided the sediment for the larvae. The sterility of the sand was achieved by heating in the oven at 160^{o}C for 2 h. The culturing procedure followed was that described in APHA (1992). The aquarium was placed in a cage with inner dimensions of 60 cm by 36 cm and whose sides and top we re covered with 0.5 mm mesh netting material in order to retain the adults after emergence. The culture medium was continuously aerated using a Sera air 550R aeration pump and the oxygen concentration was maintained at above 40%. The larvae were kept at a light: darkness ratio of 16: 8 hours and fed on food prepared by blending 5g of fish food and 1g of dry grass per litre of water twice a week (APHA, 1992). The larvae were fed on floating fishmeal manufactured by Ungachick Poultry Breeders Ltd, Kampala Uganda, blended with crushed dry grass stalks of Nandi *Setaria* obtained from the study area, where it is commonly found. The adults laid eggs in the water which were removed carefully with the aid of a blunt pipette dropper. The eggs were then placed in a 50 - mL petri dish (10 cm diameter) containing the river water until the eggs hatched. The hatched larvae were then moved to 1- L plastic bottles containing water obtained from a treatment plant before chlorination and whose nitrate content was 0.05 mgL^{-1} NO_3-N. Sterilized sand was added to the bottle to provide sediment and the water was aerated constantly to maintain an oxygen concentration of above 40%. The larvae were fed twice a week. This was done for different egg masses found on different days, therefore hatched larvae of the same age were kept in the same container.

Test Media

The test media were prepared by adding different volumes of 100 mgL^{-1} NO_3-N potassium nitrate solution to some of the river water and the volume brought to 100 mLs. Nominal co ncentrations of 3.9, 6.4, and 8.9 mgL^{-1} NO_3-N were prepared and used in the test with the control (river water) containing 1.4 mgL^{-1} NO_3-N. A replicate with nominal concentrations of 2.3, 2.8, 5.3 and 7.8 mgL^{-1} NO_3-N and a control (river water) containing 0.3 mgL^{-1} NO_3-N was carried out. Twenty, fourth instar larvae were placed in each of the test media under room temperature (18 – 20^{o}C) and light: darkness regime of 12:12 hours, the natural equatorial cycle. The control was the group of larvae exposed to t he river water only. All the containers were not provided with extra aeration during the test. No food was provided during the experiment. The tests were carried out in duplicate. After 48 h all the larvae were still alive and were tested for methemoglobin as described below. The methemoglobin levels were compared with the different serial nitrate concentrations for the specific sites.

Methemoglobin Analysis

This was carried out as described in (Khazenzi, Osano, Wakhisi, & Raburu, 2011). It involved obtaining hemolymph by gently breaking open the body walls of twenty larvae. Twenty -five microlitres of the fluid was then carefully taken, avoiding particles, and added to 2.5 mLs of the buffer in a cuvette.

The absorbance of the hemoglobin in the sample was measured using a Jenway 6505 UV/Vis Spectrophotometer over a range of wavelengths between 528.3 nm and 589.3 nm and compared to absorbance of Hemoglobin Standard (13.4 $g.dl^{-1}$, Biosystems S.A Costra Brava 30 Barcelona: Spain). The standard solution was made by taking 2.5 µLs of the standard and adding this to 3 mLs of the buffer; the absorbance was then read over a range ofwavelengths between 528.3 nm and 589.3 nm.

Acute Toxicity Test

This was a static test (APHA, 1992). The aim of the test was to find out the maximum concentration of nitrate in water for which the larvae may be used as a biomarker. Third instar chironomid larvae (based on the size of larvae) obtained from the river were exposed to different concentrations of nitrate solutions for 48 hours. They were observed for deaths over time and the percentage of dead larvae calculated. The nitrate solutions were prepared using river water as the solvent. The concentrations used were 10, 20, 30, 40, 50 and 100 $mgL^{-1}NO_3$-N. Hundred mLs of the solutions were taken in beakers and 15 larvae added to each beaker. The river water was used as the control; the nitrate concentration of the river water was 1.4 $mgL^{-1}NO_3$-N. The test was carried out in duplicate. The beakers were covered with perforated filter paper to reduce evaporation.The larvae were observed after 4 hours, 24hours and 48 hours. The larvae were not fed during this duration. The number of dead larvae was recorded each time and removed from the beaker. A criterion for death was no movement (APHA, 1992). The nitrate concentration that resulted in the deaths of half the number of larvae after 48 hours, LC_{50}, was calculated using the ProbitAnalysis (SPSS Programme).

The toxicity test was also carried out using first instar larvae that were hatched in the laboratory. Different concentrations of potassium nitrate solution made using river water as the solvent were prepared for the test. The concentrations used were 10, 30, 50, 75, 100, 150 and 200mgL^{-1} NO_3-N. Batches of fifty larvae each were counted with the help of a Gallenkamp dissecting microscope and transferred into petri dishes using an eyedropper. The larvae were then added at once into 100 mLs of the solution in a beaker for each of the different concentrations. The beakers were covered with perforated filter paper to reduce evaporation. The larvae were observed after 24hours, 48 hours, 72 hours and 96 hours. The larvae were not fed during this duration.The number of dead larvae was recorded each time, with the aid of the dissecting microscope, and removed from the beaker. A criterion for death was no movement (APHA, 1992).The nitrate concentration that resulted in the deaths of half the number of larvae after 96 hours, LC_{50}, was calculated using the ProbitAnalysis (SPSS Programme). The test was carried out in triplicate.

Data Analysis

Correlations were carried out between the nitrate concentrations and cyanmethemoglobin absorbance. The t-test was used to compare differences in absorbance. Lethal concentrations, LC_{50}, were calculated using SPSS Package.

Results

Methemoglobin Levels in Chironomid Larvae

An increase in hemoglobin absorbance with increase in nitrate concentration was observed in 72% of the 18 tests in which the larvae were placed in different concentrations of nitrate. However it was noted that in 60% of all the cases, the control group had higher hemoglobin absorbance than some groups from solutions that had higher nitrate concentration. Fig.1 shows absorbance curves obtained from hemoglobin of larvae from one site (Huruma) exposed to different concentrations of nitrate solution when mean absorbances at different wavelengths were plotted. The peak at 540 nm (when both cyanmethemoglobin and carboxyhemoglobin absorb highly) was higher than the peak at 568 – 570 nm (when only carboxyhemoglobin absorbs highly). This implies formation of methemoglobin. There was a positive correlation between the nitrate concentration and the cyanmethemoglobin absorbance. Pearson's correlation $r = 0.998$ ($p < 0.05$).The concentrations of NO_3-N given are the mean concentrations found in water from the Site. The absorbance of the standard gave a similar trend as that of the hemoglobin of the larvae.

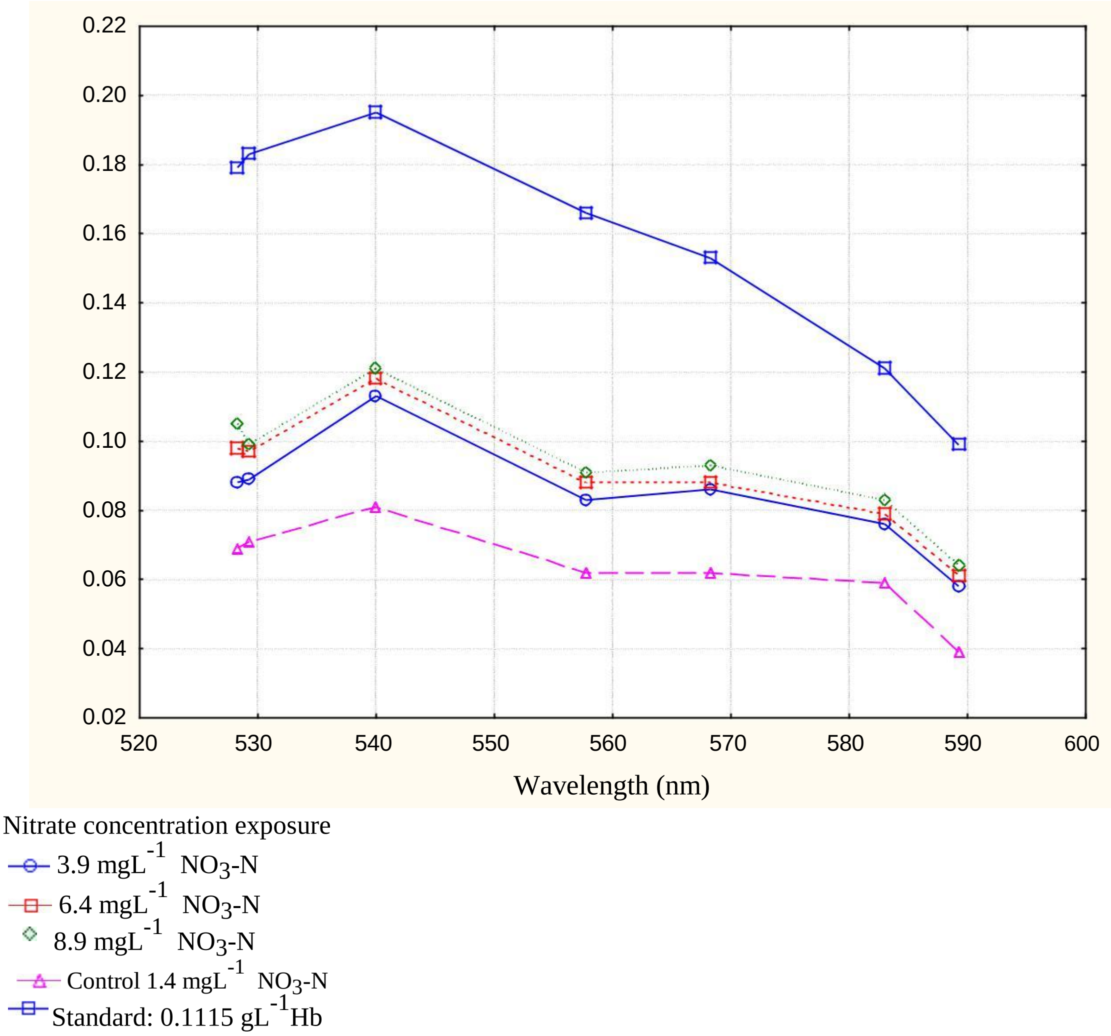
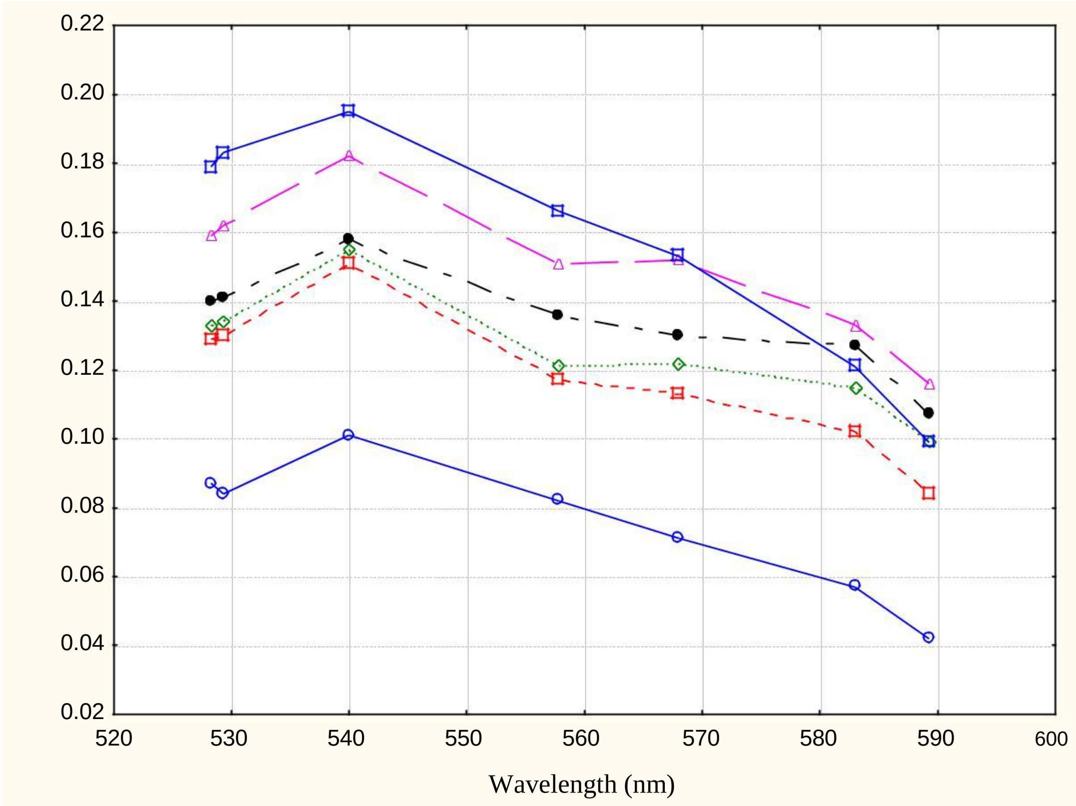


Figure 1. Absorbance of Cyanmethemoglobin at the Different Wavelengths, Obtained from Hemoglobin of Chironomid Larvae from Huruma Site, which had been Exposed to Different Nitrate Concentrations during the Study Period

Fig.2 shows absorbance curves obtained from hemoglobin of larvae from the other site (KCC) exposed to different concentrations of nitrate solution when mean absorbances at different wavelengths were plotted. A positive correlation was observed between the nitrate concentrations and the cyanmethemoglobin absorbances; Pearson's correlation $r = 0.874$ ($p = 0.05$) was used. The concentrations of NO_3-N given are the mean concentrations found at the different sites. The absorbance of the standard gave a similar trend as that of the hemoglobin of the larvae.



Nitrate concentration exposure

2.3 mgL^{-1} NO_3-N

2.8 mgL^{-1} NO_3-N

5.3 mgL^{-1} NO_3-N

7.8 mgL^{-1} NO_3-N

Control 0.3 mgL^{-1} NO_3-N

Standard: 0.1115 gL^{-1}Hb

Figure 2. Absorbance of Cyanmethemoglobin at Different Wavelengths, Obtained from Hemoglobin of Chironomid Larvae from KCC Site, that had been Exposed to Different Nitrate Concentrations during the Study Period

Acute Toxicity Test

The 48hr LC_{50} for 3rd instar larvae was calculated using the ProbitAnalysis (SPSS Programme) and was found to be 34.2 (30-39; 95% confidence limit) mgL^{-1} NO_3-N. From the same tests the 48hr LC_{90} was found to be 49.0 (43.2-60,5; 95% confidence limit) mgL^{-1} NO_3-N. The 96hr LC_{50} for 1^{st} instar larvae was calculated using the ProbitAnalysis (SPSS Programme) and was found to be 41.3 (35.9-50.0; 95% confidence limit) mgL^{-1} NO_3-N. From the same tests the 96hr LC_{90} was found to be 75.4 (68.0-85.9; 95% confidence limit) mgL^{-1} NO_3-N.

Discussion

Biomarkers

Chironomus has been used extensively as a field bioindicator because it is relatively sensitive and tolerates poorer environmental conditions than other genera (Hudson & Ciborowski, 1996). The hemoglobin it contains can also be used as a biomarker by reflecting the changes it undergoes when exposed to pollutants that can affect it. The increase in levels of methemoglobin with increase in nitrate concentration in 72% of the tests shows some potential of using it as a biomarker.

Methemoglobin Levels

Methemoglobin formation in chironomid larvae has been implied in literature where oxyhemoglobin levels were measured and found to vary after exposure to various environmental contaminants. The difference in the oxyhemoglobin levels was explained to be a result of auto-oxidation of the hemoglobin to methemoglobin (Ha & Choi, 2008). In this study, the absorbance curves obtained for larvae exposed to different concentrations of nitrate (Fig.1 and 2) imply formation of methemoglobin because of the lowering of the carboxyhemoglobin absorbance peak at between 568 and 570 nm, one of carboxyhemoglobin peak absorbances (Zijlstra & Buursma, 1997) in comparison to that at 540 nm where cyanmethemoglobin has a peak absorbance (Wylie & Lovric, 1988) as well as carboxyhemoglobin (Zijlstra & Buursma, 1997). Although this was expected to result in the lowering of the absorbance at 568-570 nm as the nitrate concentration increased, the opposite occurred for 72% of the tests. This is most likely because of increase in hemoglobin concentration that has been stimulated by low oxygen concentration in order to try and maintain oxygen delivery. This has been observed in other studies (Avilez, Altran, Aguiar, & Moraes, 2004). There was a positive correlation between the nitrate concentration and absorbance shown by Pearson‘s Correlation (at 0.05 significance). With very high nitrate concentrations (50 mgL^{-1} NO_3-N and above) it was noted that there was no significant difference in absorbance (Khazenzi, Osano, Wakhisi, & Raburu, 2011). This could have been due to a mechanism of adaptation which reacts quite rapidly to increased exposure of nitrates (Gruener & Toeplitz, 1975) such as an antioxidant system. The same has been observed in other studies (Avilez, Altran, Aguiar, & Moraes, 2004; Sadeq, Attarassi, Cherkaoui, ElAouad, & Idrissi, 2008). The differences in absorbance can be used as an indicator of the presence of nitrates in water but this can be improved by either quantifying the amount of methemoglobin formed or identifying any other substance that forms as a result of nitrate reaction with hemoglobin to be used as a marker.

In the methemoglobin tests, the absorbance readings were not able to quantify the amount of methemoglobin for different concentrations of nitrate. It was also not possible to equate a certain absorbance to exposure of a certain concentration of nitrate, although an increase in absorbance was noted at all wavelengths. This is because larvae exhibit stage -specific and tissue- specific single-chain globin synthesis throughout the four larval and the pupal stages (Weber & Vinogradov, 2001). Seasonal variability of hemoglobin content and component composition has also been found in chironomid larvae (Leyko & Osmulski, 1985). The hemoglobin concentrations in larger larvae are higher than those of smaller larvae because the larger larvae have more hemoglobin (Panis, Goddeeris, & Verheyen, 1995). This was observed in this study and hence makes them more suitable for the tests as they can be used in smaller numbers. The larvae can be used for concentrations less than 34.2 (30-39; 95% confidence limit) mgL^{-1} NO_3-N comfortably as this is the calculated LC_{50} but may go as far as the LC_{90}, 49.0 (43.2-60, 5; 95% confidence limit) mgL^{-1} NO_3-N if enough numbers are available.

Conclusion

The increase in absorbance with increased nitrate concentration in water, together with the implication of methemoglobin formation indicates the possibility of using chironomids as biomarkers of nitrate in water. The larger larvae (over 10 mm) may be better indicators than the smaller ones because of their higher hemoglobin content. The study can help in developing a fast bioassessment tool for *in situ* nitrate detection in surface waters. So far the methods available for nit rate assessment in water are all chemical. Although reliable, most of them are time consuming due to the reduction processes involved. This study opens avenues for further work using cultured larvae and other oxidants as a way of improving towards the development of the bioassessment tool.

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