Video Article

A Simple Approach to Manipulate Dissolved Oxygen for Animal Behavior Observations

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Abstract

The ability to manipulate dissolved oxygen (DO) in a laboratory setting has significant application to investigate a number of ecological and organismal behavior questions. The protocol described here provides a simple, reproducible, and controlled method to manipulate DO to study behavioral response in aquatic organisms resulting from hypoxic and anoxic conditions. While performing degasification of water with nitrogen is commonly used in laboratory settings, no explicit method for ecological (aquatic) application exists in the literature, and this protocol is the first to describe a protocol to degasify water to observe organismal response. This technique and protocol were developed for direct application for aquatic macroinvertebrates; however, small fish, amphibians, and other aquatic vertebrates could be easily substituted. It allows for easy manipulation of DO levels ranging from 2 mg/L to 11 mg/L with stability for up to a 5 min animal-observation period. Beyond a 5 min observation period water temperatures began to rise, and at 10 min DO levels became too unstable to maintain. The protocol is scalable to the study organism, reproducible, and reliable, allowing for rapid implementation into introductory teaching labs and high-level research applications. The expected results of this technique should relate dissolved oxygen changes to behavioral responses of organisms.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54430/>

Introduction

Dissolved oxygen (DO) is a key physiochemical parameter important in mediating a number of biological and ecological processes within aquatic ecosystems. Exposures to acute and chronic sub-lethal hypoxia reduce growth rates in certain aquatic insects and reduce the survival of insects exposed¹. This protocol was developed to provide a controlled method to manipulate DO levels in stream water to observe the effects on animal behavior. Since all aerobic aquatic organisms' survival depends on the oxygen concentration in order to live and reproduce, changes in the concentration of DO are often reflected in behavioral changes by organisms. More mobile aquatic invertebrates and fish have been observed to respond to low oxygen concentrations (hypoxic) by seeking locales with higher DO^{2,3}. For less mobile aquatic organisms, behavioral adaptations to increase intake of DO may be the only viable option. The aquatic macroinvertebrate order of Plecoptera (stonefly) has been noted to perform "push-up" movements to increase the flow of water, and uptake of oxygen, across their external gills^{4,5}. These adaptive behaviors have been observed in natural environments and in laboratory experiments.

Laboratory manipulation of DO in water opens up significant opportunities for animal behavior studies, but significant gaps in methodological deployment exist. For example, one study used large aquaria to evaluate the physiological response time of Largemouth bass (*Micropterus salmoides*) to hypoxic environments following gassing with nitrogen, but scant detail is given for the methodology⁷. Another study performed on Zebra fish (*Danio rerio*) described using nitrogen gas and a porous stone to deliver gas to water and reduce the DO of the water⁸. For chemistry-based applications, methods for degasification of solvents utilize specialized apparatus⁹⁻¹¹ to remove oxygen from solvents, but would not be suitable for animal behavior studies. While these studies employ methods to remove oxygen from water, no descriptive method could be identified that would allow for evaluation of animal behavior in response to DO changes.

This method described hereafter is an attempt to fully describe a protocol for manipulation of DO of water by using nitrogen gas. Further, this method was developed towards observing relationships between stonefly behavior (pushups) and DO that was employed in a freshman-level biology laboratory. One of the main benefits of this method is that it can easily be performed within a laboratory with common glassware and materials accessible to most secondary and higher education institutions. The protocol is also easily adaptable, allowing for individuals to scale the procedure to meet the objectives set forth for research or teaching applications.

Protocol

Note: This experiment did not use vertebrates and therefore did not require approval by Juniata College's Institute for Animal Care and Use Committee. However for individuals adapting this method for use with vertebrates, IACUC approval should be sought.

1. Field Sample Collection

1. Determine and evaluate potential field sites for the ability to collect, store, and transport stoneflies quickly to minimize time in transit with a maximum recommended time in transit of 1 hr.
2. Perform kick-net sampling at the selected field site following standard kick-net procedures enough times to collect at least 35 stoneflies¹².
3. Collect 50 L of stream water and rocks with a maximum diameter of 2 cm from streams.
4. Place aquariums in a refrigerator set to the temperature of the stream site. Distribute rocks collected at the stream site into aquariums and fill with 4 L of stream water per aquarium. Place 20-30 collected stoneflies per aquarium and place a bubbling stone attached to an aquarium bubbler into each tank and turn on bubblers to continuously add room air to the water.
5. Allow the stoneflies to adjust to the new environment in the aquariums for a 48 hr period.

Figure 1. Set up for dissolved oxygen manipulation. (A) 1) Fitting for copper pipe to male hose barb 2) Location of stopper seal to examine for ensuring well sealing flask. (B) 1) 2 L side-arm flask filled with 1.9 L of water 2) Gas tube and air bubbler (blue) for use in nitrogen bubbling and room air bubbling, respectively 3) Nitrogen tank and gauged values 4) 2 L flask filled with 0.4 L of water with vacuum tube submerged 5) Dissolved oxygen meter. [Please click here to view a larger version of this figure.](#)

2. Experimental Set up

1. On a bench top, connect a standard walled vacuum tube to the side arm of a 2 L side-arm flask as is shown (1 in **Figure 1B**).
2. Fill the flask with 1.9 L of stream water from 3 L plastic containers holding collected stream water in refrigerator set to 12 °C.
3. Place the flask and tubing on a tray large enough to hold an ice bath around the side-arm flask without obscuring the view of the flask interior and fill the tray with ice.
4. Drill two 3 mm diameter holes in a rubber stopper to allow the passage of 1) a copper pipe to deliver the gas to the vessel and 2) the probe of a DO meter into the 2 L side-arm flask (1 in **Figure 1B**).
5. Make a lateral incision from the edge of the stopper to one of the holes to allow seating of the wire of the DO probe into the stopper.
6. Connect a coupler with a 3 mm male hose barb to a piece of 2 mm diameter copper pipe (1 in **Figure 1A**). Ensure that this pipe is long enough to reach to within 10 cm of the bottom of the flask while reaching through the stopper.
7. Place the pipe with coupler through the second hole in the stopper until the length from the bottom of the stopper is enough to reach to within 10 cm of the bottom of the flask.
8. Connect a 0.75 m length, thin walled polyethylene gas tube with a diameter of 3 mm to the coupler on the pipe.
9. Slide both the DO probe and copper pipe into the flask and seal the flask with the stopper.

10. Check for a secure seal between the stopper and the flask, as well as a snug fit between the pipe and the probe wire within the stopper.
11. Fill a 1 L flask with 0.4 L of tap water and place adjacent to the tray with the ice bath and vacuum flask.
12. Submerge the polyethylene tube coming from the large vacuum flask into the water of the 1 L flask. Secure the tube with tape such that it will remain submerged through the experiment.
13. Connect the 3 mm diameter gas line from the vacuum flask to an aquarium room-air bubbler. Begin to bubble the water in the 2 L flask by plugging in the aquarium bubbler, which introduces room air and oxygen to the water.
14. Monitor the DO concentration and temperature of the water with the DO meter for 5 min or until equilibrium of DO is established within the chamber such that little change in DO is occurring.

3. Testing the Stability of the Experimental Set Up

1. Test each setup for DO stability prior to the addition of stoneflies.
2. Add three or four rocks to the 2 L flask so that stoneflies have substrate conducive for pushups.
3. Begin a trial manipulation of DO by disconnecting the gas tube from the bubbler and attaching it to the nitrogen gas line.
4. Start bubbling nitrogen at 20 cubic feet per hr (CFH) for approximately 40 sec to 1 min.
5. Once the DO has dropped to within 0.5 mg/L of the target concentration, reduce the flow to 15 CFH and allow the concentration to decrease to the target.
6. Cease flow of nitrogen immediately once the target concentration is reached.
7. Use the aquarium room-air bubbler to return the concentration to the target concentration if the DO decreases below the target.
8. If the DO is unstable during the testing of a set-up then check the water volume is still at 1.9 L and no water has bubbled out, water temperature is stable and not changing, and seals on all fittings appear to be tight and sealed.
9. Once three trials have been performed and the experimenter has confidence in the ability to control DO, attach the gas line to the bubbler and bubble to equilibrium again.
10. Bubble to equilibrium by attaching the 3 mm diameter gas line to the aquarium bubbler and starting the addition of room air to the water until the concentration of oxygen in the water does not increase or change for 3 min.
11. Once at equilibrium, stop bubbling and unseal the flask.

4. Stonefly Push-up Experiment

1. Divide the total number of stoneflies by the number of observers to determine the number of trials to perform.
2. Determine different DO levels between 2 and 10 mg/L to evaluate the behavioral response of stoneflies (number of pushups).
3. Set up one flask per trial and add an equal number of stoneflies as there are observers to the flask (4 stoneflies within this design), place the probe and pipe back into the flask, then reseal the flask with the rubber stopper.
Note: An initial DO concentration of 10 mg/L was chosen as the first observation point since it was the DO concentration of the stream from where the stoneflies were sampled.
4. Once the water is at 10 mg/L by bubbling following steps 2.10-2.11, record the starting water temperature and allow the stoneflies to attach to the rock substrate in the flask.
5. Assign only one observer to watch a single stonefly to ensure accurate counting of push-up behavior, which is the up and down body movement exhibited by the stonefly.
6. Count and record the number of push-ups observed over the course of a 3 min observation period.
7. Manipulate DO to the next experimental DO level and repeat 3 min observation period for the additional experimental levels.
Note: Within this experimental design, three different DO levels were evaluated.

5. Statistical Analysis

1. To perform statistical analysis use average number of push-ups across the four stoneflies across a group for a given DO trial.
2. Use the free R statistical computing software¹² to perform an Analysis of Variance (ANOVA) on the number of push-ups and the DO concentrations using the order of each experimental trial (DO level) and temperature as covariates. Analyzed DO as discrete levels of a single factor.
3. Use an Anderson-Darling normality test on residuals to check for normality¹³.
4. Perform a linear regression on the data by plotting the mean number of push-ups against DO concentrations.

Representative Results

Six trials of the described setup were performed by 24 freshmen undergraduate students in a teaching laboratory setting to quantify the number of push-ups stoneflies perform in response to different DO concentration in water. The average number of push-ups performed within a DO level and within each trial was pooled to plot push-ups against the DO level in **Figure 2**. An ANOVA was performed initially utilizing DO concentration, sequential order of trials, temperature, as well as the interactions between all variables. Results suggest that only DO concentration significantly influenced the number of push-ups performed by stoneflies (R^2 adj. = 0.322, p = 0.004) and no other variable or interaction was a significant predictor of pushups. All data used in this analysis was confirmed for normality using an Anderson-Darling test.

Figure 2. Mean number of push-ups performed by stoneflies grouped by trial plotted against dissolved oxygen concentration. This shows a significant negative relationship (R^2 adj. = 0.322, p = 0.004) between push-ups and dissolved oxygen concentration (slope of -6.063). Red numbers indicate water temperature (in °C) for a trial. The temperatures were stable across 3 min trial periods, but varied across the experiment. [Please click here to view a larger version of this figure.](#)

Supplemental Code File: R code for the statistical analyses. [Please click here to download this file.](#)

Discussion

Critical steps

This procedure provides a simple and efficient way to manipulate DO in a laboratory setting to perform behavioral studies on aquatic organisms. We found there to be several critical steps/items to be aware of when performing this experiment that directly related to the outcomes. Within a trial, it is critical to maintain the chamber pressure to avoid changes in the partial pressure of gasses above the water, and subsequent DO fluctuations. Following the steps outlined in the "testing the stability of the experimental setup" subsection of the protocol is critical. Inspecting the seal of the stopper with the flask, ensuring complete submersion of the vacuum tubing into the 1 L flask of water (to prevent backflow of room air), and seating the gas line and DO wire seal with the stopper can help maintain a stable chamber environment. Additionally, maximizing the volume of water in the chamber is necessary to improve stability of DO within a trial as we have found that too little volume results in erratic and unstable DO manipulations.

Maintaining water temperature in the chamber is imperative for good control of internal DO levels. While we were able to maintain cold-water temperatures within individual group experiments, some minimal variation in temperature across different trials was evident (**Figure 2**). Since our overall range of temperatures was low (11.8-13.5 °C) and within the typical range for stoneflies, it did not prove to be a significant predictor in our model for stonefly pushups. However, water temperature is known to effect the oxygen saturation potential of water^{14,15}, and failing to maintain chamber water temperature would have direct impacts on DO levels. With a good seal, adequate volume of water, and stable water temperature, internal chamber pressure and DO is easily maintained throughout the experiment and control of DO between trials is stable and reproducible.

Limitations and Modifications

Two potential limitations of this experiment are the size of the chamber and the length of the observation period. The volume of water (~2 L) and small opening in the neck of the chamber limits the size of organism able to be used. At this scale, the protocol would allow for easy substitution of stoneflies for other macroinvertebrates, amphibians, and smaller fishes, but would not be applicable for larger organisms (*i.e.*, predatory fish, octopi). However, it would be possible to scale this experiment up by using larger glassware, and adapting the overall protocol to meet different learning/research objectives with larger organisms. Additionally, any substrate required in the chamber for larger organisms should be taken into account when choosing glassware due to small neck size on the 2 L flask. In our experiment small river-stones were collected with stoneflies and provided ample chamber substrate for the stoneflies to perform pushups.

Internal chamber conditions over short time observation periods were maintained with minimal fluctuations, however, uncertainties remain about longer observation periods. Utilizing the 3 min observation period outlined in the protocol, chamber water temperature and DO levels were maintained at constant values. We were able to maintain DO and water temperature up to a 5 min observation period, but at a 10 min long observation period, water temperatures in the chamber began to rise. In the current protocol, it is not feasible to extend the observation period beyond 5 min. However, adaptations to the current protocol (such as using a climate controlled room) could allow for increased long-term stability of water temperatures. Further, a more robust and detailed analysis of observation time versus ambient water conditions (temperature, DO, partial pressure) would help determine limiting factors.

This protocol has the ability to be modified (as mentioned above) to meet a number of different needs and objectives. One additional modification to the existing protocol would be a modification to the bubbling system. While we utilized solely a small copper pipe to bubble the water, we did

notice that the addition of the large nitrogen gas bubbles often dislodged the stoneflies from their hold on the river stones and sent them floating around the chamber. We attempted to use a bubbling stone for more even dispersal of nitrogen gas, but found that the chamber water was not agitated enough to evenly disperse the nitrogen gas, resulting in a column of hypoxic conditions within the chamber. Further refinement of the nitrogen gas delivery system may provide useful insight, and remove the potential confound of dislodging stoneflies from substrate between DO trials.

Significance and Future Applications

This experiment was the first of its kind to include a detailed protocol development to manipulate DO levels for animal behavior observation in a laboratory setting. While other published work suggested the use of nitrogen gas to manipulate DO levels^{7,8,16}, insufficient methodological detail is given to allow for replication. Interest in this protocol development stemmed from our desire to manipulate DO levels and observe animal behavior for use in a college-level introductory ecology laboratory at Juniata College. Within the class of 24 students, this protocol proved reproducible across trials with differing DO levels and across groups of students. Moreover, this protocol provides a highly accessible and cost-effective way to manipulate DO levels for laboratory experimentation.

While this protocol was developed specifically for use with stoneflies in a teaching lab, it could easily be adapted for other objectives. More specifically, this protocol could easily be used with other small aquatic macroinvertebrates, fish, and even amphibians depending on the species of interest. For example members of the order Amphipoda that increase their locomotive activity in response to hypoxia¹⁷ could be used, or goldfish (*Carassius auratus*) that exhibit a "gulping" behavior at the water surface during hypoxic conditions¹⁶. Additionally, various life-stages of aquatic organisms could also be used with this protocol to help further our understanding of organismal oxygen demands throughout development. This protocol could also be utilized to study biochemical responses to hypoxia by experimenting with amphibians such as mudpuppies (*Necturus maculosus*)¹⁸. Further, this protocol can be scaled up or down in size to meet the needs of larger or smaller organisms and teaching or research applications. While we feel that the protocol and the specific application itself is of broad ecological interest, the greatest strength of this protocol is that it provides a great foundation development across taxonomic groups and experimental objectives.

Disclosures

The authors declare that they have no competing financial interests.

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