INTERACTIVE EFFECTS OF DIEL-CYCLING HYPOXIA, PH, AND TEMPERATURE ON GROWTH OF *FUNDULUS HETEROCLITUS*, A COMMON ESTUARY-RESIDENT FISH

by

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ABSTRACT

Growth rate (G_S) of *Fundulus heteroclitus* was examined at three temperatures in several treatments of diel-cycling DO and pH. Diel-cycling pH (ranging either from 7.2-7.8 or 6.8-8.1) at 25°, 30° and 35°C did not significantly affect growth. However, wide ranging diel cycles in DO (1-11 mg O_2 l^{-1} but not 3-9 mg O_2 l^{-1}) significantly reduced growth rates during initial 10 days of treatment at 30°C but not at 25°C. Rising temperature appears to determine whether diel-cycling DO significantly impacts growth rates of this species. *F. heteroclitus* acclimated to treatment conditions after 10 days, with initial differences in growth rate between treatments and control disappearing. In a separate experiment, *F. heteroclitus* did not show a statistically significant level of growth compensation when fish exposed to 10 days of treatment (1-11 mg O_2 l^{-1}, 6.8-8.1 pH) were returned to normoxia and control pH. However, the high level of individual growth variation following return to normoxia and control pH makes a definitive conclusion on growth compensation difficult.

Chapter 1

INTERACTIVE EFFECTS OF DIEL-CYCLING HYPOXIA, PH, AND TEMPERATURE ON GROWTH OF FUNDULUS HETEROCLITUS, A COMMON ESTUARY-RESIDENT FISH

Introduction

Estuaries are highly variable environments important to a wide variety of fishes (Weinstein, 1979; Peterson & Ross, 1991; Peterson *et al.*, 2000). Many fish species use estuaries as nursery habitat because of the favorable physico-chemical conditions, high prey abundance and relatively low predation (Lubbers *et al*., 1990; Peterson & Ross, 1991; Able, 1999: Minello, 1999). The physico-chemical fluctuations in estuaries can influence the growth and survival of resident fishes (Stierhoff *et al*., 2003). Therefore, the long-term survival, growth and reproductive success of fish populations is dependent on their ability to tolerate changing environmental conditions (Wu & Woo, 1982; Eby & Crowder, 2002; Elliot & Quintino, 2007). Estuarine habitats exhibit swift, daily shifts in dissolved oxygen concentration (DO), pH, salinity and temperature (Boynton *et al*., 1996; Wong, 1998; Stierhoff *et al*., 2009a; Tyler *et al*., 2009; Howarth *et al*., 2011). Anthropogenic influences such as increasing greenhouse gases, nutrient loading and ocean acidification are impacting estuarine water quality variation as the normal shifts in water quality become more prolonged and dramatic (Diaz, 2001; Cai *et al*., 2011).

Hypoxia is increasingly prevalent in estuarine and coastal seas worldwide (Diaz & Rosenberg, 2008; Rabalais *et al*., 2009) with severe hypoxia and anoxia becoming more common during summer months (Bricker *et al*., 1999; Stierhoff *et al.*, 2009b). Periods of hypoxia that result from day/night swings in DO (diel-cycling hypoxia) are common in highly productive shallows of estuarine tributary systems (Beck & Bruland, 2000; Tyler *et al*., 2009). Cycles of photosynthesis and respiration in estuarine algal communities drive diel-cycling hypoxia by causing DO to fluctuate between hyperoxia (> 15 mg O_2 l^{-1}) during the day and hypoxia or anoxia at night (Kemp & Boynton, 1980; D'Avanzo & Kremer, 1994; Tyler *et al*., 2009).

Sub-lethal exposure to static and diel-cycling hypoxia reduces fish growth rates and increased temperature exacerbates these effects (Beida *et al*., 1992; McNatt & Rice, 2004; Stierhoff *et al*., 2006). However, growth rate reduction in the field is greater than that predicted by laboratory investigations (Stierhoff *et al*., 2009a). Potential synergistic effects of hypoxia with other physico-chemical properties in the estuary (not investigated in the laboratory) may contribute to this difference.

Increased photosynthesis during daylight hours coupled with continuous rates of respiration cause diel cycling of aqueous CO_2 and pH. Photosynthetic rates, CO_2 uptake, oxygen production and DO all diminish during the night. Respiration rates remain constant throughout the night therefore aqueous CO_2 concentrations increase, resulting in decreased pH (Green *et al*., 2009; Howarth *et al*., 2011). During summer months, diel-cycling DO in estuarine tributaries can range from <1 mg O_2 l^{-1} to >20 mg O_2 l^{-1} while corresponding diel-cycling pH can range from 6.5 to 8.5 (Tyler *et al*.,

2009; MD DNR, 2012). Effects of this covariance between diel-cycling DO and pH on fish growth and survival, particularly in shallow estuarine waters, have not been investigated. In addition, rising temperature, rising atmospheric CO_2 and increased ocean acidification threaten to exacerbate and prolong hypoxic events and declining pH worldwide (Broecker *et al*., 1979; Diaz, 2001; Feely *et al*., 2004). Because estuaries are vital habitats, understanding how the interaction of hypoxia and pH affect growth and survival of estuarine organisms is essential.

The mummichog (*Fundulus heteroclitus*) is a eurytopic resident of shallow estuarine and coastal waters from Newfoundland (Canada) to Florida (USA) (Able & Fahay, 1998). At 25°C, diel-cycling hypoxia (1-11 mg O_2 l^{-1}) has no effect on growth; mummichogs exhibit reduced growth rates only when DO falls to a static 1 mg O_2 l^{-1} (Stierhoff *et al*., 2003**).** However, they are capable of acclimating to static 1 mg O_2 l^{-1} after two weeks of exposure (Rees *et al*., 2012). There are no studies analyzing the synergistic impact of diel-cycling hypoxia and pH on growth rates or the influence of higher environmental temperatures (30° and 35°C) on growth rate in the mummichog. The objectives of this study were: (1) to quantify the individual and potential combined effects of diel-cycling hypoxia and pH on growth rates of mummichog, (2) to determine whether increasing temperature affects the growth response of mummichogs to the prescribed hypoxia/pH treatments and (3) to determine whether mummichogs are capable of acclimating to diel-cycling hypoxia and pH or showing compensatory growth after return to normoxia and control pH.

Materials and Methods

Laboratory Set-Up

Specific growth rates of *F. heteroclitus* were measured in a series of laboratory experiments. Fish were held under controlled temperature, photoperiod, DO and pH conditions in a recirculating aquarium system (see Appendix A for full details). This system is an adaptation of the DO-controlling aquarium apparatus (Grecay & Stierhoff, 2002) previously used for hypoxia studies on estuarine fishes (Stierhoff *et al.* 2006, Stierhoff *et al*., 2009b). The system is capable of controlling DO and pH in five separate recirculating aquaria (~415 l each). A computer program regulates DO and pH by bubbling compressed air, CO_2, N_2, and O_2 as needed to maintain desired treatment conditions.

Each treatment aquarium consists of a tray topped with airtight glass lids. Within each tray are 10 clear polyethylene replicate tanks (18 l). A centrifugal pump propels water from a polyethylene sump tank to each replicate tank via a distribution manifold pipe. These replicate tanks overflow into the tray. A bulkhead fitting at one end of the tray allows water to overflow via a 4" PVC pipe into the sump.

Fish Collection & Acclimation

F. heteroclitus were collected in minnow traps from Canary Creek (38°46' N, 75°09' W) in the Great Marsh Preserve adjacent to the University of Delaware's College of Earth, Ocean and Environment campus in Lewes, Delaware, USA. Fish were transported to 350 l recirculating aquaria where they were acclimated to laboratory conditions of constant temperature (25^o, 30^o or 35^oC), salinity (12ppt) and

normoxia for ≥ 14 d. Fish were held under a 14L:10D photoperiod and fed mysid shrimp (*Mysis relicta*) *ad libitum* once per day. Following acclimation, 50 fish (40±4 mm SL) were randomly assigned to replicate tanks for growth trials.

Growth Trials

DO and pH treatment ranges simulated the range and periodicity observed in the shallow estuarine environments. Utilizing water quality data from Pepper Creek (tributary of Indian River Bay, DE) and the Chesapeake Bay (Tyler *et al*., 2009; MD DNR 2012), a set of appropriate DO/pH treatments were developed that would reflect natural DO/pH ranges and allow for proper statistical analysis.

A 3x3 factorial design was established to examine growth effects resulting from the interaction of diel-cycling of DO and pH. Three treatment levels of DO were established; (1) DO that cycled between 1 and 11 mg O_2 l^{-1} (Wide-DO Cycle; "W-DO"); (2) DO that cycled between 3 and 9 mg O_2 l^{-1} (Narrow-DO Cycle; "N-DO"); (3) DO held continuously at 7.5 mg O_2 l^{-1} (Static DO Control; "Control-DO"). Crossed with the above treatments were three levels of pH: (1) pH that cycled between 6.8 and 8.1 (Wide-pH Cycle; "W-pH"); (2) pH that cycled between 7.2 and 7.8 (Narrow-pH Cycle; "N-pH"); (3) pH held continuously at 7.5 (Static pH Control; "Control-pH"). Minimum and maximum DO concentrations in the diel-cycling treatment coincided with the beginning of the light (07:00 h) and dark (21:00 h) periods, respectively.

For all experiments there were ten replicate fish per treatment for each combination of DO and pH. (Table 1; for graphs of treatments see Appendix B). The

experimental design outlined above was repeated at three temperatures (25°, 30° and 35°C); these temperatures span the range of temperatures normally encountered by mummichogs during summer months (Abraham, 1985). At 25°C, the duration of the experiment was 10 days. At 30° and 35°C, the duration was extended to 30 days to investigate the potential for long-term acclimation to treatments (Rees *et al*., 2012).

Following transfer to the replicate tanks, fish were given 7 days to acclimate to control DO and pH levels of 7.5 mg O_2 l^{-1} and 7.5, respectively (Stierhoff *et al*., 2009b). Mass (±0.01g) of each fish was recorded at the beginning of experiments, just prior to feeding to minimize effects of stomach content on mass (Stierhoff *et al*., 2003). At each temperature, two sets of five treatments were run successively. Therefore, 8 experimental treatments were run once with the control treatment being run twice (once for each successive run).

Fish were fed frozen mysids twice per day (09:00h and 17:00h). Experiments at 30° and 35°C were divided into sequential ten-day growth periods: on days 10, 20 and 30, each fish was re-weighed (prior to morning feeding). Every day, before feeding at 17:00h, uneaten *M. relicta* were removed from the tanks to minimize water quality degradation. Ammonia, nitrite and nitrate concentrations were monitored throughout treatment. When necessary (ammonia>0ppm; nitrite>0ppm; nitrate>40ppm), 30% water changes were performed to improve water quality. Mortalities were recorded daily.

Recovery Trials

An additional experiment was conducted to examine the potential for compensatory growth. Fish were initially exposed to one diel-cycling treatment ("W-DO"/"W-pH") and a control treatment ("Control-DO"/"Control-pH" treatment) at 30°C for 10 days, after which control conditions (DO = 7.5 mg O_2 l^{-1}; pH = 7.5) were reestablished for an additional 10 days (Table 1). Acclimation to treatment aquaria and feeding/measuring regimen were identical to the previous experiments. Growth rates during this recovery trial were compared between the initial ten days of treatment and the subsequent ten days of normoxia and control pH to determine whether growth compensation occurred when control conditions returned (Bejda *et al.*, 1992).

Data Analysis

Daily specific growth rate (G_S) is the percent change in body mass per day. It is calculated as $G_S = 100(e^G - 1)$ where G (instantaneous growth rate) = $[(\ln M_2 - \ln M_1)/(t_2 - t_1)]$ where M_2 and M_1 were masses at times t_2 and t_1 respectively (Ricker, 1975; Stierhoff *et al*., 2003; Rees *et al*., 2012). Data were checked for normality with a 1-sample Kolmogorov-Smirnov test with the Lilliefors option. A few gravid females were eliminated from the analysis as outliers (confirmed with boxplot analysis).

Because there were 5 treatment aquaria (each containing 10 replicate tanks), for full comparisons it was necessary to run two series of experiments sequentially at each temperature. Thus, there was a control treatment for each of the two experimental runs. All G_S data for each run were normalized relative to its respective control by dividing mean G_S at each treatment level by the mean G_S of the respective control.

This division yields a measure of daily specific growth relative to the control (G_S*); G_S* of the control group is equal to 1 and the G_S* measurements of other treatments are relative to 1. The two control groups were combined for the sake of analysis yielding a control group with twice as many of fish as the other treatments, thus, a Type III SS was necessary. For each temperature, mean G_S* in all treatments was compared during each ten-day growth period, with two-way Type III SS ANOVA for the main effects of DO and pH, as well as interaction. Significant differences among treatments were determined using Dunnetts' tests. For long-term growth trials examining the potential for acclimation, mean G_S* was compared among treatments for each sequential ten-day growth period to determine whether acclimation occurred over time. For recovery trials, an analysis of covariance (ANCOVA) was used to analyze the effect of treatment type ("Control-DO"/"Control-pH" or "W-DO"/"W-pH") on the growth rate change between the initial ten-day treatment period and the subsequent ten-day recovery period (ΔG_S) using initial fish mass as the covariate.

Results

Experimental Conditions

The recirculating aquarium system was capable of rapid and reliable regulation of DO and pH. Raising pH by bubbling compressed air prevented target oxygen supersaturation until the target pH was achieved and air bubbling was halted (see Appendix B for plots of DO and pH recordings for each treatment). Once target pH was reached, supersaturation of oxygen was easily achieved by bubbling oxygen.

Specific Growth Rate

Initial body mass of *F. heteroclitus* was similar across all treatments (2.1 ± 0.54 g; n=300) and G_S results were not correlated with initial fish mass (Pearson correlation coefficient; r(96)= - 0.157 , p = 0.28). In all ANOVA analyses of G_S*, there was no interaction between DO and pH, therefore, data were analyzed for main effects alone.

25°C Experiment:

During this 10 day experiment, there were significant differences in G_S* among DO treatments ($F_{2,83}$= 3.31, p = 0.04). Mean G_S* among pH treatments did not differ ($F_{2,83}$= 1.05, p = 0.35). However, Dunnett's tests failed to detect difference in G_S* between the control and any other treatment (Figure 1). Mean G_S for the control treatment was 3.89 (% body mass growth/day) and mean G_S for the "W-DO"/"W-pH" treatment was 3.67. No mortalities occurred.

30°C Experiment:

During the initial 10 days there were significant differences in G_S* among DO treatments ($F_{2,93}$= 10.6, p < 0.001). Mean G_S* among pH treatments did not differ ($F_{2,93}$= 1.97, p = 0.14). Mean G_S* was significantly reduced compared with the control for both the "W-DO"/"W-pH" treatment (p < 0.001; Dunnett's test) and the "W-DO"/"Control-pH" treatment (p < 0.05; Dunnett's test)(Figure 2). A 3D interaction diagram illustrates the decline of G_S* with increased range in diel-cycling of DO during the initial 10 days (Figure 3). Between day 10 and day 20, there were no differences in G_S* between treatments for either DO ($F_{2,88}$= 0.04, p = 0.96) or pH

($F_{2,88}$= 0.18, p = 0.83)(Figure 4). Similarly, from day 20-30 there were no differences in G_S* between treatments for either DO ($F_{2,89}$= 0.07, p = 0.94) or pH ($F_{2,88}$= 1.02, p = 0.36)(Figure 5). While there was an initial growth rate reduction in the "W-DO"/"W-pH" and "W-DO"/"Control-pH" treatments relative to the control during the first ten days of treatment, this difference disappeared between days 10 and 30. Mean G_S for the control treatment was 3.91 (% body mass growth/day) for days 0-10, 1.52% for days 10-20 and 0.94% for days 20-30. Absolute growth rate declined over the 30 days for all treatments including the control but the differences between treatments and the control disappeared after ten days. Despite there being no difference in growth rate after the initial ten days, at the end of the 30 day experiment, the absolute growth rate of the "W-DO"/"W-pH" treatment was only 55% of the control. There was a single mortality in the "W-DO"/"N-pH" treatment between days 20 and 30. Two other fish escaped from their holding tanks and were eliminated from analysis (one fish in the "W-DO"/"N-pH" treatment and one in the "Control-DO"/"W-pH" treatment).

35°C Experiment:

During the initial ten days, G_S* did not differ among DO treatment ($F_{2,92}$= 1.14, p = 0.32) or pH treatment ($F_{2,92}$= 1.21, p = 0.30)(Figure 6). For the control, mean body mass increased by 1.72 % per day during the initial ten days. After the initial ten days, the two sequential controls diverged drastically in mean G_S. Therefore it was not possible to adjust G_S with respect to mean control growth for statistical analysis of growth during days 10-20 and day 20-30. Three mortalities occurred between days 10 and 30 (one fish in the "W-DO"/"W-pH" treatment (between days 10-20), one in the

"Control-DO"/"N-pH" (between days 20-30) and one in the first "Control-DO"/"Control-pH" treatment (between days 20-30)).

Recovery Trial

Absolute growth rate (G_S) declined between the initial ten day period and the subsequent recovery period for both the control and the "W-DO"/"W-pH" treatment at 30°C. Mean G_S for the "W-DO"/"W-pH" treatment (1.57%) was lower than that of the control (2.05%) during the ten-day treatment period. However, during the subsequent ten-day recovery period, mean G_S was higher for the "W-DO"/"W-pH" treatment (0.66%) compared to the control (0.45%). The ANCOVA analyzing the effect of treatment group and initial mass (the covariate) on change in growth rate between the ten-day treatment period and the ten-day recovery period (ΔG_S) found that the interaction term was nearly significant (α=0.05, p=0.053). A reduced ANCOVA model, which excluded the interaction term, found neither treatment group nor initial mass to have a significant effect on ΔG_S. Another ANCOVA analyzing the effect of treatment group and mass at the end of the treatment period (Day 10 mass) on ΔG_S found a marginally significant interaction term (α=0.05, p=0.048), but neither treatment nor Day 10 mass had a significant effect. There was more variation within the "W-DO"/"W-pH" group during the recovery period (CV=149%) than the control group (CV=109%), preventing stronger analysis. At the end of the 20 day experiment, the overall absolute growth rate of the "W-DO"/"W-pH" treatment was 94% of the control. There were no mortalities in either the treatment or control groups.

Discussion

Growth rates of *Fundulus heteroclitus* experiencing diel-cycling DO conditions are consistent with conclusions of previous studies using static DO treatments (Wannamaker & Rice, 2000; Stierhoff *et al*., 2003; Rees *et al*., 2012): mummichogs are extraordinarily tolerant of all but the most extreme low DO concentrations. Only widely diel-cycling DO conditions (1-11 mg O_2 l^{-1}) caused growth limitation in this species and only at 30°C for the initial ten days of treatment. Fundulids, such as *F. heteroclitus*, possess dorsally oriented mouths and dorso-ventrally flattened heads that enable them to employ aquatic surface respiration (ASR)(Lewis, 1970; Stierhoff *et al*., 2003; Richards *et al.*, 2009). Use of ASR can mitigate the impact of hypoxia on growth in this species (Stierhoff *et al*., 2003).

Diel-cycling pH did not significantly affect growth rate of *F. heteroclitus* at any temperature. Furthermore, there was no interaction between diel-cycling DO and diel-cycling pH. Although there was no statistically significant effect of pH on growth rate (atα=0.05) at any temperature, the suggestion of an influence of pH independently at 30°C ($p = 0.14$) was greater than at 25°C ($p = 0.35$). Just as the effect of low DO on fish physiology is species-specific, pH may have a greater impact on growth rates of more environmentally sensitive species.

It was expected that any negative effects of DO on growth rate of *F. heteroclitus* would increase with temperature. In fact, growth effects of DO were found to be dependent on both temperature and the range of the diel-cycle. It appears

that rising temperature does determine whether diel-cycling DO significantly impacts growth rates of *F. heteroclitus*.

Growth rates of the "Control-DO"/"Control-pH" and "W-DO"/"W-pH" treatment were comparable to results from Stierhoff *et al.* (2003) at 25°C, who found G_S to be near 4% body mass growth/day during both a static treatment of 7 mg O_2 l^{-1} and a severe diel-cycling DO treatment (1-11 mg O_2 l^{-1}). Likewise, we found no significant differences in growth between diel-cycling DO treatments and normoxic conditions, regardless of pH.

With an increase in temperature to 30°C, the severity of diel-cycling DO had a significant role in growth rate reduction ($p < 0.001$). No previous studies of the effects of hypoxia on *F. heteroclitus* growth rate have been conducted at this higher temperature. It appears that increased temperature suppresses optimal growth when DO fluctuates widely. Growth was significantly reduced relative to the control only in the "W-DO" treatments (1-11 mg O_2 l^{-1}) but not under the "N-DO" treatments (3-9 mg O_2 l^{-1}). Thus, growth of *F. heteroclitus* is not negatively affected until conditions become severely hypoxic (near 1 mg O_2 l^{-1}) and, furthermore, subsequent hyperoxic conditions do not ameliorate the effect of hypoxia. Fish in the 30°C "W-DO" treatments were observed to be generally less active and less excited by food during the oxygen minima portion of treatment (morning) than fish in treatments where DO did not fall below 3 mg O_2 l^{-1}. The growth reductions seen at 30°C may be due to corresponding reductions in feeding rate. Stierhoff *et al.* (2003) found reduced feeding and growth rates in F. heteroclitus exposed to constant 1 mg O_2 l^{-1}. In the field, *ad*

libitum feeding may not always occur, due to density-dependent food limitation (Kneib, 1981) or in habitat types that reduce access to optimal foraging space (such as *Phragmites australis*)(Hagan *et al*., 2007). Fish incapable of *ad libitum* feeding may experience further growth reductions under widely fluctuating DO cycles than were observed in the laboratory.

Significant growth rate reductions were observed only during the initial ten days of treatment. During the subsequent twenty days of treatment, the growth rates of the diel-cycling treatment groups did not differ from the control, illustrating that *F. heteroclitus* are capable of acclimating to the diel-cycling conditions after an initial ten days exposure. These results are consistent with those of a previous study (Rees *et al.,* 2012) in which growth rates were significantly reduced during an initial exposure to static severe hypoxia (1 mg O_2 l^{-1}) for two weeks, but during subsequent two-week exposure, growth rates no longer differed from the normoxic control. These results and our data at 30°C illustrate that *F. heteroclitus* have the capacity to acclimate to pronounced hypoxia when these conditions persist beyond 10-14 days.

When it was found that higher temperature affected the growth-limiting effect of diel-cycling DO, a third experiment, at 35°C, was conducted to determine whether the effects of DO and pH fluctuation would become exacerbated at a higher temperature. This temperature is encountered by *F. heteroclitus* in shallow estuarine tributaries during the hottest months of the summer (Abraham, 1985**).** Neither DO nor pH, significantly impacted growth rate relative to the control during the initial ten days. However, at this high temperature, absolute growth rates were considerably

lower than at lower temperatures (the control exhibited 1.72% growth per day during the initial ten days as opposed to 3.89% and 3.91% at 25° and 30°C respectively). Furthermore, fish appeared to be generally sluggish throughout all treatments at this high temperature. Therefore, it appears that the stress of high temperature masked any effects of both DO and pH on growth. The initial ten-day results suggest that fluctuation in DO or pH at this higher temperature challenged the ability of *F. heteroclitus* to maintain normal growth rate, resulting in an overall reduction (see Figure 6). It is likely that at temperatures as high as 35°C, additional stressors may greatly increase individual variability in growth rates. Metabolism of *F. heteroclitus* has been found to vary significantly among individuals (Crawford & Oleksiak, 2007). Furthermore, this species varies in thermal tolerance due to both physiological acclimation and genetic adaptation (depending on whether the fish are from northern or southern populations)(Crawford & Powers, 1989; Fangue *et al*., 2006). As our fish were from the area in the Mid-Atlantic where the populations mix (Morin and Able, 1983); it is probable that tolerance of 35°C would be higher in more southern *F. heteroclitus* populations.

The 30 day growth trial at 30°C showed that the initial reduction in growth rates disappear over time. However, the effect of initial growth rate reduction is still felt after growth rates recover to control levels; the absolute growth rate of the "W-DO"/"W-pH" group was 55% that of the control over the first 20 days of treatment. During the recovery trial, when normoxia and control pH conditions were reestablished after 10 days of treatment, fish in the "W-DO"/"W-pH" treatment

averaged a higher growth rate (G_S) than the control fish. Over the 20-day recovery trial, absolute growth of the "W-DO"/"W-pH" treatment was 94% of the control, in contrast with the 55% found over 20 days of continuous treatment [in the 30°C growth trial]. ANCOVA results found a nearly significant effect of interaction (p=0.053) between initial mass and treatment on how fish growth rates changed when normoxia and control pH returned, but there was no significant of treatment alone on growth rate (p=0.411) during the recovery period. Another ANCOVA found a marginally significant effect of the interaction between treatment and Day 10 mass on growth rate change (p=0.048); however, the effect of treatment remained insignificant (p=0.16). However, due to the high level of variability of the "W-DO"/"W-pH" group during the normoxia period a definitive conclusion on growth compensation following return to normoxia and control pH is difficult. We can only conclude that there was a high level of individual variation in the recovery group and any effect that a return to normoxia and control pH may have had on growth is small relative to this level of individual variation.

F. heteroclitus is a eurytopic species capable of acclimating to long-term, widely fluctuating DO/pH conditions and recovering from growth rate reductions induced by short term hypoxic stress. Further research will reveal whether other less hardy fish species are capable of the same level of tolerance, acclimation, and compensation.

Table 1: Treatment conditions and durations

Growth Trial Treatments	DO Treatment (mg O_2 l^{-1})	pH Treatment	Treatment Length
W-DO/W-pH	1-11	6.8-8.1	10 days (at 25^{o}C); 30 days (at 30^{o}C & 35^{o}C)
W-DO/N-pH	1-11	7.2-7.8	
W-DO/Control-pH	1-11	7.5	
N-DO/W-pH	3-9	6.8-8.1	
N-DO/N-pH	3-9	7.2-7.8	
N-DO/Control-pH	3-9	7.5	
Control-DO/W-pH	7.5	6.8-8.1	
Control-DO/N-pH	7.5	7.2-7.8	
Control-DO/Control-pH	7.5	7.5	
Recovery Trial Treatments			
W-DO/W-pH	1-11	6.8-8.1	10 days treatment; followed by 10 days Control conditions (DO: 7.5 mg O_2 l^{-1}, pH: 7.5) (at 30^{o}C)
Control-DO/Control-pH	7.5	7.5	

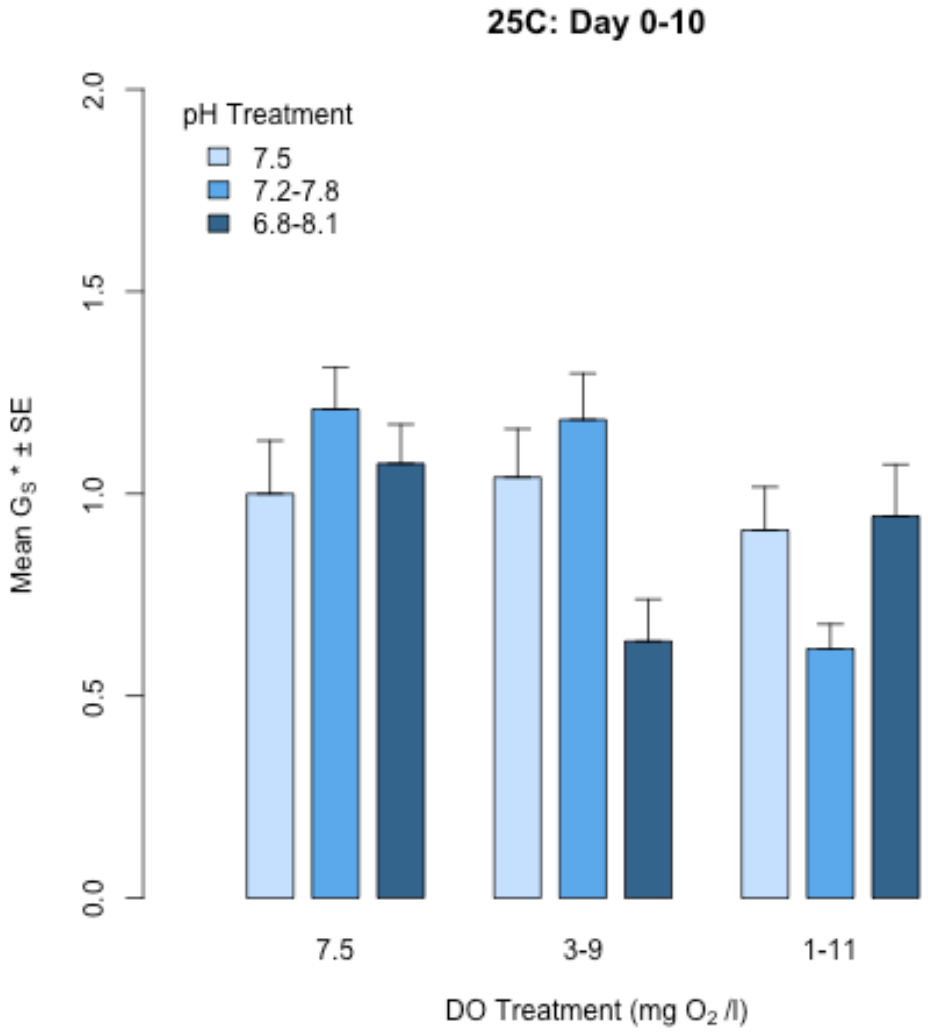

Figure 1: Mean G_S* (G_S relative to control group mean G_S) for each treatment for Days 0-10 at 25°C. No significant differences were found between diel-cycling treatment groups and the control.

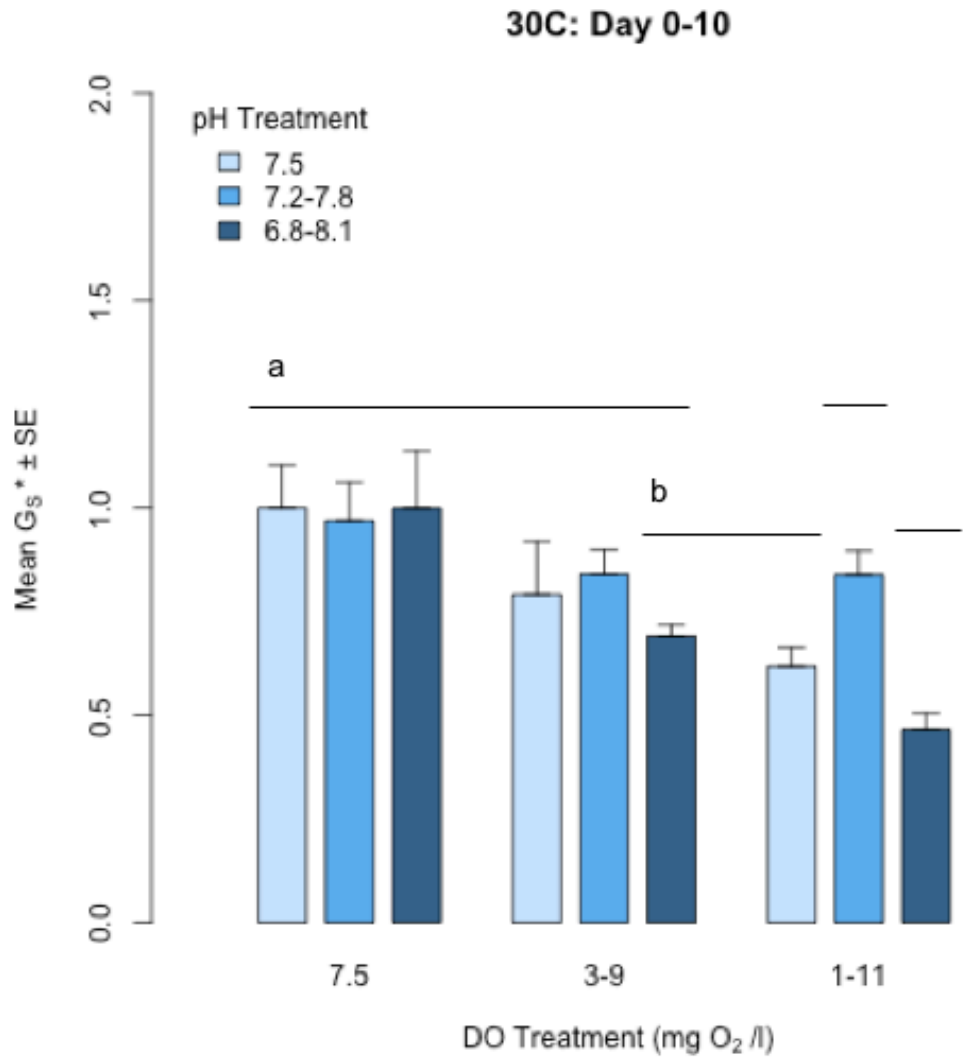

Figure 2: Mean G_S* (G_S relative to control group mean G_S) for each treatment for Days 0-10 at 30°C. Treatments under the significance level group "a" are not significantly different from the control. Treatments under the significance level group "b" are significantly different from the control and show significantly reduced specific growth rates relative to the control group.

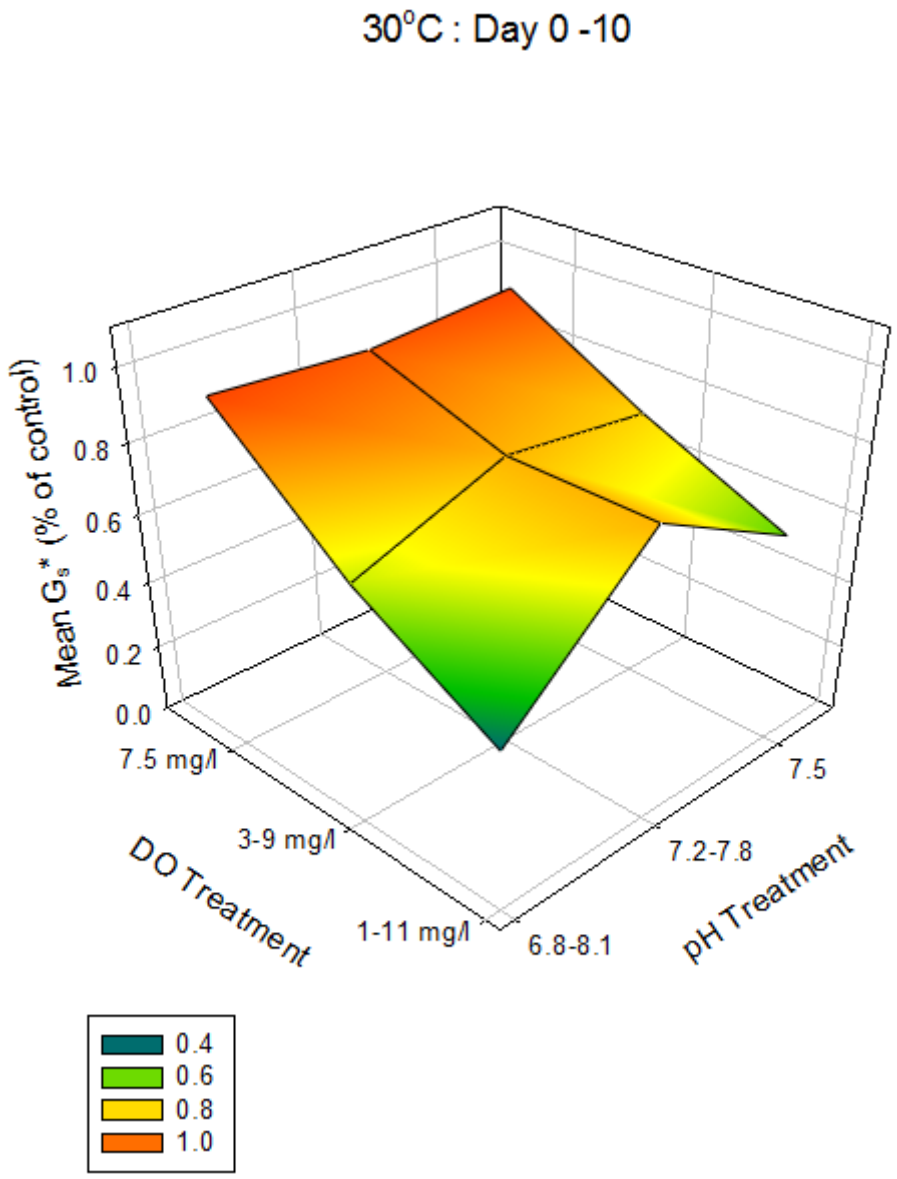

Figure 3: Interactive effects of diel-cycling DO treatment and diel-cycling pH treatment on relative specific growth rate (G_S*) of *F. heteroclitus* (Day 0-10; 30°C).

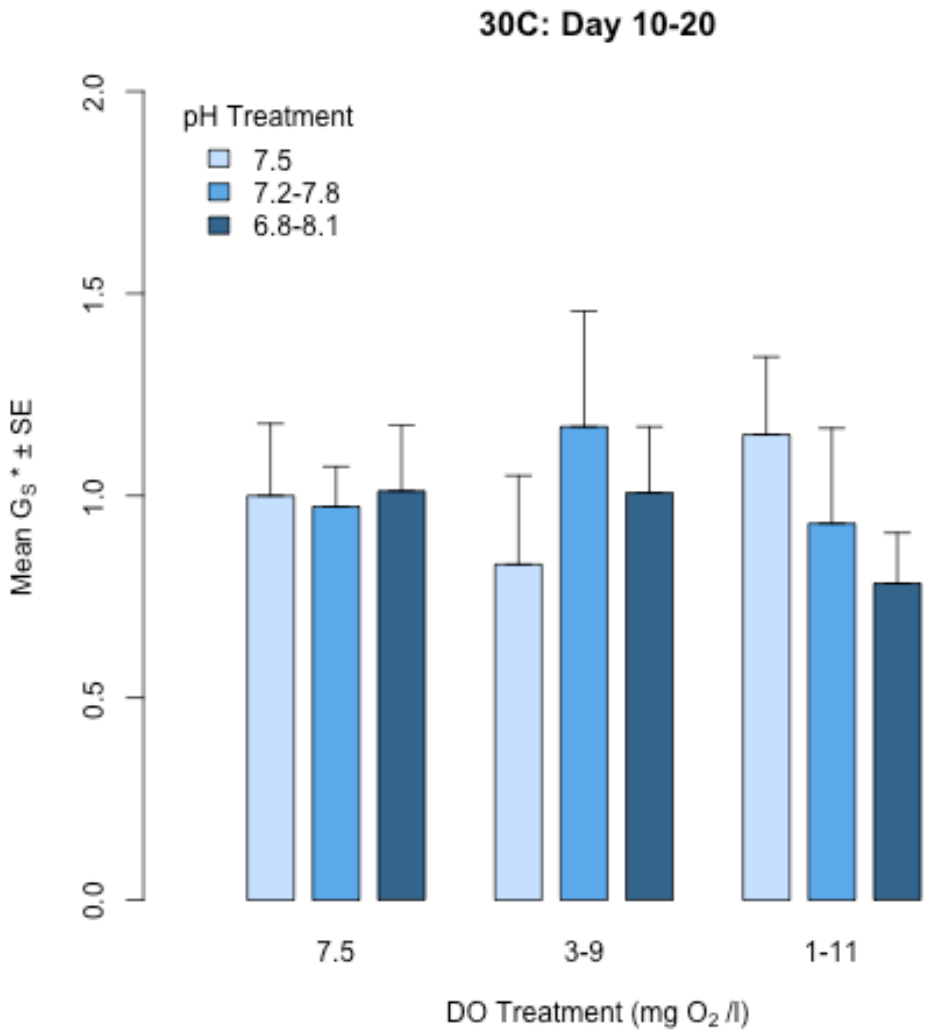

Figure 4: Mean G_S* (G_S relative to control group mean G_S) for each treatment for Days 10-20 at 30°C. No significant differences were found between diel-cycling treatment groups and the control.

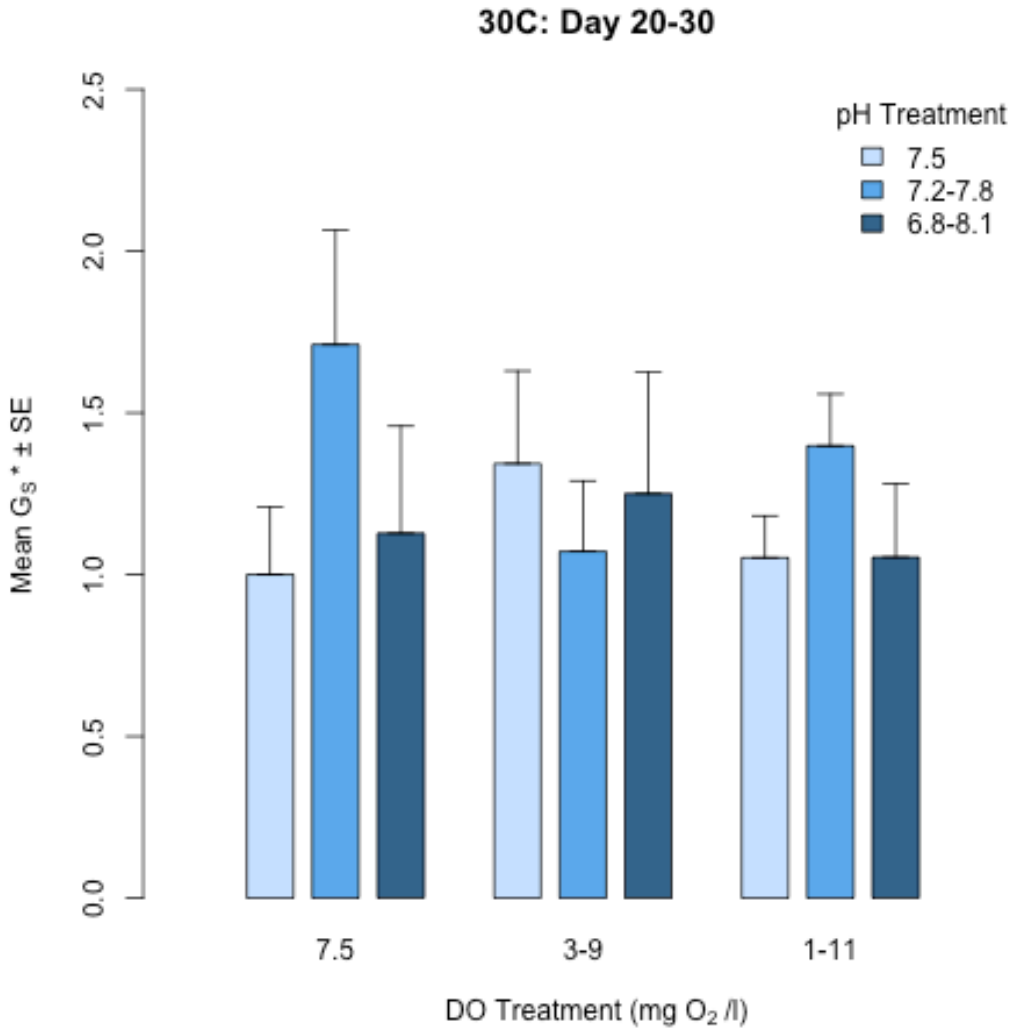

Figure 5: Mean G_S* (G_S relative to control group mean G_S) for each treatment for Days 20-30 at 30°C. No significant differences were found between diel-cycling treatment groups and the control.

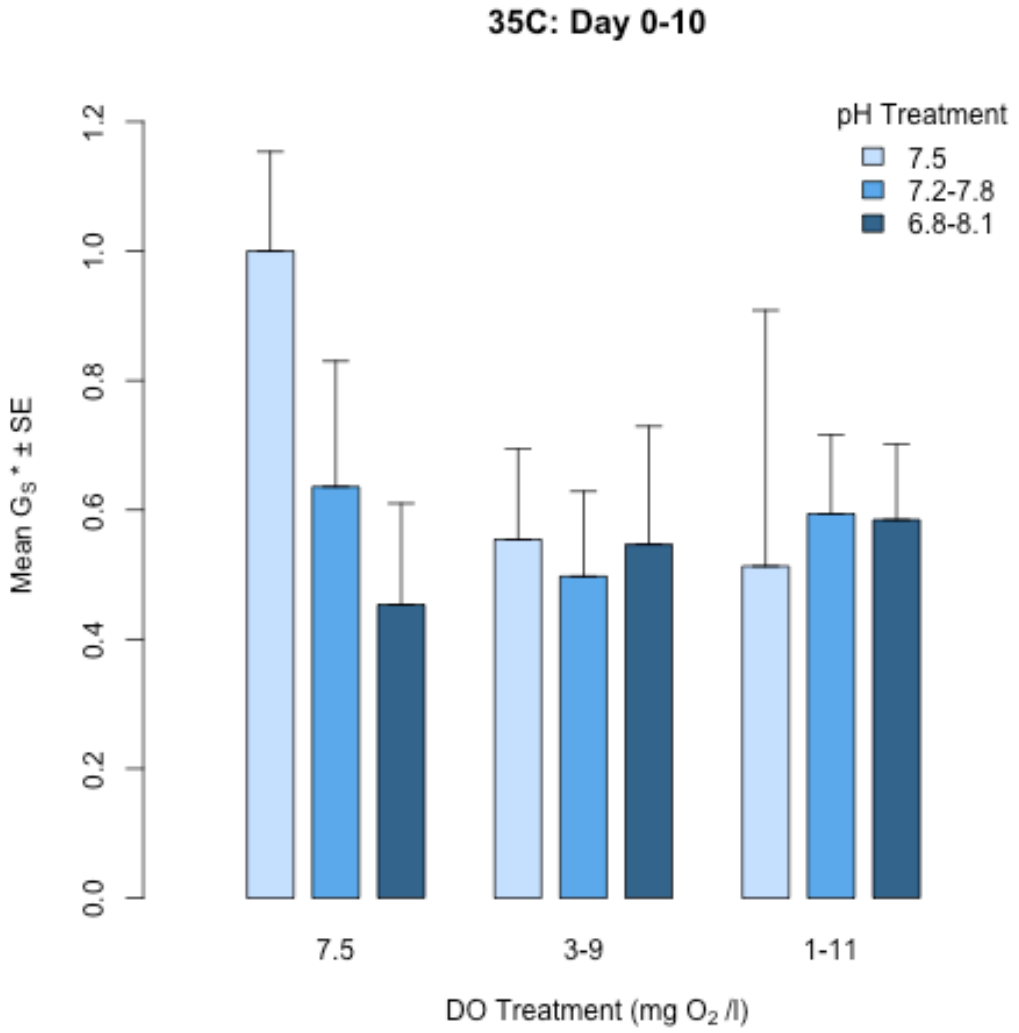

Figure 6: Mean G_S* (G_S relative to control group mean G_S) for each treatment for Days 0-10 at 35°C. No significant differences were found between diel-cycling treatment groups and the control however all treatment groups grew notably slower than the control (all p-values fell between 0.6 and 0.1).

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Appendix A

AQUARIUM SYSTEM

Below are details on the computer-controlled, recirculating aquarium apparatus (Figure A1) used to monitor growth rates of *F. heteroclitus* under diel-cycling DO/pH treatments.

Dissolved oxygen and pH were controlled in five separate recirculating treatment systems. Each system consisted of an oblong tray (L~208.25 x W~68.5 x H~31.75; Vol. ~415 l) within which were ten replicate polyethylene tanks (18 l). A centrifugal pump constantly propelled water from a polyethylene sump to supply the replicate tanks via a distribution manifold inside the tray. Thus, each replicate chamber received a steady supply of water. The replicate tanks were continuously supplied with water, which overflowed into the tray. Holes drilled near the top of each replicate tank ensured that water levels in the tanks were always higher than the level in the tray; therefore, there was constant water flow from the replicate tanks into the tray. One end of the tray was fitted with a large bulkhead fitting through which water overflowed via a 4" PVC pipe into the sump where it was again propelled to the replicate tanks. In this way constant recirculation within the system was achieved. The treatment tray was fitted with glass lids, which could be lifted for feeding and cleaning of the replicate tanks. A port positioned over the sump allowed the input of

compressed gases used to adjust and control the dissolved oxygen and pH. Gases could be selectively introduced to alter the air in the system and lids were fitted with gasket material to ensure that the gas mixture within the system was sequestered from the atmosphere (Figure 1A).

A LabVIEW program enabled the investigator to specify a desired DO as well as a desired pH. The desired DO and pH values for all treatments, as well as any desired patterns of either static or cycling in DO and pH were initialized before the experiment begins and stored in a file for use by the program. The desired DO/pH could be held constant for static treatment, or programmed to change in half hour intervals to create a desired pattern of diel fluctuation.

For each treatment system, the program began by first actuating solenoid valves which allowed the water in the system to flow over the sensing surfaces of a Hach LDO dissolved oxygen probe and a differential pH/ORP sensor. When a stable reading of DO and pH was attained, it was compared with the desired values. Whenever DO or pH deviated from desired values, the program actuated appropriate solenoid gas valves to alter the DO and pH of the water and compensate for the deviation. Compressed N_2 was injected to strip dissolved oxygen and reduce DO; compressed oxygen was injected to raise DO. Likewise, compressed CO_2 was injected to reduce pH and air was bubbled in the sump tank to raise pH.

Because compressed air was used to raise pH the water became saturated with DO whenever pH was being increased. Whenever both supersaturated levels of DO and increased pH were desired it was necessary to first increase pH before

supersaturation of DO could be achieved (see Appendix B for graphs of DO/pH levels over time for each treatment). Bicarbonate/carbonate salts were added until the carbonate alkalinity of the water reached 8 dKh to maintain pH of stability the water. This was equivalent to the carbonate alkalinity of water sampled from Canary Creek where the *F. heteroclitus* were collected.

The sequence described above for a single treatment system was sequentially repeated to maintain DO/pH control in all five systems. The program and apparatus operated continuously as it sequentially measured and adjusted DO and pH thus providing prolonged control throughout the experiments. The resulting patterns of actual DO and pH values together with the desired DO and pH values were collected by the program and saved as a .csv file.

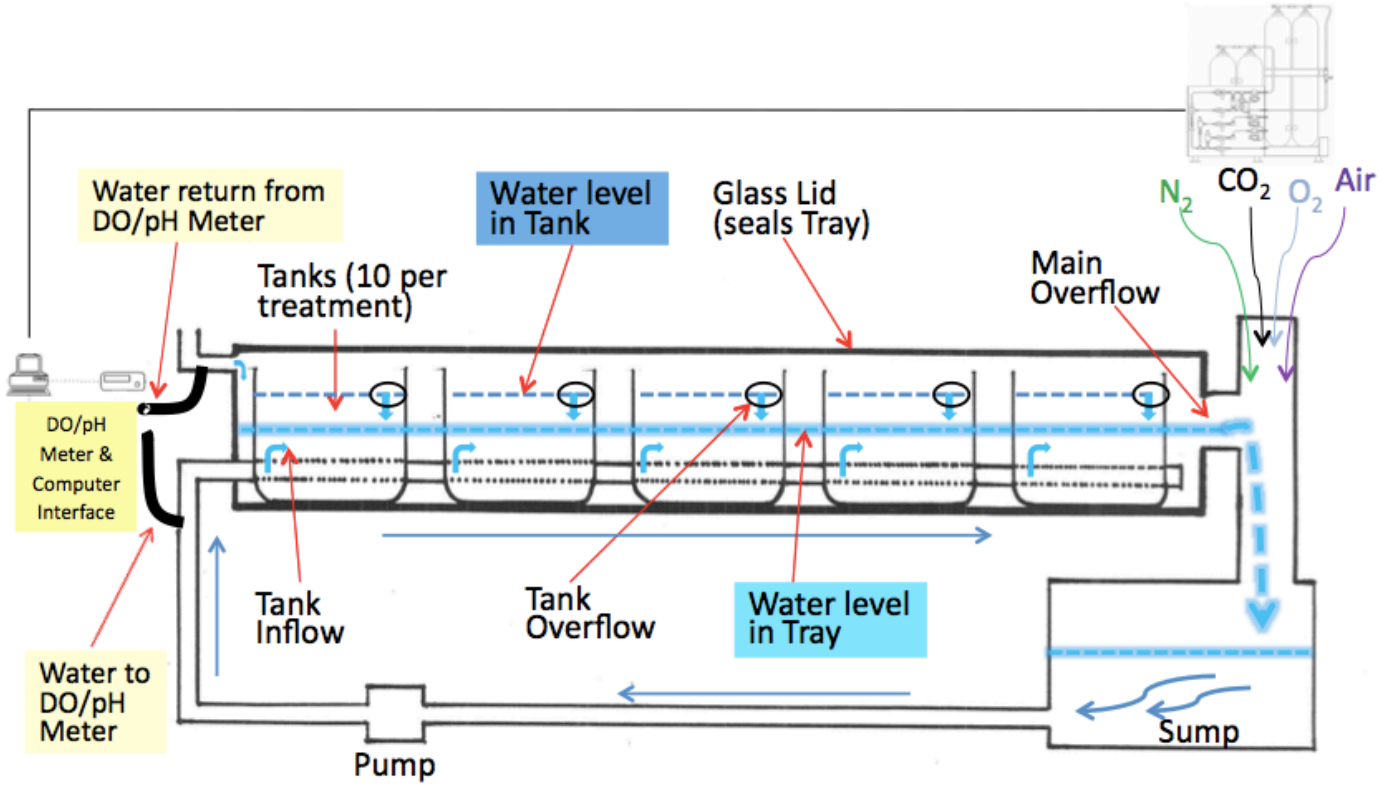

Figure A1: A side-view representation of a single aquarium component of the computer-controlled recirculating aquarium system. Water flows from the sump, driven by the pump to the tank inflow where it is dispersed into 10 replicate holding tanks (2 rows of 5 tanks within each tray). Then tanks drain through the tank overflow into the surrounding tray, which drains back to the sump. When LabVIEW initiates water sampling, a series of solenoids open, allowing water to flow through a line past the DO/pH Meter and back into the tray. The DO/pH Meter, interfaced with the PC and LabVIEW, takes a reading and the program determines which combination of gases (N_2, O_2, CO_2, or air) to bubble into the sump water. There is a single continous atmosphere between the sump and the tray; glass lids seal the top of the tray, isolating the interior atmosphere save for a small relief valve.

Appendix B

TREATMENT READINGS

The following pages contain figures detailing the actual DO and pH records (over a 24 hr cycle) as compared to the programmed target values for each of the nine growth trial treatments.

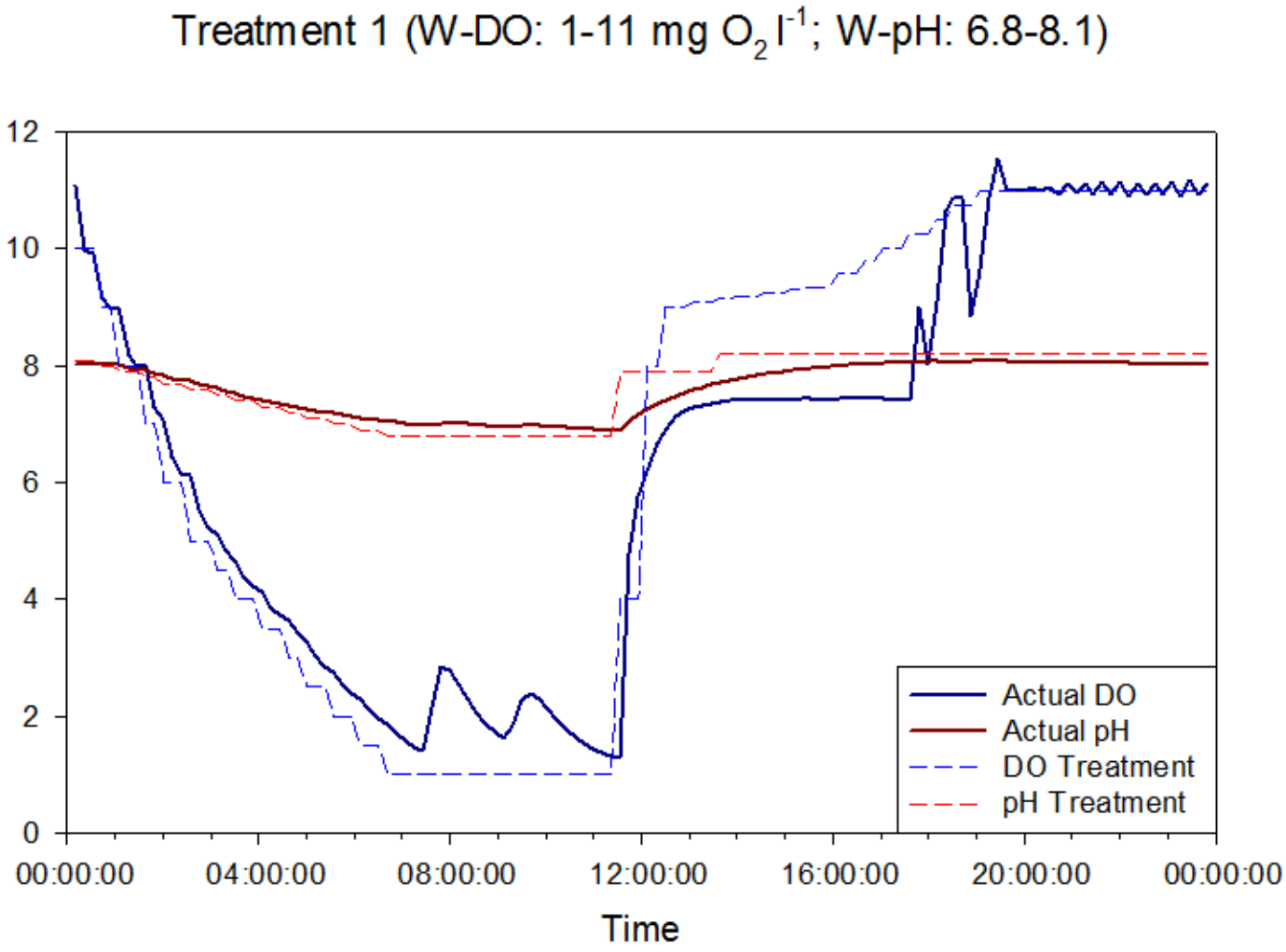

Figure B1: Treatment 1 (W-DO/W-pH); actual recorded and targeted values over 24 hrs.

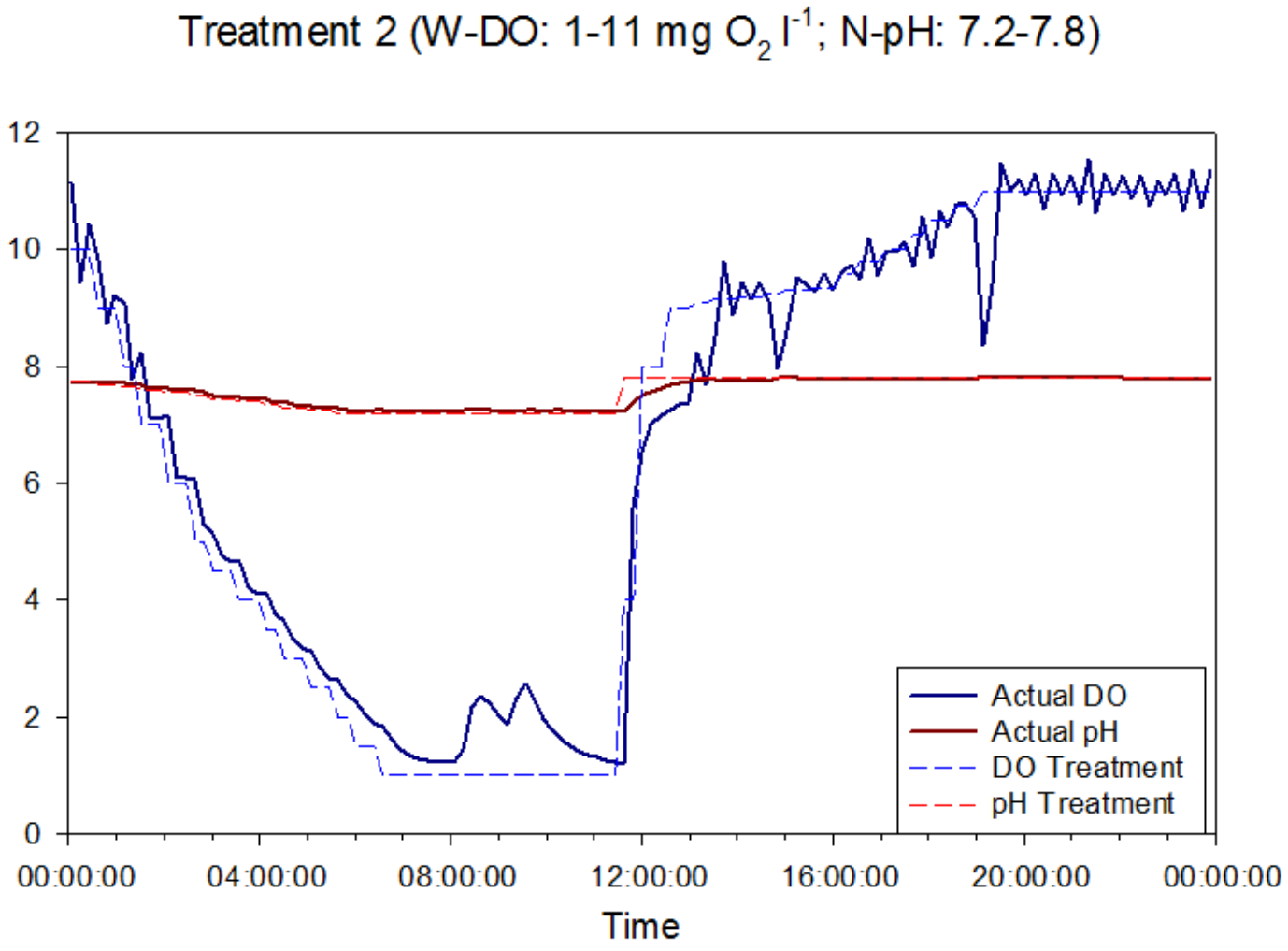

Figure B2: Treatment 2 (W-DO/N-pH); actual recorded and targeted values over 24 hrs.

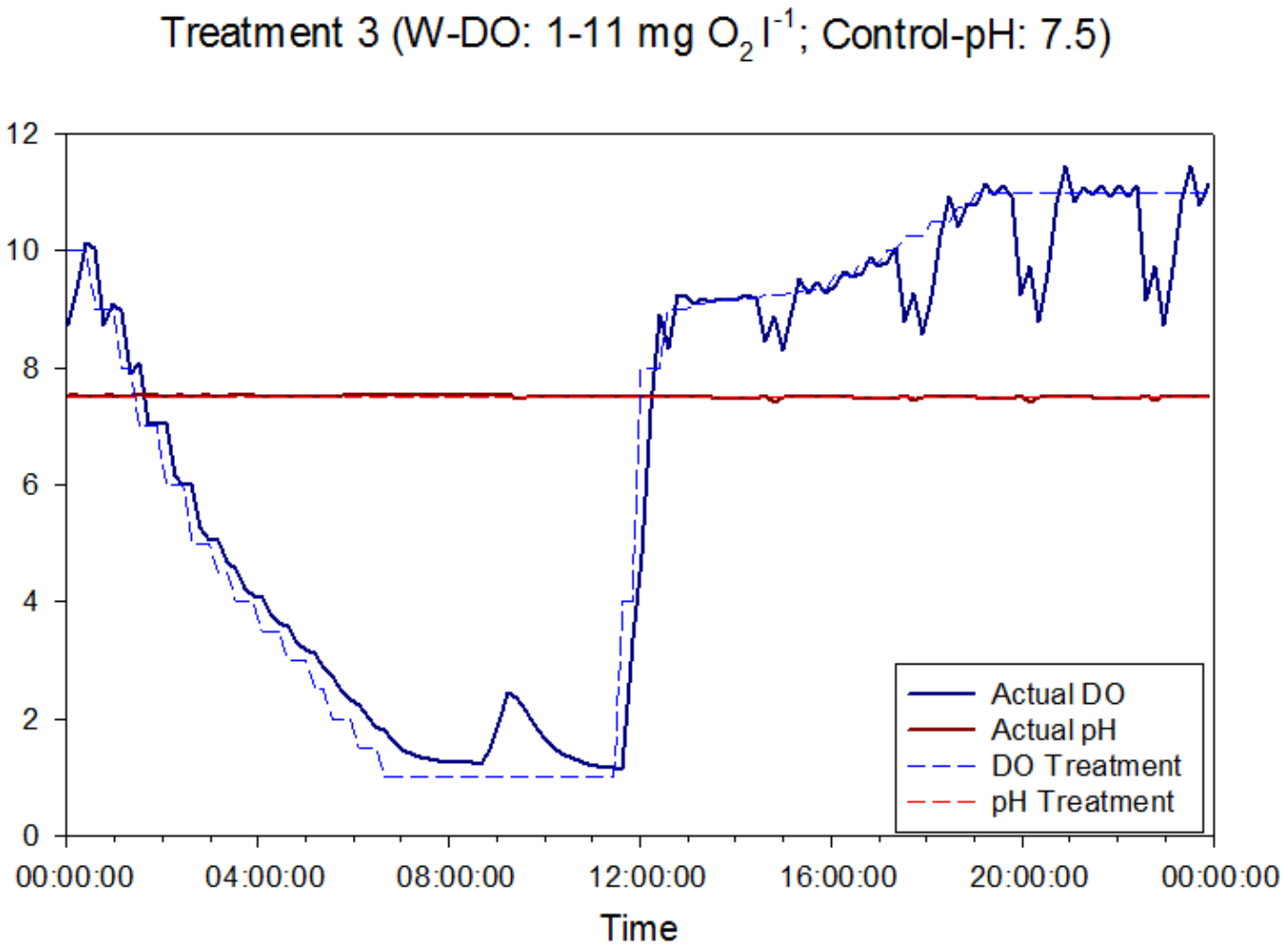

Figure B3: Treatment 3 (W-DO/Control-pH); actual recorded and targeted values over 24 hrs.

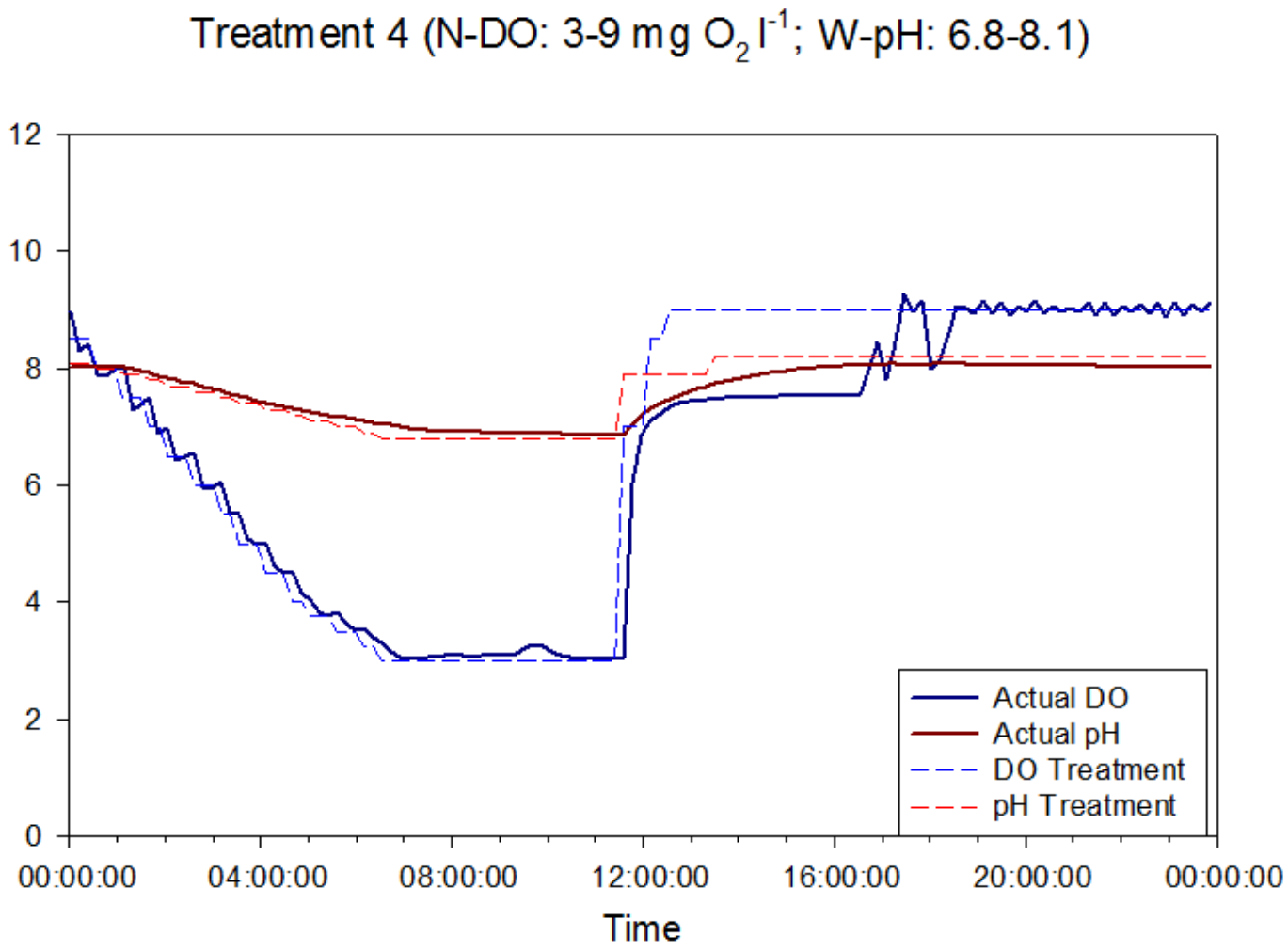

Figure B4: Treatment 4 (N-DO/W-pH); actual recorded and targeted values over 24 hrs.

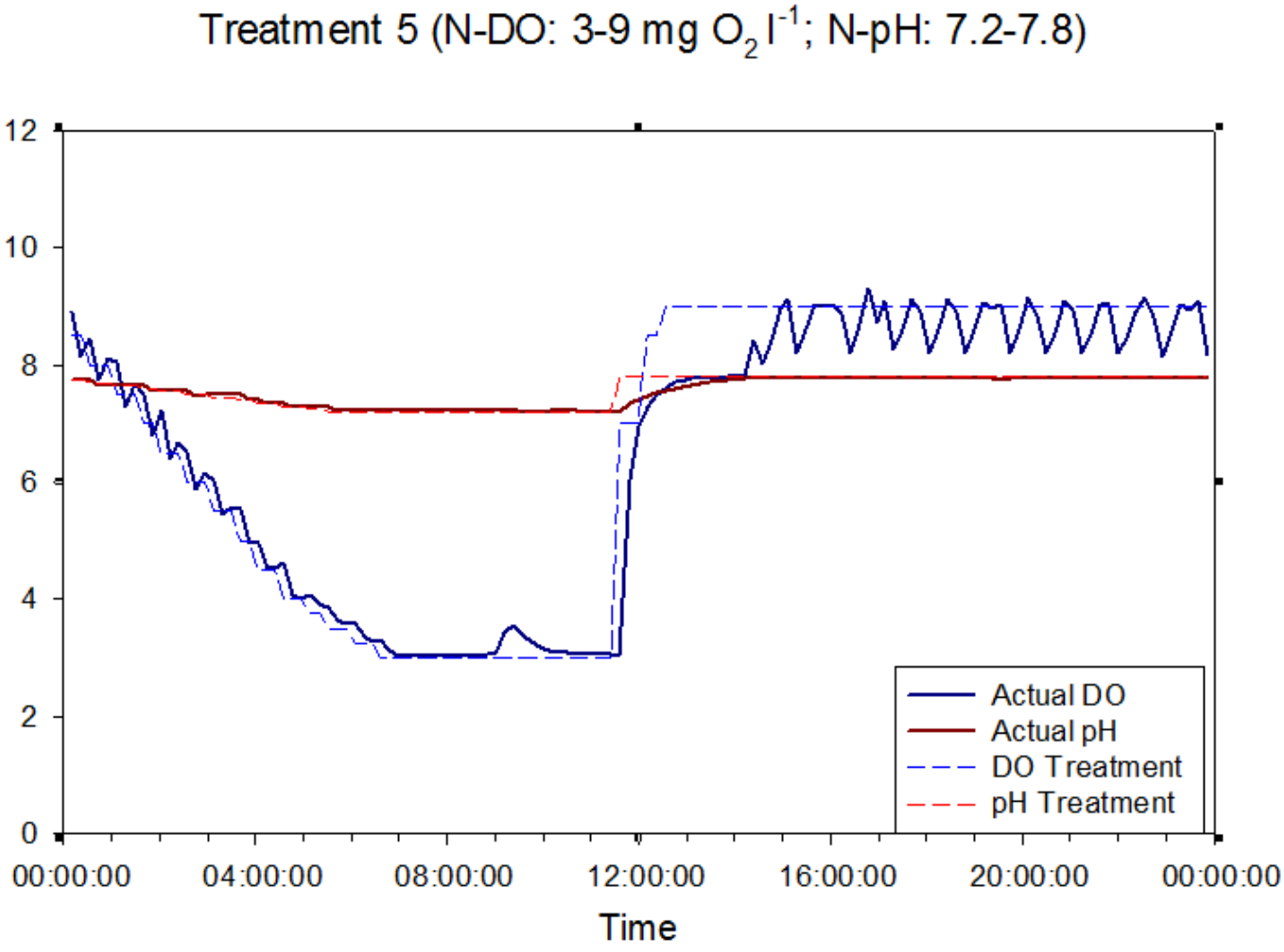

Figure B5: Treatment 5 (N-DO/N-pH); actual recorded and targeted values over 24 hrs.

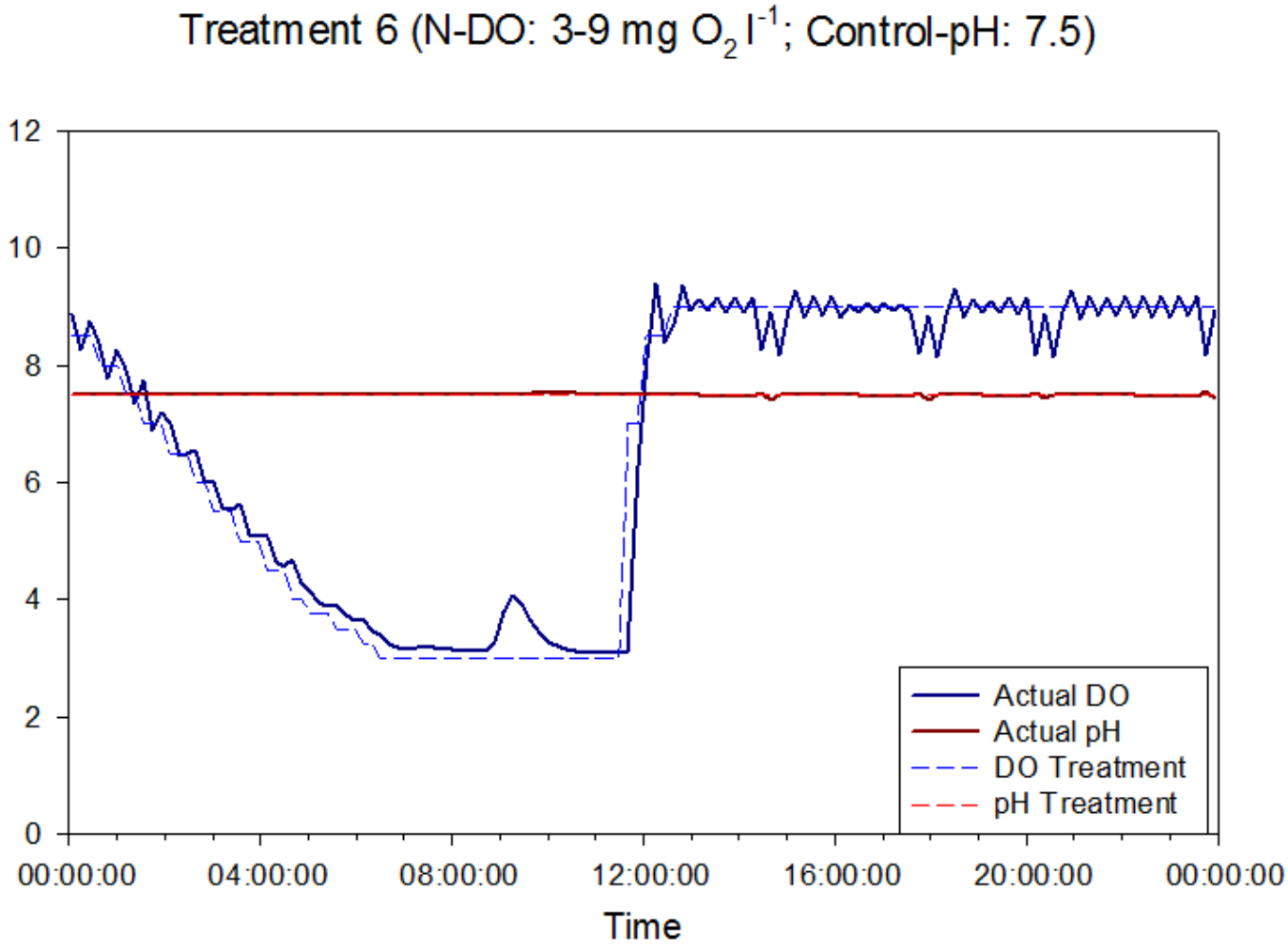

Figure B6: Treatment 6 (N-DO/Control-pH); actual recorded and targeted values over 24 hrs.

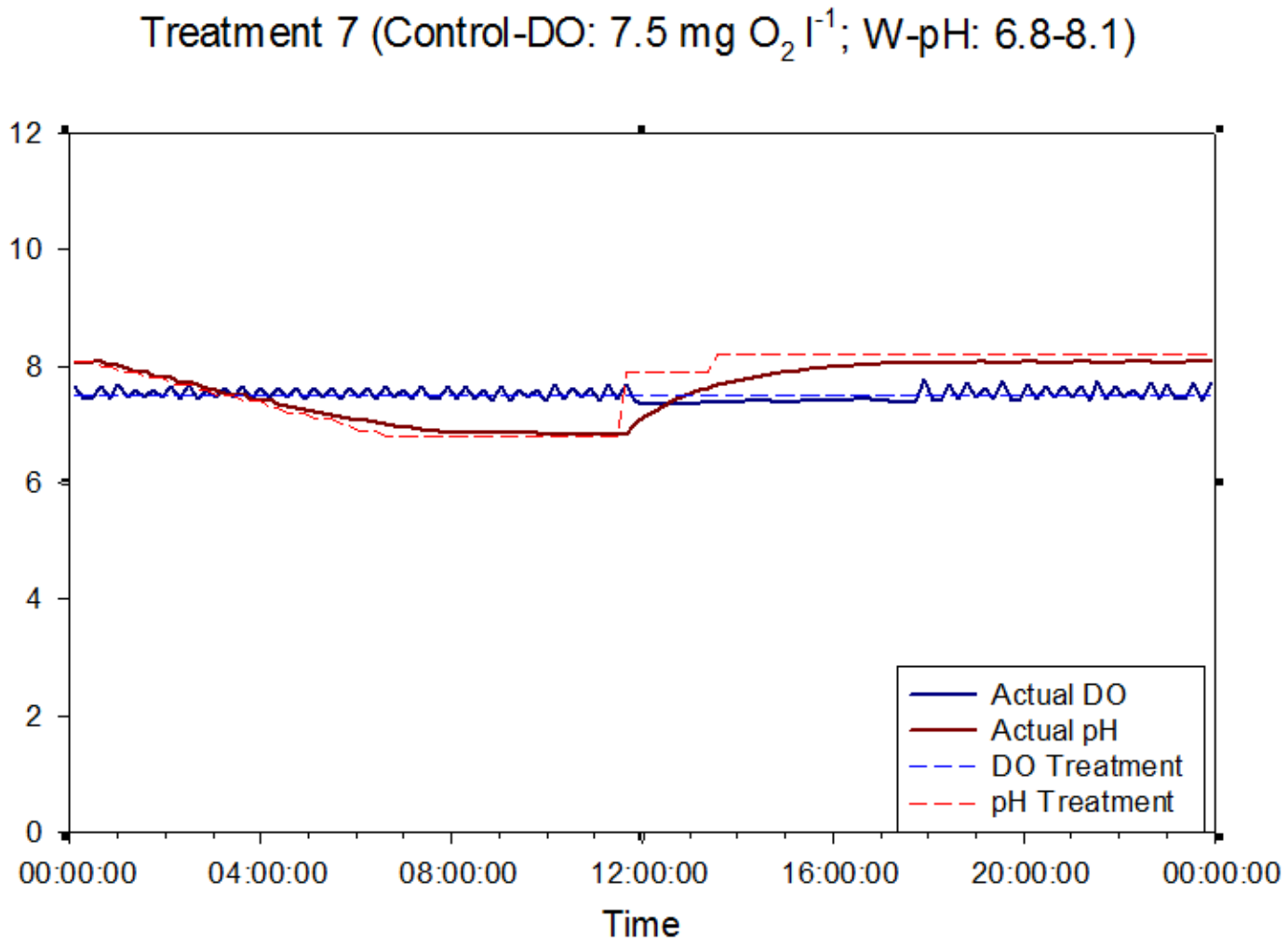

Figure B7: Treatment 7 (Control-DO/W-pH); actual recorded and targeted values over 24 hrs.

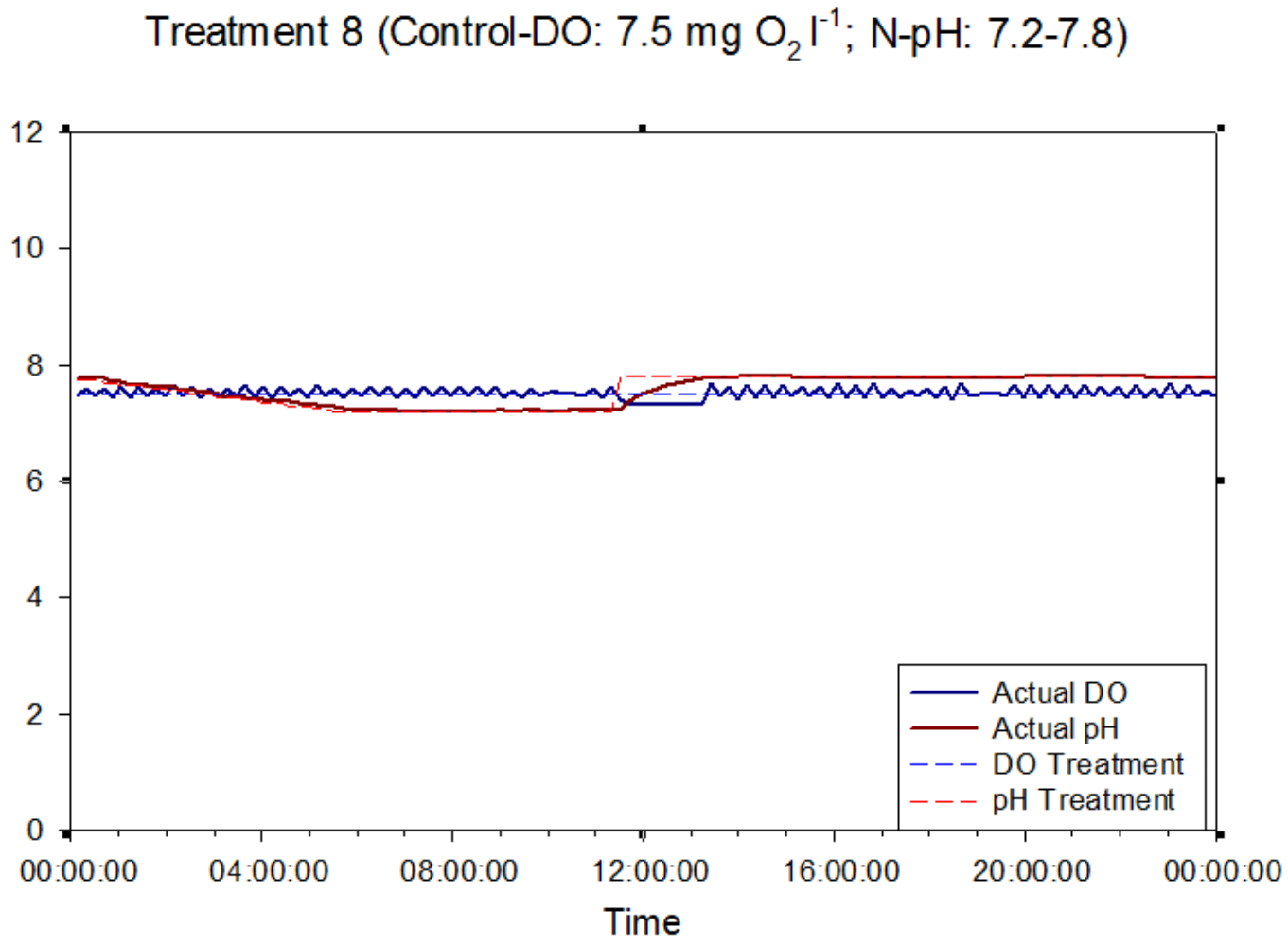

Figure B8: Treatment 8 (Control-DO/N-pH); actual recorded and targeted values over 24 hrs.

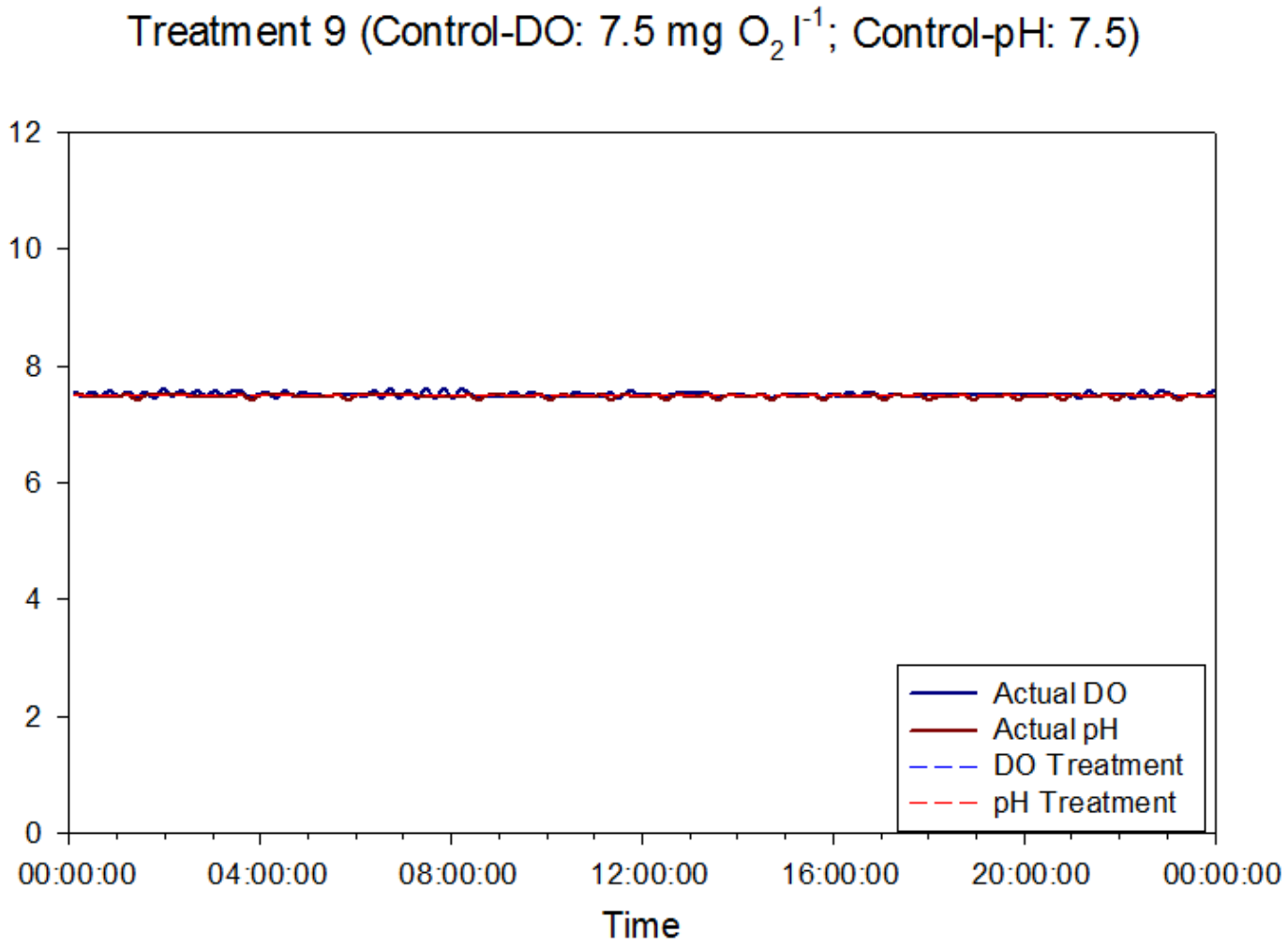

Figure B9: Treatment 9 (Control-DO/Control-pH); actual recorded and targeted values over 24 hrs.