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Design and Fabrication of a Hypoxia-Inducing Chamber for Simulation Studies in Environmental Bio-monitoring Using *Chironomus* larvae

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Research Article

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ABSTRACT

The larvae of the aquatic *Chironomid midge* (*Diptera, Chironomidae*) are known to survive in freshwater habitats exposed to stress especially that of hypoxic stress. Hence, a study of the effect of alterations in the levels of dissolved oxygen on the physiology of the larvae could help in establishing them as potential sensitive bio indicators for monitoring such freshwater habitats. A simple inexpensive and non-cumbersome experimental set-up unlike commercially available sophisticated set-ups was designed and fabricated in the lab. The validation of this set-up was carried out and alterations in the hemoglobin levels in the larvae of the tropical midge species *Chironomus ramosus* exposed to hypoxic conditions were studied. It was observed that there was an increase in the level of hemoglobin on exposure to hypoxic conditions and the findings were further validated by studying the expression of hemoglobin gene at various time points during exposure to hypoxia. These findings suggest that the device designed could be used as an inexpensive and effective method for generating and maintaining the desired levels of dissolved oxygen as compared to several of the chemical and physical methods generally used. Further, this would be useful in studies to be done for the monitoring of hypoxia in freshwater habitats using *Chironomus* hemoglobin as a biomarker.

INTRODUCTION

Hypoxia in freshwater ecosystems is a frequent threat to life forms thriving in such aquatic habitats. Hypoxic or low dissolved oxygen conditions could arise as a result of increase in temperature, deposition of effluents, discharges from sewage treatment plants and several such factors [1,2]. Introduction of nutrients which cause eutrophication eventually lead to hypoxic conditions in the water body due to the decomposition of the dead algal mass [3]. Thus, in the current scenario of a steady increase in the global ecological threats to freshwater ecosystems, the fluctuations in the dissolved oxygen content in aquatic ecosystems is becoming a pertinent issue [4-8]. In this context, arthropods, especially the aquatic insects have emerged as suitable models for investigating hypoxic stress and global warming led climatic changes [9-12].

Amongst the aquatic ectotherms, chironomid midge larvae have proved to be a useful model organism and constitute an important group of globally existing macro benthos. The non-biting midge of the genus *Chironomus* is one of the most abundant group of insects found in freshwater ecosystems [13-15]. While the terrestrial adults of the midge have a short life span of a couple of days, the aquatic larvae constitute the longest phase of the midge life cycle. Since these larvae coexist with other aquatic life forms for several days, they form a very important component of the aquatic food chain. They are fed upon by several freshwater fish and aquatic beetles as well as other predatory insects. In the context of environmental bio monitoring, the Chironomid larvae have particularly gained importance owing to their ability to survive in hypoxic environments [16-19]. Hence studies on the density fluctuation of the larval population, increased incidence of mortality and other morphological, physiological and biochemical changes in the larvae could provide an insight into the ecological conditions of such habitats. The ability of the larvae to survive in aquatic habitats with low dissolved oxygen content has been attributed to the presence of a high content of the respiratory pigment hemoglobin in the hemolymph [20-23]. Studies on the larval hem lymph have indicated that the hemoglobin constitutes approximately 90% of the total hem lymph proteins [24]. The hemoglobin synthesis in the larvae occurs in the fat body and it is

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then secreted into the hemolymph. Thus the larval hemoglobin is found free-flowing in the hemolymph unlike most of the higher vertebrate organisms. Moreover, the larval hemoglobin is predominantly found as monomers and dimers in the larval hemolymph [25,26]. A few seminal studies could be found in the literature where Chironomus hemoglobin (ChHb) has been shown as an indicator of environmental toxicants and hypoxia [27,28]. Although there have been attempts to develop devices to be used for monitoring hypoxic water samples, none of these devices are user-friendly and most of them are labor-intensive [29-31]. Such lab studies to monitor the changes occurring in the organisms due to hypoxic conditions necessitate the need for a chamber to provide steady simulated experimental conditions. The commercially available nitrogen gassing apparatus are cumbersome and expensive to use at a small scale for studies involving juvenile stages of invertebrates such as insect larvae. Hence the need for a chamber to suit the specific requirement was felt. The design and fabrication of such a hypoxia-inducing cum bio monitoring and simulation chamber is reported in this paper. The fabricated chamber was validated and tolerance of test organisms to varying levels of dissolved oxygen (DO) was monitored. Since DO levels below 2 ppm is termed as hypoxic for aquatic habitats [32], survival at ppm levels of 2 ppm and below were tested. Changes in the hemoglobin content and the transcriptional level of ChHb gene due to hypoxia over time were monitored using this chamber and the results have been reported. These lab-based simulation studies could help in exploiting the midge larvae as potential indicators of hypoxia in aquatic freshwater ecosystems.

MATERIALS AND METHODS

Organisms

Third instar larvae of *Chironomus ramosus*, a tropical insect midge species was used in the present study. Isofemale lines of *C. ramosus* were maintained at 25 ± 2°C and 14 h light: 10 h darkness cycle. The larvae were supplemented with food as described by Nath and Godbole [33].

Design of the Hypoxia Set-up

A rectangular polypropylene box of 10 inches × 7 inches × 4 inches (l × b × d) dimensions was used as a chamber to house the larvae during treatment. The chamber had a capacity of 5 litres **(Figure 1)**. However, 2 litres of distilled water was used for the experiments reported in this paper. Nitrogen gas under controlled pressure could be introduced into the aquatic system by the tube connected to the Nitrogen cylinder fitted with pressure valves. A diffuser was used at the point of introduction of nitrogen into the water. An opening to allow the introduction of a dissolved oxygen meter probe was made available in the wall of the chamber. The rectangular box was provided with a lid which could be closed and materials such as parafilm could be used to seal the point of contact between the box and the lid such that the oxygen levels achieved can be sustained for longer periods.

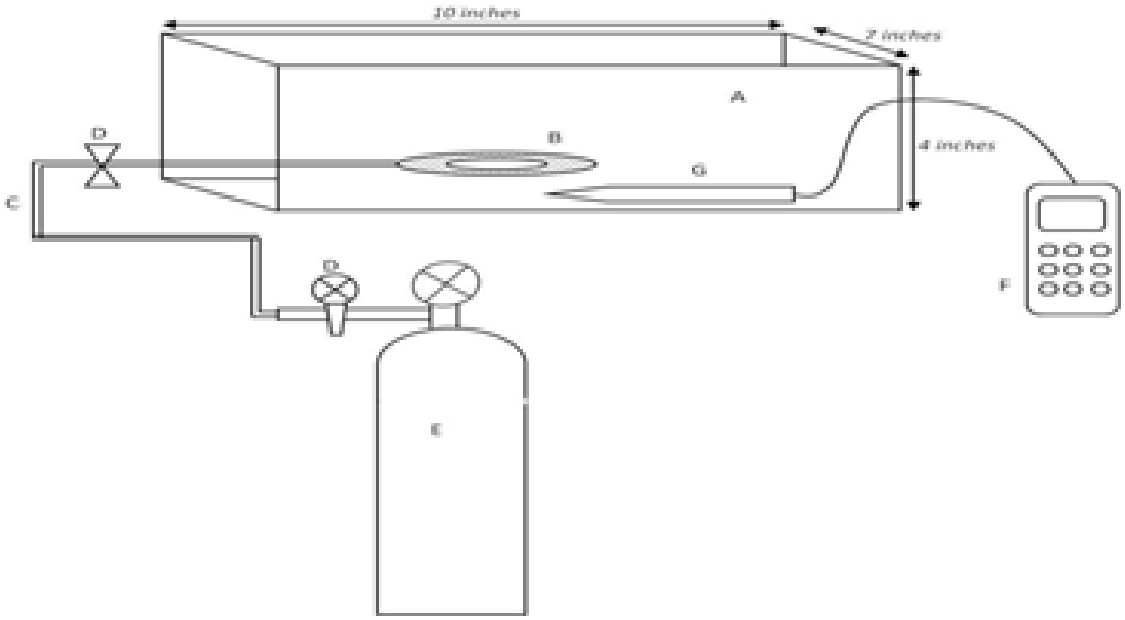

Figure 1. Schematic diagram illustrating the hypoxia-inducing chamber: (A) Polypropylene chamber (B) Gas diffuser (C) Pipeline for introduction of N_2 gas (D) Pressure valves (E) Nitrogen cylinder (F) Dissolved oxygen (DO) meter (G) DO probe.

Tolerance of the Test Organism to Hypoxia

The tolerance and survival of the larvae to DO levels of 2 ppm, 1 ppm, 0.5 ppm, 0.25 ppm and 0 ppm was studied. The study was carried out by introducing larvae into the test chamber containing water with the DO level to be tested. Survival of larvae over varying time periods upto 24 hours of exposure to hypoxia was recorded. Larvae exhibiting movement as well as responding to tactile stimuli were taken as indication of the larvae being alive. 30 larvae were used for each experiment and all experiments were carried out in triplicates.

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Measurement of Hemoglobin Content

The hemoglobin content as a function of time of exposure of larvae to hypoxia was carried out by measuring the Soret peak (λmax=415 nm) [34-36] of hemoglobin present in the hemolymph of the larvae. The hemolymph was obtained by homogenizing 30 larvae, of a given time point of exposure, in phosphate buffer (pH 7). The homogenate was centrifuged at 12000 rpm for 20 minutes while maintaining the temperature at 4°C. The absorbance values of the supernatant at 415 nm were measured by spectrophotometry using an ELISA plate reader (Biotek Instruments, Inc, Vermont, USA).

RNA Isolation and RT-qPCR

Whole larvae (n=5) were homogenized in 700 µl of TRIsoln reagent (Merck) and total RNA was isolated as per the manufacturer's standard protocol. At the end of the procedure, RNA obtained was resuspended in 50 µl nuclease free water. Quantification was carried out spectrophotometrically using plate reader (Biotek Instrument Inc., Vermont, USA). Post quantification, 2 µg total RNA was used to synthesise cDNA using cDNA synthesis kit (SD Prodigy) by following the manufacturer's protocol. The total volume of the cDNA reaction was 20 µl. At the end of cDNA synthesis, 30 µl of nuclease free sterile water was added. 2 µl of cDNA was used for RT-qPCR analysis using primer sequences previously described by Lee et al. [27]. Hb (GenBank accession no. X56272 - F: 5'TTGAGATTCCACGGTTGTGA3'; R: 5'AAGTTGACATCCTTGCTGCC3') and actin (GenBank accession no. AB073070 - F: 5'GATGAAGATCCTCACCGAACG3'; R: 5'CCTTACGGATATCAACGTCGC3'). Of the several isoforms of Hb present in the *Chironomus* larvae, one of the isoforms was studied for expression. 2 µl of cDNA template was mixed with 2 µl each of the forward and reverse primers and 10 µl of the SsoFast EvaGreen Supermix (BioRad). The volume of the reaction mixtures was made up to 20 µl by the addition of 4 µl nuclease free sterile water. The reaction conditions used during the RT-qPCR were as follows: 2 min for initial denaturation at 95°C, 40 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 60°C and 30 sec extension at 72°C. A 5 min final elongation step at 72°C was the last step in the reaction. All samples were analysed as triplicates and the Ct values were calculated. Expression of the actin gene was used as reference for normalization. mRNA levels in the samples were represented as fold induction relative to the control value (set to 1).

Statistics

Statistical analysis was done using the IBM SPSS Version 20 Software.

RESULTS AND DISCUSSION

Test of Hypoxia-Inducing Efficacy of the Chamber

It was observed that nitrogen gassing at a steady pressure of 1 psi led to a gradual decrease in the dissolved oxygen content in the test chamber. At the start of the experiment a DO level of 5.73 ppm was recorded. In about 10 minutes from the start of gas introduction, the DO content reached a value of 1.14 ppm and within the next 20 mins it further dropped to 0.35 ppm **(Figure 2)**. Five replicates were carried out and it was observed that a hypoxic condition of about 0.3 ppm could be reached within 30 mins of the start of the experiment. A similar set-up without the nitrogen bubbling was used as a control. The findings have indicated that the fabricated experimental set-up could serve as a hypoxia-inducing chamber for simulation experiments. The gas flow can be controlled in a manner such that DO content in a required range can be maintained using the set-up over time. Thus, it provides a possibility to carry out simulation studies for aquatic ecosystems varying in oxygen concentrations.

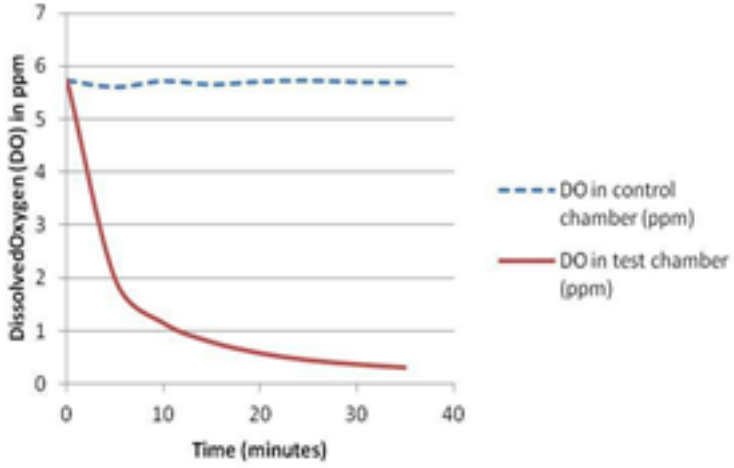

Figure 2. Testing of the hypoxia-inducing chamber expressed as changes in the dissolved oxygen (DO) content in the control and hypoxia-inducing (test) chambers over time.

Tolerance of the Test Organism to Hypoxia

Test organisms (n=30) were exposed to varying DO content and the survival of the larvae was monitored at various time

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points upto 24 hr. The survival plot of the larvae at various experimental DO content of 2 ppm, 1 ppm, 0.5 ppm, 0.25 ppm and 0 ppm shows that the lowest concentration of DO at which the larvae show maximum survival is 0.5 ppm whereas the death rate increases at 0.25 ppm and 0 ppm. The plot showing the survival of larvae re-establishes the fact that the larvae of *Chironomus* are able to survive in hypoxic conditions for prolonged periods of time **(Figure 3)** as documented in many of the past studies [14,16,19]. Since the lowest DO at which maximum tolerance was observed was 0.5 ppm, exposure to 0.5 ppm was selected for further studies.

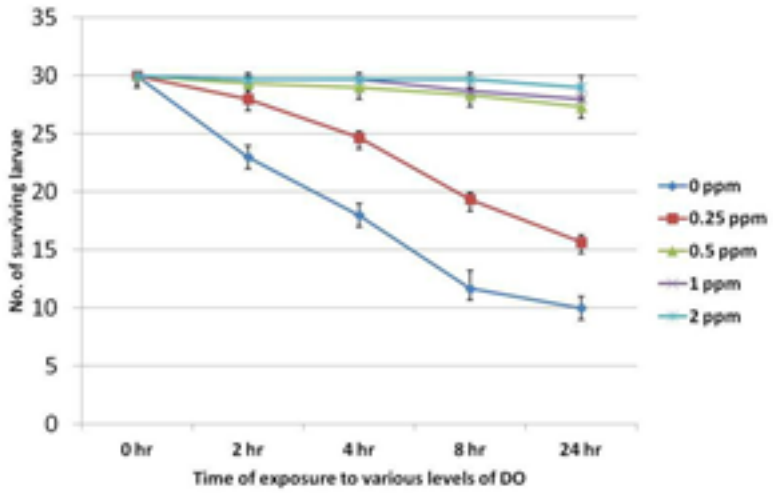

Figure 3. Survival plot of larvae of *C. ramosus* exposed to varying DO.

Alterations in the Hb Content as a Function of Time of Exposure to Hypoxic Conditions

Measurement of the hemoglobin content in the larvae exposed to hypoxia (0.5 ppm) showed a significant change of about 25% at the end of a 24-hr period as compared to larvae exposed to normoxia for the same time period ($p<0.005$) **(Figure 4)**. The hemoglobin content has been expressed as µg per mg larva. Hb measurements were done for larvae exposed to the respective conditions for 2, 4, 8 and 24 hours. The hemoglobin content for larvae exposed to hypoxia shows an increase as compared to larvae under normoxic conditions for every time point.

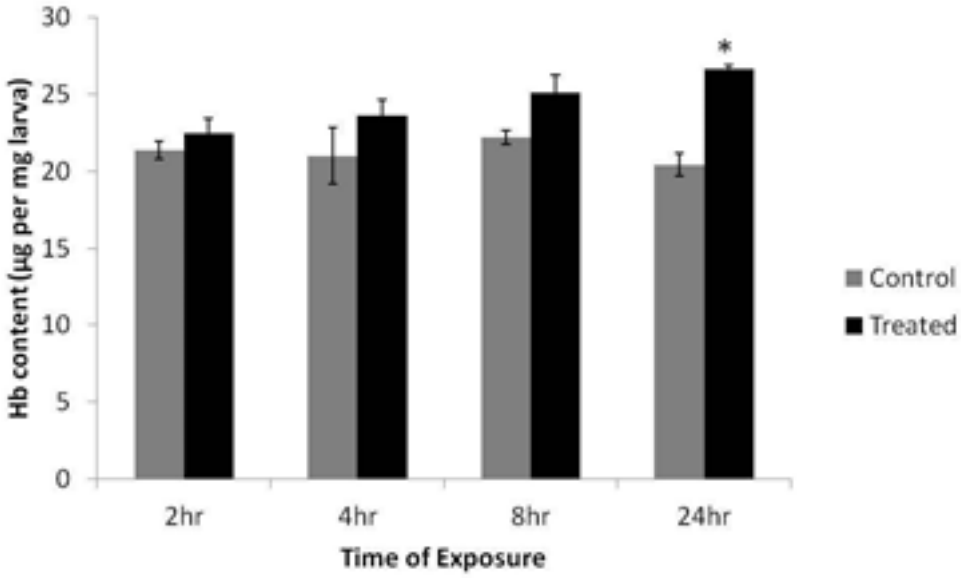

Figure 4. Rt qPCR analysis for detection of Hb expression at various time periods after exposure to hypoxia; *indicates change is significant ($p<0.05$).

Effect of Hypoxia on Hb Gene Expression

RT qPCR analysis revealed that there was an increase in the transcriptional level of the ChHb gene expression when the larvae were exposed to hypoxia for increasing time periods upto 24 hr. The results indicate that there is an increase in the levels of expression of the Hb at all-time points of exposure to hypoxia with the increase being significant at 4 hr and 24 hr ($p<0.05$) **(Figure 5)**.

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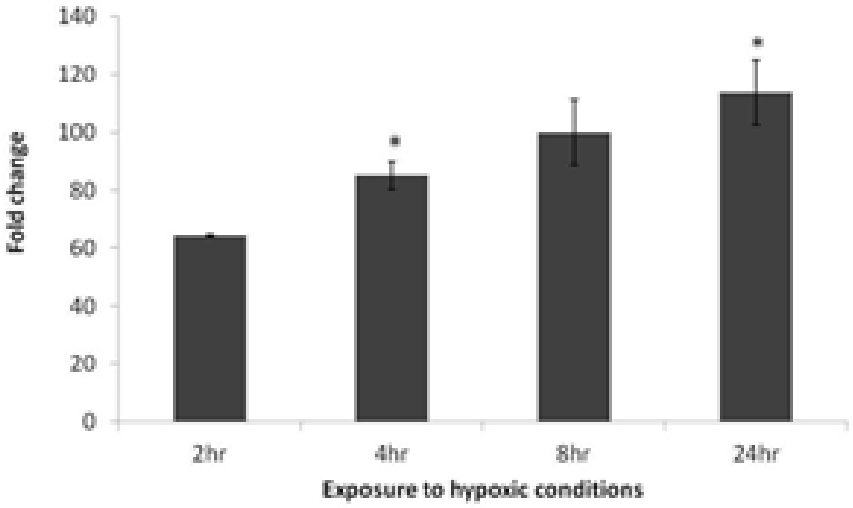

Figure 5. Hemoglobin content (µg/mg larva) of *C. ramosus* larvae at various time points under control (normoxic) and treated (hypoxic) conditions; ** indicates change is significant relative to the corresponding control value ($p < 0.005$).

CONCLUSIONS

The larvae of *Chironomus* popularly known as bloodworms due to the presence of high content of hemoglobin in the hemolymph have been documented to survive in aquatic habitats with low oxygen content. Field studies on these larvae have been carried out in several instances with emphasis on the effluents that are introduced into these naturally occurring water bodies. However, in order to establish specific effects of changes in the levels of dissolved oxygen in the aquatic surrounding in which these larvae survive, one needs to carry out such experiments under controlled conditions in the lab. A chamber to carry out such experiments where the DO content can be easily altered would facilitate such studies. The efficacy of the hypoxia-inducing chamber designed and fabricated was evaluated and the effectiveness of the chamber in acquiring and maintaining low DO content in an aqueous body was demonstrated. The effect of hypoxia on a tropical midge species *Chironomus ramosus* showed that the larvae were tolerant to DO as low as 0.5 ppm. Exposure of the larvae to 0.5 ppm over time was studied with respect to physiological, biochemical as well as molecular parameters. There was a steady increase in the Hb content during the initial hours of treatment which was confirmed by gene expression studies at the transcriptional level of the ChHb gene. The test performance indicated that such a chamber mimics natural hypoxic aquatic habitats leading to alterations in the Hb of *Chironomus* larvae which reaffirm the studies on the effect of hypoxia that have been reported in the past. Hence it could be used as a reliable simulatory aid for studying such habitats using ChHb as a parameter. To conclude, the hypoxia-inducing chamber fabricated and described here is inexpensive and effective equipment for generating and maintaining hypoxia for bio monitoring studies on aquatic larvae. This method also provides a good alternative to the traditionally used cumbersome chemical and physical methods of hypoxia induction.

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CONFLICT OF INTERESTS

The authors declare that the work reported here has no conflict of interest of any kind.

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