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The effects of carbon dioxide on performance and histopathology of rainbow trout *Oncorhynchus mykiss* in water recirculation aquaculture systems

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ABSTRACT

Chronic exposure to elevated levels of dissolved carbon dioxide (CO_2) has been linked to reduced growth, physiological disturbances and negative health outcomes in intensively reared fish. Although pumping to a degassing tower can lower concentrations of dissolved CO_2 in water recirculation aquaculture systems (WRAS), pumping can be a significant cost for operators. A 6-month trial was conducted to compare the effects of high (24 ± 1 mg/L; partial pressure = 8.79 mm Hg) and low (8 ± 1 mg/L; partial pressure = 2.91 mm Hg) dissolved CO_2 concentrations on rainbow trout *Oncorhynchus mykiss* performance and health in replicated WRAS operated at low-exchange rates (0.26% of the total recirculating flow). Rainbow trout (62 ± 1 g) were randomly stocked into six replicated WRAS and into three small tanks within a flow-through system to provide a physiological comparison. All study fish were maintained at densities between 25 and 80 kg/m^3, at water temperatures of approximately 13–14 °C, and at dissolved oxygen concentrations of approximately saturation. A 24-h photoperiod was provided and all fish groups were fed equal portions every 2 h during the study period. Fish health and performance were assessed with daily mortality and monthly length and weight data collection, as well as multiple tissue samplings for histopathological assessment. At the study's end, percentage survival for both groups was high (>97%). No significant ($p < 0.05$) differences in growth or survival were observed between CO_2 treatments. No nephrocalcinosis or related pathologies were noted. Skin and gill pathologies were common in both treatment groups; however, there were few statistically significant differences between groups for any of the tissue types evaluated: high CO_2 fish were more likely to exhibit lymphocytic portal hepatitis, while the low CO_2 treatment group exhibited greater gill epithelial hyperplasia. None of the pathologies observed were substantive or likely to cause mortality. The results of this study indicate that raising rainbow trout to market size in WRAS with CO_2 concentrations of 24 mg/L does not significantly affect their overall health and performance.

1. Introduction

Fish produce carbon dioxide (CO_2) as a normal outcome of aerobic metabolism and excrete this gas through the gills into the surrounding water. Dissolved CO_2 levels can become elevated in water recirculation aquaculture systems (WRAS) that use pure oxygen supplementation, as these systems support high feed loading rates, the biofilters produce free acid that results in a significant net increase of dissolved CO_2 (Summerfelt and Sharrer, 2004), and degassing techniques are sometimes insufficient in stripping the quantities of CO_2 produced (Summerfelt et al., 2000). Fish avoid areas of high dissolved CO_2 (Clingerman et al., 2007) and previous research using salmonids has demonstrated that elevated dissolved CO_2 can be associated with reduced feed intake (Smart, 1981), reduced growth (Danley et al., 2005; Fivelstad et al., 2007), reduced condition factor (Fivelstad et al., 1998, 2003a,b), and nephrocalcinosis (Landolt, 1975; Fivelstad et al., 1999; Hosfeld et al., 2008), presumably due to stress, hypercapnia, i.e. elevated blood CO_2, the resultant acidosis, and impaired oxygen–hemoglobin binding (Colt et al., 1991; Wedemeyer, 1996). Recommended upper limits for dissolved CO_2 in intensive salmonid aquaculture usually range between 10 and 20 mg/L (Boyd, 1979; Portz et al., 2006; MacIntyre et al., 2008), although other species can tolerate much higher CO_2 levels in their rearing environment (Crocker and Cech, 1998). Establishing species-specific CO_2 limits is complicated by various factors that influence the toxicity of this gas, such as oxygen saturation (Wedemeyer, 1997; Hosfeld et al., 2008), alkalinity (Eshchar et al., 2006) and water temperature (Fivelstad et al., 2007); metals (e.g. aluminum) can become more soluble, and therefore more available to exert

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toxic effects, in high CO_2 water due to the resultant decrease in pH (Fivelstad et al., 2003b).

Although the potential fish health and performance benefits from raising stocks in low dissolved CO_2 environments may be attractive for producers, the economics of lowering CO_2 in WRAS must also be considered. Assuming all else to be equal, the dissolved CO_2 concentration allowed to accumulate in culture tanks is inversely proportional to the water flow rate that exchanges through the tank. Pumping more water flow through a culture tank to decrease dissolved CO_2 levels in the tank will increase the variable cost and to a lesser extent the fixed equipment cost in inverse proportion to the drop in dissolved CO_2 concentration that is required (Summerfelt and Vinci, 2004). It is therefore more expensive to operate at lower culture tank CO_2 concentrations, which would only be beneficial if lower concentrations resulted in improved fish health and performance.

Previous observations at The Freshwater Institute have indicated that in high feeding and low flushing WRAS (i.e. 1.3–2.0 kg feed/day per m^3/day of makeup water flow), rainbow trout *Oncorhynchus mykiss* mortality increases due to undetermined causes. This elevated mortality could represent an important barrier to operating WRAS with high feeding rates during periods of limited makeup water availability, but research aiming to understand this mortality phenomenon has not been successful in reproducing the initial mortality event (Good et al., 2009). The study mentioned was performed at CO_2 concentrations averaging 10 mg/L; however, during the initial mortality event CO_2 concentrations were >20 mg/L, and therefore CO_2 toxicity alone or in combination with other water quality parameters (fine suspended solids and/or dissolved organic matter or metals accumulated in WRAS) could have caused the previously observed decline in fish health, and thus warranted investigation. Danley et al. (2005) reported that dissolved CO_2 concentrations of 34.5 ± 3.8 mg/L decreased rainbow trout growth compared to 22.1 ± 2.8 mg/L of CO_2; however, the authors did not evaluate lower dissolved CO_2 concentrations, and furthermore these studies were carried out in flow-through systems as opposed to WRAS, which could explain why overall fish survival was not affected at these CO_2 levels.

The objective of the present study was to assess rainbow trout performance and health in relation to elevated (24–25 mg/L) dissolved CO_2 concentrations in low-exchange WRAS.

2. Materials and methods

The replicated WRAS used for experimentation at The Freshwater Institute have been described in detail elsewhere (Davidson et al., 2009). Briefly, six identical 9.5 m^3 WRAS, with 5.3 m^3 circular dual-drain tanks, fluidized sand biofilters, degassing columns placed over low head oxygenators (LHOs), and 60-μm drum filters, were employed in this study. Total recirculation flow was set at 380 L/min, with water exchange rate controlled at 0.26% of the total recirculation flow; total system volume was exchanged once every 6.7 days. Three WRAS were randomly selected to receive additional CO_2 to attain dissolved concentrations >20 mg/L, and this was achieved by co-transferring pure CO_2 feed gas with O_2 feed gas within the LHO. Dissolved CO_2 in these systems was increased slowly over a 2-week period after stocking for acclimation; final dissolved CO_2 levels were 24 ± 1 mg/L. The remaining three WRAS received no additional CO_2, such that levels in these systems were at normal, low levels (8 ± 1 mg/L). In addition to the six experimental WRAS, three small (0.5 m^3) circular tanks within a flow-through system in a separate building were used for a comparison fish group. Although no 5.3 m^3 flow-through tanks were available for comparison, fish were maintained in these smaller tanks at identical densities and feeding rates as the six experimental WRAS. All WRAS makeup water, as well as water supplying the flow-through comparison tanks, originated from a common spring source.

Rainbow trout, acquired as eyed eggs from a commercial producer from single spawn, and incubated and raised on-site at The Freshwater Institute, were stocked at 63.7 ± 1.2 g in size (800 fish per WRAS and 100 fish per flow-through comparison tank) and were maintained for 6 months at a density range of 25–80 kg/m^3. Throughout the study period, a constant 24-h photoperiod was provided. All fish groups were fed equal portions (every second hour) using automated feeders (T-drum 2000CE, Arvo-Tec, Finland). Feeding rates were based on standardized feeding charts and on observations of feeding activity and wasted feed. A slow-sinking trout feed (Zeigler Brothers, Inc., Gardners, PA, USA) with a 42:16 protein-to-fat ratio was used. Mean feed loadings of 4.1 kg/day per m^3/day makeup water were maintained for the high and low CO_2 treatments.

Fish performance was assessed through monthly length and weight samplings, and mortalities were removed and recorded daily to assess survival in each tank using the following formula:

$$\frac{\text{initial number} - \text{cumulative mortalities}}{\text{initial number}} \times 100$$

Length/weight sample size ranged from 50 to 120 fish and was calculated with the following formula:

$$n = (Z \times (\text{stdev}_{.\text{g}}/\text{accepted error}_{\text{g}}))^2$$

where $Z = 1.65$ (relative to a 90% confidence interval), and the accepted error was 5 g. From the length and weight data, growth curves were generated, feed conversion was assessed, and thermal growth coefficients were calculated using the following formula (Iwama and Tautz, 1981):

$$\frac{\text{FBW}^{1/3} - \text{IBW}^{1/3}}{\sum[T \times D]} \times 100$$

where FBW, final body weight; IBW, initial body weight; *T*, water temperature; and *D*, number of days. Differences in growth between the two treatments were assessed statistically in SYSTAT (Cranes Software International, Bangalore) using a repeated measures approach. Survival was assessed with a Kruskal–Wallis test due to unequal variances in mortality data between the high and low CO_2 groups.

Weekly water samples were collected from the side drain of each WRAS and from the outflow of each flow-through comparison tank. Each water sample was assessed for the following parameters: alkalinity, CO_2, carbonaceous biochemical oxygen demand ($cBOD_5$), dissolved organic carbon (DOC), nitrite nitrogen, nitrate nitrogen, phosphorous, true color, total ammonia nitrogen (TAN), total organic carbon (TOC), total suspended solids (TSS), and UV transmittance. Weekly particle analyses (counts and size distributions) were carried out using a 2200 PCX Particle Counter (Hach Company, Loveland, CO, USA). The water quality parameters listed above were measured in accordance with the methods described in APHA (2005) and Hach (2003). Dissolved oxygen, pH, and temperature were monitored on a continuous basis employing a SC100 Universal Controller (Hach Company, Loveland, CO, USA). To prevent depletion of alkalinity in the WRAS over time, sodium bicarbonate ($NaHCO_3$) was added to each system at 0.15 kg $NaHCO_3$ per kg feed. All water quality measurements taken throughout the study (41 separate samplings at regular intervals) were averaged to create grand means for each WRAS, and statistical analyses (ANOVA) were carried out to determine significant water quality differences between the WRAS high and low CO_2 groups ($n = 3$ grand means for each treatment). Flow-through tank water quality data were collected for descriptive comparison only.

To assess general fish health, samples of six separate tissues were collected from five fish per tank at the study's end, and were preserved in histological grade 10% formalin solution (Fisher Scientific) for 1 week prior to shipment to the Washington Animal Disease Diagnostic Laboratory (Pullman, WA) for processing and histopathological evaluation. Sampled tissues were skin, gill, heart, liver, spleen, and anterior and posterior kidney, with all tissues sampled at the same anatomical region within a given fish, e.g. all gills were sampled on the left side, taking the middle third of the second gill arch. The evaluating pathologist was blinded to the treatment group origins of all sampled specimens. A 0–5 point grading scale was developed to quantify the severity of each lesion type, with 0 representing normal tissue and 5 representing lesions affecting essentially 100% of the tissue examined (Dr. Kevin Snekvik, Washington Disease Diagnostic Laboratory, pers. comm.). Histopathological data for each tissue type and for each specific lesion observed were analyzed with STATA 9 software (StataCorp, College Station, TX) using bivariable ordered logistic regression models, with treatment (high or low CO_2) as the independent variable in each model and specific lesion score as the ordinal dependent variables. These regression analyses were repeated to compare fish in WRAS (both high and low CO_2 treatment groups combined) with comparison fish from the flow-through tanks.

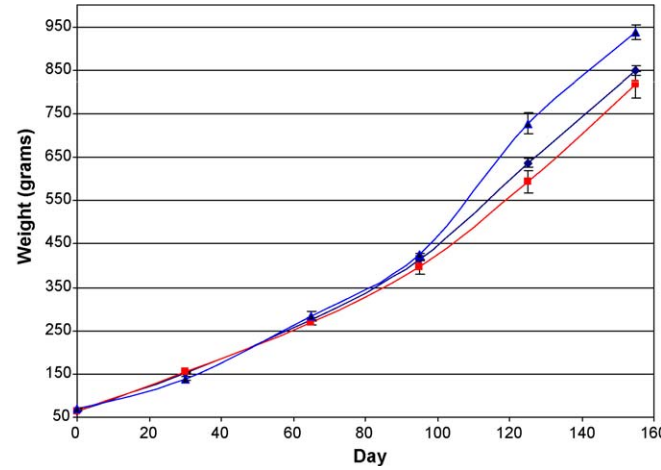

Fig. 1. Growth curve comparison for rainbow trout raised in high (◆) and low (■) CO_2 water recirculation aquaculture systems and in flow-through tanks (▲) during the study period.

3. Results

Differences in water quality between the high and low CO_2 treatment groups were related to experimental treatment: higher CO_2 WRAS had significantly ($p < 0.05$) higher dissolved CO_2, with resultant (significantly) lower pH and unionized ammonia (Table 1). All other measured water quality parameters were within published safe ranges for raising salmonids.

Mean final rainbow trout weights (331 days post-hatch) for the high and low CO_2 conditions were not significantly different: 849 ± 7 and 817 ± 43 g, respectively (Fig. 1). Flow-through comparison fish had a final weight of 938 ± 17 g. Feed conversion ratios (FCR) for the high and low CO_2 treatment groups were 1.25 ± 0.10 and 1.25 ± 0.05, respectively, and calculated thermal growth coefficients (TGC) for these two treatments were 2.56 ± 15 and 2.55 ± 12, respectively; neither FCR nor TGC was significantly different between the two treatments. There was no significant difference in mortality between treatments, with mean survival for the high and low CO_2 treatments being 98.3 ± 0.2 and 96.5 ± 1.8%, respectively. Flow-through comparison fish had perfect (100%) survival over the study period (which is anomalous compared to previous on-site fish survival in flow-through systems).

Analyses of histopathology evaluations revealed statistically significant differences in the prevalences of two specific lesion types: fish from the high CO_2 cohort were more likely to exhibit portal lymphocytic hepatitis, while fish from the low CO_2 group were more likely to have branchial epithelial hyperplasia (Table 2). The remaining lesions noted in the skin, gill, heart, liver and kidney were not significantly different between the two treatment groups, and all pathologies noted were subclinical in nature. In comparing sampled fish from the WRAS (both treatment groups combined) and flow-through tanks, hyperplasia of the gill epithelium was more prevalent in the WRAS group, while other specific subclinical pathologies displayed no significant differences in prevalence between the recirculation and flow-through fish (Table 3).

4. Discussion

The major outcome of this study was a demonstration that rainbow trout are able to perform and survive approximately as well in an elevated (24 mg/L) CO_2 WRAS environment compared to trout raised in lower (8 mg/L) CO_2 systems (partial pressures of 8.79 and 2.91 mm Hg, respectively (Colt, 1984)). Reduced growth and feed intake, as reported elsewhere (Smart, 1981; Danley et al., 2005), were not observed in our rainbow trout raised to market size while exposed to a constant 24 mg/L dissolved CO_2. The results of this study therefore suggest that pumping water to decrease dissolved CO_2 levels by decreasing tank hydraulic retention time in WRAS is, to an extent, unnecessary when considering rainbow trout performance if tank CO_2 levels remain below 24 mg/L, and if other water quality parameters (e.g. temperature, dissolved oxygen, and alkalinity) are similar to those reported in this study.

Table 1
Summary of measured water quality parameters in the high and low CO_2 treatment groups and flow-through comparison tanks.

Parameters	High CO_2 ($n=3$)	Low CO_2 ($n=3$)	Flow-through ($n=3$)
TAN (mg/L)	0.35 ± 0.01	0.35 ± 0.02	0.18 ± 0.00
Unionized ammonia (mg/L)*	0.001 ± 0.000	0.004 ± 0.000	0.001 ± 0.000
Nitrite nitrogen (mg/L)	0.054 ± 0.013	0.051 ± 0.008	0.019 ± 0.005
Nitrate nitrogen (mg/L)	46 ± 0	50 ± 2	3 ± 0
Alkalinity (mg/L)	202 ± 2	202 ± 2	267 ± 2
CO_2 (mg/L)*	24 ± 1	8 ± 1	9 ± 1
$cBOD_5$ (mg/L)	5 ± 0	6 ± 1	1 ± 0
True color (Pt–Co units)	42 ± 1	48 ± 3	3 ± 0
UV transmittance (%)	70 ± 0	67 ± 1	98 ± 0
TSS (mg/L)	7.99 ± 0.37	9.21 ± 1.30	2.23 ± 0.19
Total particles (0–60 μm)	1.8×10^4	2.3×10^4	4.4×10^3
Temperature (°C)	13.9 ± 0.1	13.7 ± 0.1	12.9 ± 0.0
pH*	7.17 ± 0.01	7.61 ± 0.03	7.61 ± 0.00
DO (mg/L)	10.1 ± 0.0	10.1 ± 0.0	10.5 ± 0.0

* Significant ($p < 0.05$) differences between high and low CO_2 systems.

Table 2
Summary of histological lesions observed in sampled tissues from high and low CO_2 treatment groups, and results of statistical analysis using ordered logistic regression.

Tissue/lesion type	Lesion prevalence		Odds ratio (95% C.I.)	*p*-Value
	High CO_2	Low CO_2		
Skin (all lesions)	11/15	10/14	1.10 (0.21, 5.86)	0.911
Lymphocytic dermatitis	1/15	1/14	0.93 (0.07, 12.7)	0.956
Lymphocytic epidermitis	11/15	10/14	1.64 (0.26, 10.4)	0.597
Gill (all lesions)	14/15	15/15	–	–
Lymphocytic branchitis	14/15	15/15	–	–
Epithelial hyperplasia	5/15	9/15	0.31 (0.11, 0.89)	0.030
Epithelial hypertrophy	0/15	3/15	–	–
Capillary thrombosis	0/15	1/15	–	–
Heart (all lesions)	4/15	4/15	1.00 (–)	1.000
Lymphocytic epicarditis	4/15	4/15	0.91 (0.42, 1.97)	0.818
Lymphocytic/histocytic myocarditis	1/15	1/15	–	–
Liver (all lesions)	9/15	8/15	1.31 (0.50, 3.45)	0.581
Lymphocytic hepatitis, portal	9/15	5/15	3.00 (1.11, 8.11)	0.030
Lymphocytic hepatitis, random	0/15	1/15	–	–
Hepatic lipidosis	0/15	3/15	–	–
Kidney (all lesions)	3/15	5/15	0.50 (0.04, 5.68)	0.576
Tubular hydropic degeneration	2/15	4/15	0.42 (0.05, 3.49)	0.424
Increased melanomacrophages	1/15	1/15	0.93 (0.07, 13.2)	0.958

Table 3
Summary of histological lesions observed in sampled tissues from water recirculation aquaculture systems (high and low CO_2 treatment groups combined) and flow-through comparison tanks, and results of statistical analysis using ordered logistic regression.

Tissue/lesion type	Lesion prevalence		Odds ratio (95% C.I.)	*p*-Value
	Recirc.	Flow-through		
Skin (all lesions)	21/30	15/15	–	–
Lymphocytic dermatitis	2/30	0/15	–	–
Lymphocytic epidermitis	21/30	15/15	0.19 (0.03, 1.04)	0.055
Gill (all lesions)	29/30	15/15	–	–
Lymphocytic branchitis	29/30	15/15	–	–
Epithelial hyperplasia	14/30	4/15	2.47 (1.03, 5.95)	0.043
Epithelial hypertrophy	3/30	1/15	1.64 (0.22, 12.0)	0.626
Capillary ectasia	0/30	1/15	–	–
Capillary thrombosis	1/30	2/15	0.22 (0.02, 2.01)	0.182
Heart (all lesions)	8/30	6/15	0.55 (0.22, 1.36)	0.193
Lymphocytic epicarditis	8/30	5/15	0.77 (0.20, 2.94)	0.703
Lymphocytic/histocytic myocarditis	1/30	1/15	0.51 (0.03, 7.42)	0.621
Liver (all lesions)	17/30	6/15	1.96 (0.76, 5.05)	0.162
Lymphocytic hepatitis, portal	14/30	5/15	1.75 (0.52, 5.84)	0.362
Lymphocytic hepatitis, random	1/30	1/15	0.48 (0.03, 7.01)	0.594
Hepatic lipidosis	2/30	1/15	1.43 (0.17, 12.2)	0.742
Kidney (all lesions)	8/30	6/15	0.55 (0.14, 2.12)	0.381
Tubular hydropic degeneration	6/30	6/15	0.38 (0.11, 1.23)	0.106
Increased melanomacrophages	1/30	1/15	–	–

Reduced variable and fixed costs related to a reduction in water pumping to control dissolved CO_2 could assist in increasing the profitability of commercial facilities employing WRAS to raise rainbow trout.

Numerous histopathological lesions were noted in the tissue types sampled, and it should be noted that the number and range of lesion types observed were typical of other histopathological surveys that the authors have carried out on-site and at other facilities, and represent very low-level pathology in general. Although slight differences in histopathology were noted between the two groups, all lesions detected through tissue evaluations were subclinical and did not affect survival or performance during the 6-month study period. It is interesting to note that nephrocalcinosis, commonly considered to be associated with elevated CO_2 (Harrison, 1979; Hosfeld et al., 2008), was not observed in any of the sampled fish from the high or low CO_2 treatment groups or from the flow-through comparison tanks; typical histological lesions with this condition include renal tubular dilation and degeneration with concretions of calcium salts (Harrison and Richards, 1979). Fivelstad et al. (2007) also reported an absence of observable nephrocalcinosis in Atlantic salmon parr exposed for 47 days to elevated dissolved CO_2 (mean values > 30 mg/L), and therefore the pathogenesis of this condition clearly requires other host (species, life-stage) and, in particular, environmental (diet composition, water chemistry, e.g. hardness) factors beyond elevated CO_2 and resultant acidosis in order to develop, as is suggested in previous studies (e.g. Hicks et al., 1984; Richardson et al., 1985; Dzuvic et al., 1986). Additional data on blood gas and chemistry would have provided useful information to compare with the histological findings of this study; however, a leased (human blood) analyzer employed during the final sampling event failed to reliably measure such parameters using rainbow trout blood samples.

Lymphocytic portal hepatitis was observed to be significantly higher in prevalence in the high CO_2 group relative to the low CO_2 group, although the prevalence of liver lesions in general was not different between these two cohorts. Lymphocytic portal hepatitis is an inflammatory disease of the liver of unknown etiology, and has not been adequately studied in fish, although it is thought to be an autoimmune disorder in certain mammalian species (Day, 1998). The reasons for a significantly higher prevalence of this condition in fish chronically exposed to elevated CO_2 are at present unknown, and as mentioned previously the observed lymphocytic portal hepatitis was subclinical in nature, with no clinical signs (e.g. anorexia, weight loss) being noted during the study period. Likewise, while no signs of respiratory distress were observed in either cohort over the 6-month study, the significantly higher prevalence of gill epithelial hyperplasia in the low CO_2 cohort cannot be adequately explained by the data collected. Gill epithelial hyperplasia is generally an unspecific lesion related to numerous infectious and environmental conditions (e.g. bacterial gill disease, high levels of irritants in the water, etc.), and the resultant increased thickness of the respiratory surface leads to an increased distance for gas and ionic exchange (Ferguson, 1989), which in clinical cases can lead to signs such as increased ventilation rates, lethargy, and flared operculae. The more acidic environment of the high CO_2 WRAS could theoretically result in this pathological response; however, the opposite was observed in this study, and the reasons for this finding are unknown at present.

Finally, it is interesting to note that despite the differences in water quality between the experimental WRAS and the flow-through tanks, the prevalences of a variety of lesions putatively associated with reduced water quality were in fact similar, if not higher, in sampled flow-through fish relative to those sampled from the WRAS (both treatment groups combined). In general, the flow-through comparison tanks had lower TAN, nitrite, TSS and total particles, yet fish from these tanks demonstrated a comparable or higher prevalence of lesions (all lesion types combined) in tissues that directly contact the water (i.e. gill and skin). This finding demonstrates that the relatively poor water quality in low-exchange WRAS is not in itself necessary to produce skin and gill inflammation, and that either unmeasured water quality parameters common to both WRAS and flow-through systems, or common physiological changes (e.g. response to diet) in both cohorts, may have been related to the histological lesions observed. In any case, the comparable pathologies noted in fish from WRAS and flow-through tanks are a novel finding and warrant further investigation to understand why these lesions types also occur in a relatively good water quality environment.

5. Conclusions

Rainbow trout grew and survived equally well in high vs. low CO_2 WRAS over 6 months to market size, and while several histological differences were noted between the two treatment groups none of these findings were associated with clinical disease and mortality. Engineers designing WRAS can set water pumping rates to control CO_2 accumulation at 24 mg/L, which could reduce fixed and variable costs and improve a facility's profitability (compared to operating at 10 mg/L CO_2) without compromising overall fish performance. Further research is warranted to examine fish health and performance in high and low CO_2 WRAS with low alkalinity, e.g. 50 mg/L.

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