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Use of aquatic plants to create fluctuating hypoxia in an experimental environment

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Abstract. In freshwater systems, dissolved oxygen (DO) saturation frequently fluctuates, falling at night and rising during the day in response to respiration and photosynthesis, respectively, of aquatic biota. Low DO (hypoxia) is a common cause of fish kills in freshwater systems around the world. Laboratory studies on responses of fish to fluctuating DO are currently limited, and require techniques that produce a realistic cycle of DO depletion and replacement. Artificial DO-depletion mechanisms frequently used for hypoxia studies may underestimate the field effects of hypoxia on fish because of the lack of the naturally occurring synergistic effect of lower pH, and seldom allow fish to employ behavioural adaptations to hypoxia, such as aquatic surface respiration. We demonstrate proof-of-principle for an alternative method of creating fluctuating hypoxia in an experimental environment, using the natural rhythms of photosynthesis and respiration of aquatic plants to create realistic conditions. A range of volumes of aquatic macrophytes were used alone and in combination with fish to lower DO saturation in sealed freshwater aquaria, and achieved DO saturations as low as 1.3%. This cost-effective method can be deployed over long periods with minimal effort in comparison to traditional methods of DO reduction.

Additional keywords: barramundi, *Ceratophyllum demersum*, *Lates calcarifer*.

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Introduction

Hypoxia (low DO saturation) is a major cause of fish deaths and reduced fish diversity worldwide (e.g. Townsend *et al.* 1992; Hamilton *et al.* 1997; Hernández-Miranda *et al.* 2010). Hypoxia occurs naturally in marine, estuarine and freshwater environments and can be exacerbated by anthropogenic nutrient sources such as agricultural runoff (e.g. Bonsdorff *et al.* 1997; Martin and Saiki 1999; Collins *et al.* 2000), urban runoff (Tucker and Burton 1999), industrial effluents (Winn and Knott 1992) or wastes from aquaculture facilities (Hargrave *et al.* 1993; Bonsdorff *et al.* 1997).

Hypoxia has been defined as DO less than 2 mg L^{-1} (~18% in seawater), or below the point that sustains most animal life (Diaz 2001; Rose *et al.* 2009), so its definition depends on the context of the study (Farrell and Richards 2009). We understand the lethal and sublethal effects of hypoxia largely from numerous studies on marine and freshwater fish from cold and temperate regions (e.g. Kramer 1987; Miller *et al.* 2002; Richards 2011). Comparatively little information exists on the sublethal effects of hypoxia in tropical freshwater systems, with some notable exceptions from South America (e.g. Rantin *et al.* 1992; Fernandes *et al.* 1995), Africa (e.g. Chapman *et al.* 1995; Chapman and Chapman 1998; Corrie *et al.* 2008) and northern Australia (Pearson *et al.* 2003; Butler *et al.* 2007).

Moreover, studies of hypoxia rarely involve testing fluctuating hypoxia, whereby in the presence of high plant biomass, DO saturation varies over a diel cycle, falling at night owing to the respiration of aquatic organisms, and rising during the day, through production of oxygen by photosynthesis (Brady *et al.* 2009). The upper and lower saturations reached during the cycle vary with conditions that include nutrient status, plant and microbe abundance, abundance of particulate and dissolved organic material, temperature and flow. In agricultural regions in tropical northern Queensland, fluctuating hypoxia is common (Pearson *et al.* 2003). Studies on responses of fish to fluctuating hypoxia require techniques that produce a cost-efficient, low-maintenance system for DO depletion and replacement.

There are hundreds of published studies reporting the effects of hypoxia on fish of various species, spanning at least 60 years. Many studies have used laboratory experiments to identify these effects, employing a variety of methods to achieve depleted DO concentrations, including addition of nitrogen gas, vacuum

Table 1. Experimental oxygen-depletion methods used in marine and freshwater studies

Dissolved oxygen (DO) depletion methods are recorded from a sample of 80 studies. All references were either technical methods papers, or examined the effects of hypoxia on fish. Marine/freshwater: M = marine/estuarine, F = freshwater; type of hypoxia: C = chronic and/or acute hypoxia, F = fluctuating hypoxia, G = DO saturation gradually altered to a maximum or minimum level (progressive hypoxia); lethal/sublethal: L = experiment to determine lethal level, S = experiment on sublethal effects

Technique	Studies using this technique	Location of study	Marine/ freshwater	Type of hypoxia	Lethal/ sublethal
Addition of nitrogen gas (51 of 80)	Fry 1951	Toronto	–	–	–
	Downing 1954	UK	F	G	L
	Downing and Merkens 1955	UK	F	G	L
	Whitmore *et al.* 1960	Oregon	F	C	S
	Davis *et al.* 1963	Oregon	F	C	S
	Dahlberg *et al.* 1968	Oregon	F	C	S
	Siefert and Spoor 1974	Minnesota	F	C	L & S
	Swift and Lloyd 1974	UK	F	C	S
	Johnston 1975	UK	F	C	S
	McDonald and McMahon 1977	Canada	F	C	S
	Kramer and Mehegan 1981	[A]Trinidad	F	C	L & S
	Drewett and Abel 1983	UK	F	C	L
	Petersen and Petersen 1990	Denmark	M	C	L & S
	Pihl *et al.* 1991	Virginia	M	C	L & S
	Kaufmann and Wieser 1992	Austria	F	C	S
	Schurmann and Steffensen 1994	Denmark	M	G	S
	Cech and Massingill 1995	California	F	G	S
	Fernandes *et al.* 1995	Brazil	F	C	S
	Thomason *et al.* 1996	UK	M	C	S
	Crocker and Cech 1997	California	F	C	S
	Schurmann and Steffensen 1997	Denmark	M	G	S
	Dalla Via *et al.* 1998	[A]Italy	M	G	S
	Plante *et al.* 1998	Quebec	M	C	L
	Chabot and Dutil 1999	Quebec	M	C	S
	Jones and Reynolds 1999*a*, 1999*b*, 1999*c*	UK	M	C	S
	Renshaw and Dyson 1999	Australia	M	C	S
	Tallqvist *et al.* 1999	Finland	M	C	L & S
	Geiger *et al.* 2000	Florida	M	C	S
	Pichavant *et al.* 2000	France	M	C	S
	Pichavant *et al.* 2001	France	M	C	S
	Richardson *et al.* 2001	New Zealand	F	C	S
	Taylor and Miller 2001	North Carolina	M	C & F	S
	Pearson *et al.* 2003	Australia	F	C	L & S
	Ishibashi *et al.* 2005	Japan	M	G	L
	Shimps *et al.* 2005	North Carolina	M	C	L
	Ishibashi *et al.* 2007	Japan	M	G	L
	Landry *et al.* 2007	Mississippi	M	C	S
	Ripley and Foran 2007	Virginia	M	C	S
	Hassell *et al.* 2008	Australia	M	C	L & S
	Sloman *et al.* 2008	British Columbia	M	G	S
	Wang *et al.* 2008	Taiwan	F	C	S
	Brady *et al.* 2009	Delaware	M	F	L & S
	Lefrançois *et al.* 2009	Italy	M	G	S
	Martínez *et al.* 2009	Uganda	F	C	S
	Brady and Targett 2010	Delaware	M	F	S
	Vanlandeghem *et al.* 2010	Illinois	F	C	S
	Cheek 2011	Texas	M	F	S
	Laursen *et al.* 2011	[A]UK	F	G	S
	Tzaneva *et al.* 2011	Canada	F	G	S
Fish respiration (11 of 80)	Hunn 1969	Wisconsin	F	G	S
	Courtenay and Keenleyside 1983	[A]Central America	F	G	S
	Vig and Nemcsok 1989	Hungary	F	G	S
	Kakuta and Murachi 1992	Japan	F	G	S
	Kakuta *et al.* 1992	Japan	F	G	S

(*Continued*)

Table 1. (Continued)

Technique	Studies using this technique	Location of study	Marine/ freshwater	Type of hypoxia	Lethal/ sublethal
	van Raaij *et al.* 1994	Netherlands	F	G	S
	Thetmeyer *et al.* 1999	Germany	M	C & F	S
	Waller *et al.* 2000	[A]British Columbia	M	C	S
	Cerezo Valverde *et al.* 2006	Spain	M	G	L & S
	Lays *et al.* 2009	Norway	M	G	S
	Barnes *et al.* 2011	Australia	F	G	L
Vacuum degassing (8 of 80)	Mount 1961	Ohio	F	G	L
	Carlson and Herman 1978	[A]Wisconsin	F	C & F	S
	Carlson *et al.* 1980	Minnesota	F	C & F	L & S
	Scott and Rogers 1980	Alabama	F	C	S
	Bejda *et al.* 1987	New Jersey	M	G	S
	Pouliot *et al.* 1988	Quebec	F	C	S
	Pouliot and de la Noüe 1989	Quebec	F	C	S
	Miller *et al.* 2002	East USA	M	C	L
Sodium sulfite – including when used in conjunction with nitrogen gas (7 of 80)	Chapman *et al.* 1995	[A]Lake Victoria	F	G	S
	Gee and Gee 1995	Australia	M	C	S
	Chapman and Chapman 1998	[A]Lake Nabugabo	F	G	S
	Schofield and Chapman 2000	[A]Lake Nabugabo	F	G	S
	Melnychuk and Chapman 2002	[A]Lake Kabaleka	F	G	S
	Schofield *et al.* 2007	Florida	F	G	L
	Corrie *et al.* 2008	[A]Uganda	F	G	S
Cages in the field (3 of 80)	Moore 1942	Minnesota	F	C	L
	Dunson and Dunson 1999	Florida	M	C & F	S
	Ruggerone 2000	Alaska	F	C	L

[A]Location the fish were sourced from.

degassing, addition of sodium sulfite and sealing the experimental containers so that the fish's own respiration removes oxygen from the water (Table 1). Other studies have aimed to achieve realism by inserting cages or mesocosms into low-oxygen environments in the field (Dunson and Dunson 1999; Ruggerone 2000).

We assessed 80 published laboratory studies that examined the effects of hypoxia on fish. The most common method of DO depletion in these experiments was the displacement of oxygen gas by bubbling water with nitrogen gas (64% of papers; Table 1). This technique is very effective and has the advantage that nitrogen gas is biologically inert. However, some aspects of this method are unnatural, including the presence of a 'nitrogen atmosphere' above the water's surface, preventing fish from effectively employing aquatic surface respiration (ASR; Kramer and Mehegan 1981) or facultative air-breathing.

In natural waterways, fluctuating hypoxia is often caused by abundant macrophyte growth, encouraged by high light conditions or nutrient concentrations (Kaenel *et al.* 2000). During the night, respiration by organisms in the water body removes oxygen from the water and replaces it with carbon dioxide, causing pH levels to drop (except in extremely hard water) (Burnett 1997). Adding nitrogen gas to water causes the reverse effect because nitrogen displaces both oxygen and carbon dioxide from solution. This artificial situation is not ideal, given that the low pH caused by high concentrations of dissolved carbon dioxide in field situations can disrupt the acid–base balance and gas transfer across fish gills (Cruz-Neto and Steffensen 1997), thereby lowering the efficiency of oxygen uptake (Dahlberg *et al.* 1968). This artificial effect may be avoided by bubbling carbon dioxide gas into the water (e.g. Pearson *et al.* 2003), or by adjusting pH using buffers. However, the disadvantage is that such methods make experiments more expensive, time-consuming and difficult to control, particularly for long-running experiments.

Here, we demonstrate the use of aquatic plants to create conditions of hypoxia, thereby exposing test organisms to diel cycling of DO and pH, which replicates natural environments and avoids some of the problems associated with other methods. We present a cost-effective laboratory method, which can be deployed over long periods of time with minimal effort in comparison to some traditional methods of DO reduction.

Materials and methods

We used *Ceratophyllum demersum* (commonly known as hornwort), a cosmopolitan aquatic macrophyte, as our test species. Hornwort is a non-rooted plant that absorbs nutrients from the surrounding water, has high photosynthetic performance (Blüm *et al.* 1997) and produces increasing DO saturation with increasing light intensity (Pearson *et al.* 2003). It grows quickly in high-nutrient waters, can tolerate a wide range of water hardness, and can become a noxious weed in areas where it is exotic (Global Invasive Species Database 2006). It is commonly used in the aquarium trade, making it freely available

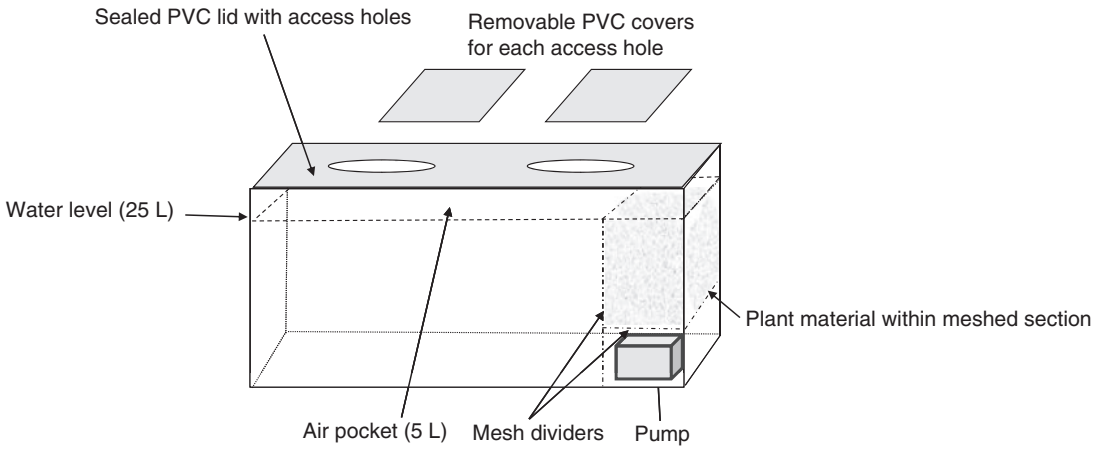

Fig. 1. Diagram of experimental aquaria. Polyvinyl chloride (PVC) lids were sealed to the tops of the aquaria by using high-vacuum silicon grease. Plastic mesh (5 mm) was used to divide plant material and pump compartment from the large fish compartment. Aquaria were of 30-L capacity, filled with 25 L of water, leaving a 5-L air pocket between the air–water interface and the lid.

as a test subject. Plants were collected from weir pools in Ross River, Townsville (19°18′S, 146°45′E), and held in carbon-filtered (0.5 μm) tap water in 500-L white plastic mesocosms in open sunlight. Plants were maintained with a nutrient mix of aquarium plant food, sodium nitrate and potassium dihydrogen orthophosphate, and kept outside for 5 days before using in experiments.

The trials were conducted in a light- and temperature-controlled room at a constant water temperature of 29°C, using 30-L glass aquaria filled with 25 L of carbon-filtered water. The aquarium design left a 5-L pocket of air between the water surface and the lid that was accessible to fish. Each aquarium was sealed with a PVC plastic lid and silicon grease (Fig. 1). Sealable ports in the lid allowed access for measuring DO. Plant volume was measured as the amount of water displaced in a graduated measuring cylinder. Treatments consisted of different volumes of hornwort (50–300 mL) added to the aquaria, and controls (0 mL plant material). Only healthy parts of the plants, especially the dense green tips, were used in the experiments. A submersible pump (Resun SP-600: 5 W, 220 V, 60/50 Hz, 250 L h^{-1} delivery, Shenzhen, China) in each aquarium maintained water circulation. Trials with fish used juvenile barramundi (*Lates calcarifer*) of 40–50-mm total length.

In all trials, DO, pH and temperature were regularly monitored with a WTW pH/Oxi 340i meter (Wissenschaftlich-Technische Werkstatten, Weilheim, Germany), in combination with a WTW CellOx 325-3 DO probe and WTW SenTix pH probe. Both probes were calibrated daily. The readings were taken by inserting the probes through a resealable opening in the lid of each aquarium.

Presented here are the results of three experiments investigating (1) the relationship between plant biomass and minimum DO saturation achieved overnight, (2) the use of fish alone to decrease DO saturation overnight and (3) the effectiveness of the plants to reduce DO saturation overnight in the presence of fish for prolonged time periods. Graphical illustration of data and regression analyses were carried out in SigmaPlot 11.0 (Systat Software Inc. 2008, Chicago, IL).

Experiment with plants

Hornwort (0-mL plant material = control, and 50-, 100-, 150-, 200- and 300-mL treatments, $n = 2$ replicates per treatment) was used to deplete DO saturation in the aquaria over 18 h, during which time aquaria were kept in complete darkness. Minimum DO saturation in each aquarium and pH data were analysed by simple linear regression with 95% confidence intervals.

Experiment with fish

Reduction in DO saturation owing to fish respiration was tested using sealed aquaria, each containing four barramundi ($n = 2$ replicates). This trial was carried out concurrently with the plant experiment detailed above, and under the same conditions.

Experiment with fish and plants

This experiment aimed to create diel reductions in DO concentration that were as low as possible while remaining sublethal to fish, to demonstrate the concentrations to which DO could be successfully and repeatedly lowered using a combination of fish (in this case barramundi) and plants (hornwort). The best combination of plants and fish to achieve this aim was found to be four barramundi and 275 mL of hornwort. Four barramundi were placed into each of two experimental aquaria, each of which also contained 275 mL of hornwort. Aquaria were sealed and kept in the dark for 16 h, during which time one aquarium was bubbled with compressed air, as a control, whereas the other one was not. In initial trials, it was found that 18 h of darkness (as used in the tests with plants and fish alone, above) resulted in excessive DO depletion, so 16-h dark periods were employed. During the 8-h 'day', when overhead fluorescent lights were switched on in the experimental room, additional aquarium lamps (Hagen Aqua Glo – 55 lux, 18 000 K, Montreal, Canada) were switched on behind the aquaria, closest to the plants, to encourage photosynthesis, and compressed air was provided to enhance DO renewal.

For 20 days, DO, pH and temperature were recorded every morning (when lights were switched on at 0900 hours) and evening (before lights were switched off at 1700 hours) in both

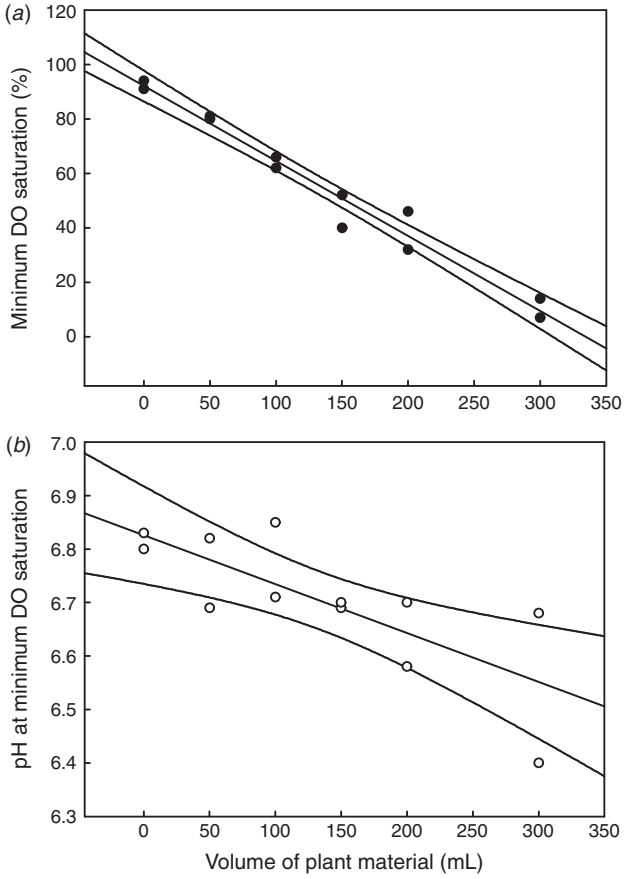

Fig. 2. Magnitude of (*a*) dissolved oxygen and (*b*) pH depletion in experimental aquaria containing varying amounts of plant material after 18 h of darkness. Each aquarium contained different volumes of hornwort, as shown on the *x*-axes (two aquaria per treatment). There were no fish in any aquaria.

aquaria. Maintenance of fish during this time included daily feeding as lights came on at 0900 hours, and a daily 50% water change using carbon-filtered tap water (DO saturation >90%), which was carried out 1 h before lights were switched off. By this time, DO concentration had returned to normoxia. Hornwort in experimental aquaria was replaced every 3 days with fresh material that had been kept in large outdoor mesocosms (as described above) for at least 5 days.

Results

Experiment with plants

Plant material alone substantially reduced the concentration of DO in aquaria overnight, and percentage DO saturation recorded in the morning decreased with increased volume of plant material (Fig. 2*a*, $R^2 = 0.970$, $P < 0.001$, 95% CI). Maximum DO before depletion was constant across treatments (98.3% ± 2.4% (s.d.)). The two control aquaria showed small decreases in DO saturation overnight (Control 1: from 98% to 94%; Control 2: from 99% to 91%).

The pH levels recorded in the morning, when DO was at a minimum, decreased with increased volume of plant material (Fig. 2*b*, $R^2 = 0.578$, $P < 0.001$, 95% CI). In the evening, when DO was at a maximum, pH levels were relatively constant across treatments (7.13 ± 0.12). In the control aquaria, pH fell slightly overnight (Control 1: from 7.2 to 6.8; Control 2: from 7.21 to 6.83).

Water circulation in the experimental aquaria was found be to be effective, with variation within the water column of <0.5% DO saturation.

Experiment with fish

The respiration of four barramundi depleted DO saturation in the sealed experimental aquaria by little more than for the control aquaria (described above), to a maximum of 9% (Fish treatment 1: from 98% to 89%; Fish treatment 2: from 98% to 92%). Concurrently, the change in pH in the treatment aquaria was similar in magnitude to that of the control aquaria (Fish treatment 1: from 7.98 to 7.45; Fish treatment 2: from 7.9 to 7.41).

Experiment with fish and plants

When 275 mL of hornwort were used to create fluctuating hypoxic conditions in an aquarium containing four barramundi, the resulting DO and pH reductions were highly repeatable over 20 days (Fig. 3). In the treatment aquarium, the mean minimum ('day') DO was 4.8% ± 2.9% (s.d.) saturation, and the mean pH at this time was 6.50 ± 0.09. The mean maximum ('night') DO saturation in the same aquarium was 89.4% ± 5.4%, and the mean pH at this time was 7.36 ± 0.16. The control aquarium during this test had a 'day' DO saturation of 97.5% ± 4.1% and pH of 7.50 ± 0.11. The 'night' DO saturation in the control tank was 94.1% ± 5.6% and the pH averaged 7.51 ± 0.14.

Discussion

In the present study, different volumes of aquatic plants (hornwort) in aquaria were used successfully to create diel cycles in DO saturation, to various levels of hypoxia. Aquaria containing barramundi alone achieved minor DO reduction overnight. A higher ratio of fish to water volume would be required to substantially deplete DO using fish respiration alone (e.g. Lays *et al.* 2009), but for some species, this might cause additional stress to fish through density effects on behaviour and physiology.

When plants were added to aquaria containing barramundi, DO depletion was rapid and repeatable, and followed a natural diel cycle. The DO cycling in the treatment aquarium containing barramundi and hornwort was similar to that under field conditions in areas where there is a high level of plant and algal material in still water. For example, in a lentic habitat in northern Queensland, Pearson *et al.* (2003) found that DO frequently cycled between 2% and 85% saturation over 24 h, and similar diel DO fluctuations have also been reported from other areas (e.g. Gulf of Mexico, Cheek *et al.* 2009; Florida, Bunch *et al.* 2010).

The extremely low DO concentrations (as low as 1.3% and frequently <5% saturation) survived by juvenile barramundi in the present study demonstrated an even greater capacity to withstand hypoxic stress than has been shown in previous studies. For example, Flint (2005) reported a lethal concentration of ~2% DO saturation for barramundi of 50–70-mm total length when oxygen is gradually depleted (noting that study and the present study were not designed to test lethal limits), and

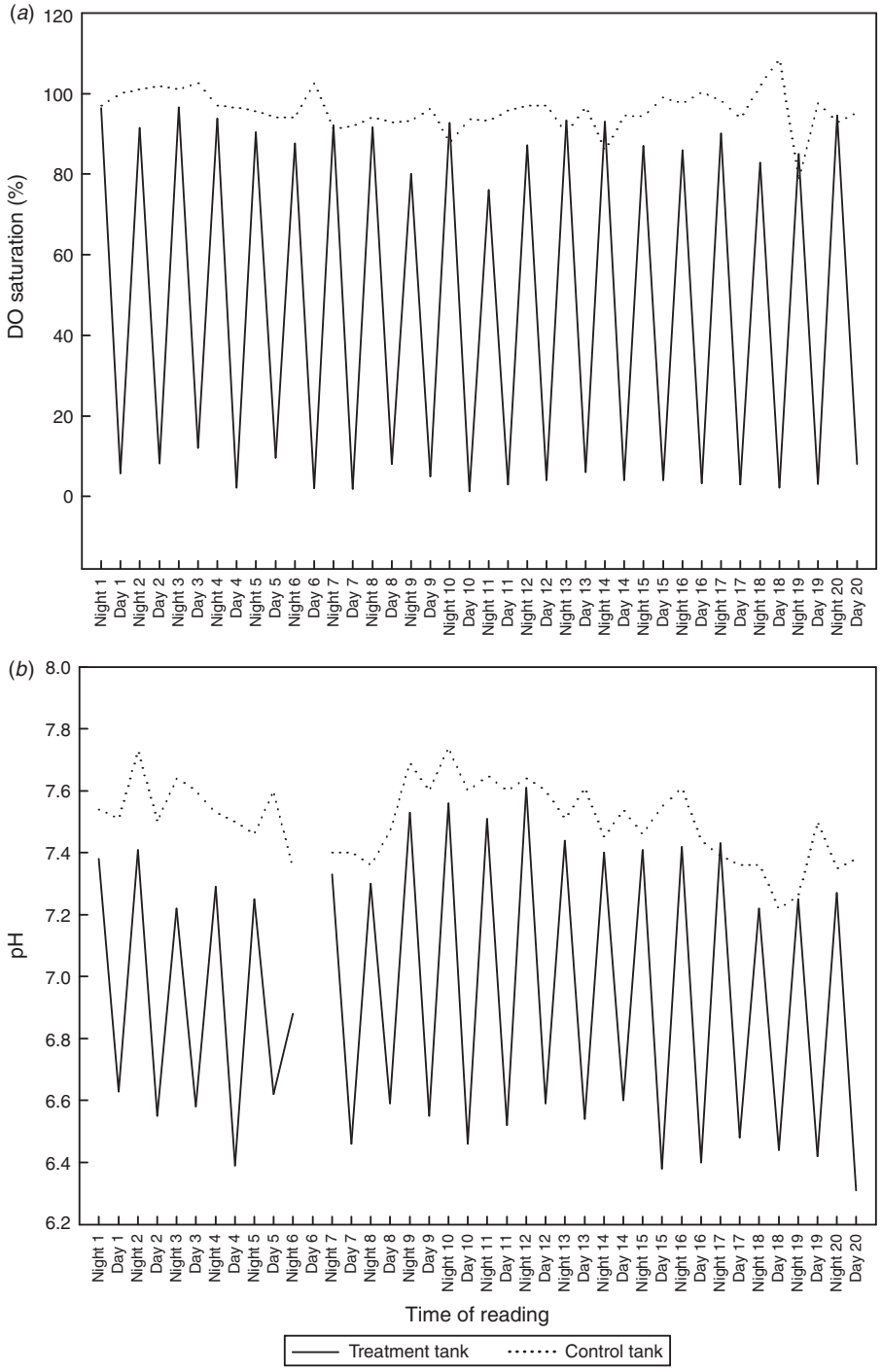

Fig. 3. Dissolved oxygen (DO) cycling using fish and plants to reduce saturation overnight. (*a*) DO saturation (%) and (*b*) pH in two aquaria each containing four barramundi fingerlings, and 275 mL hornwort. The treatment aquarium was sealed and not aerated for 16 h overnight and then aerated for 8 h during the day. The control aquarium was lightly sealed and aerated continuously. The absence of pH data at Day 6 was due to equipment malfunction. Readings were taken in the morning (day) and evening (night) only, and were not continuous. Continuous lines are for illustrative purposes only.

Pearson *et al.* (2003) identified a 24-h lethal concentration of ~11–15% saturation at 29°C for fish of 85–105-mm total length. In the present study, oxygen depletion was gradual, and animals had access to the air–water interface, and so were likely to have access to more highly oxygenated water than the minimum concentrations measured within the water column, as would often be possible in field situations (e.g. McNeil and Closs 2007; Riesch *et al.* 2010).

Aquaria containing both plants and fish showed some variability in the minimum DO saturation reached in a set time. The use of larger aquaria would probably reduce this effect. Conducting experiments under natural light may increase plant productivity and reduce the amount of plant material required to deplete DO to a required concentration, and reduce the need to regularly replace used plants.

The use of plants as the primary oxygen consumer in laboratory experiments creates natural fluctuations in dissolved carbon dioxide, and hence pH, and allows fish access to the higher oxygen concentrations in surface waters, such that aquatic surface respiration (ASR) (Kramer and Mehegan 1981; Kramer and McClure 1982) and facultative air breathing (e.g. Geiger *et al.* 2000) may be effectively utilised. This advantage is in contrast with other methods commonly used to create hypoxic conditions, such as vacuum degassers and nitrogen replacement, where access to higher oxygen saturations at the air–water interface may be unavailable. In situations where surface access is not desired for an experiment, a simple plastic mesh divider could easily be incorporated into the aquarium design to physically prevent fish from accessing the air–water interface. The method described here produces DO cycling regimes that are cost-effective and repeatable over several weeks and that simulate field conditions.

Limitations of the method described here include the time involved in changing water daily and plant material every 3 days, and the necessity to maintain stands of plants for use in the experiment. The regular water changes that were required may also cause stress to fish. Despite these limitations, the technique described here is easily implemented and could replace other methods as a DO-depletion mechanism. It would be difficult to use this method to maintain DO saturation at a minimum level for a long period of time, as is necessary for experiments on the effects of chronic hypoxia (e.g. Miller *et al.* 2002; Landry *et al.* 2007; Wang *et al.* 2008), but this method is particularly appropriate to simulate diel DO cycling or progressive DO depletion to a non-sustained minimum. Several factors may influence variability in results, including aquarium size, the amount of natural light available, temperature, water chemistry and the health of plants used. However, this method offers an alternative to traditional methods of DO depletion and should be considered as a means of creating natural diel cycles in DO saturation in experimental situations.

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