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Comparing the effects of high vs. low nitrate on the health, performance, and welfare of juvenile rainbow trout *Oncorhynchus mykiss* within water recirculating aquaculture systems

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ABSTRACT

Previous research indicates that rainbow trout *Oncorhynchus mykiss* begin to exhibit health and welfare problems when cultured within water recirculating aquaculture systems (WRAS) operated at low exchange (6.7 days hydraulic retention time) and a mean feed loading rate of 4.1 kg feed/m^3 daily makeup flow. These studies could not conclusively determine the causative agent of the health and welfare issues, but accumulation of mean nitrate nitrogen (NO_3-N) to approximately 100 mg/L was determined to be a potential cause of abnormal swimming behaviors such as "side swimming" and rapid swimming velocity. A subsequent controlled, 3-month study was conducted to determine if NO_3-N concentrations of 80–100 mg/L resulted in chronic health issues for rainbow trout. Equal numbers of rainbow trout (16.4 ± 0.3 g) were stocked within six replicated 9.5 m^3 WRAS. Three WRAS were maintained with a mean NO_3-N concentration of 30 mg/L ("low") resulting from nitrification, and three WRAS were maintained with a mean concentration of 91 mg/L ("high") via continuous dosing of a sodium nitrate stock solution in addition to nitrification. All six WRAS were operated with equal water exchange (1.3 days mean hydraulic retention time) and mean feed loading rates (0.72 kg feed/m^3 daily makeup flow), which provided enough flushing to limit the accumulation of other water quality concentrations. Rainbow trout growth was not significantly impacted by the high NO_3-N treatment. Cumulative survival for fish cultured within the high NO_3-N WRAS was lower and bordered statistical significance, which resulted in total rainbow trout biomass that was significantly lower for this group at study's end. In addition, a significantly greater prevalence of side swimming rainbow trout occurred in the high NO_3-N treatment, as was observed during previous research. Swimming speeds were generally greater for rainbow trout cultured in the high NO_3-N treatment, but were not always significantly different. Although most water quality variables were controlled, significant differences between treatments for the concentrations of other water quality parameters inhibited definitive conclusions regarding the effect of NO_3-N. However, due to the unlikely toxicity of confounding water quality parameters, study results provided strong evidence that relatively low NO_3-N levels, 80–100 mg/L, were related to chronic health and welfare impacts to juvenile rainbow trout under the described conditions.

1. Introduction

Land-based water recirculation aquaculture systems (WRAS) are becoming increasingly utilized due to water resource limitations; more stringent waste discharge standards; and the need for increased environmental control, biosecurity, and reduced environmental impacts from fish farms (Summerfelt and Vinci, 2008). These systems are often operated intensively in a semi-closed manner with minimal water exchange, which reduces the system make-up water requirement and allows for effective treatment of relatively small, concentrated waste streams (Summerfelt and Vinci, 2008). However, as the exchange rates of WRAS are reduced and feed loading rates subsequently increased, the concentrations of a variety of water quality constituents accumulate (Davidson et al., 2009, 2011a, 2011b).

There is increasing evidence that accumulating water quality concentrations within low exchange WRAS can negatively impact cultured species (Deviller et al., 2005; Davidson et al., 2009, 2011a; Martins et al., 2009a, 2009b). In particular, mounting evidence suggests that relatively low NO_3-N concentrations, once considered to be harmless (Russo, 1985; Wedemeyer, 1996; Colt and Tomasso, 2001; Timmons et al., 2001; Colt, 2006), could cause chronic toxicity to various species cultured in WRAS that

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are operated with minimal water exchange. For example, Hamlin (2005) concluded that NO_3-N concentrations accumulating within WRAS could be of concern for Siberian sturgeon *Acipenser baeri*. Hamlin (2005) determined that the 96-h LC_{50} for Siberian sturgeon (7–700 g) ranged from 397 to 1098 mg/L NO_3-N and cited anecdotal evidence that concentrations as low as 90 mg/L NO_3-N resulted in increased mortality. Van Bussel et al. (2012) found that the growth of juvenile turbot *Psetta maxima* was negatively impacted by NO_3-N concentrations ≥125 mg/L and that health and feed efficiency was reduced at ≥250 mg/L. In a study evaluating the potential effect of 200 mg/L NO_3-N on hybrid striped bass *Morone chrysops* × *M. saxatalis*, Hrubec (1996) reported increased mortality, decreased immune function, and physiological changes consistent with pathology; such as gill hyperplasia and blood chemistry alterations. Recently, Schram et al. (2012) found that feed intake and growth rates decreased for African catfish *Clarias gariepinus* exposed to NO_3-N concentrations >140 mg/L. In addition, Davidson et al. (2011a) suggested that approximately 100 mg/L NO_3-N was a potential causative agent of abnormal rainbow trout swimming behaviors such as rapid swimming velocity and side swimming, and that NO_3-N concentrations >400 mg/L were potentially related to more severe physiological effects such as spinal deformities and increased mortality. Davidson et al. (2011a) could not conclusively determine the parameter that created the fish health issues, but statistical correlation analysis indicated that NO_3-N accumulation was a potential culprit.

In general, research to evaluate chronic NO_3-N toxicity to cultured species across various life stages is limited (Camargo et al., 2005). Several studies have evaluated acute NO_3-N toxicity to rainbow trout. Westin (1974) reported a 96-h LC_{50} of 1364 mg NO_3-N/L and a 7-day LC_{50} of 1068 mg NO_3-N/L for rainbow trout fingerlings. Despite the relatively high NO_3-N levels reported for acute toxicity, Westin (1974) recommended a maximum allowable concentration of approximately 57 mg NO_3-N/L for chronic exposure and only 5.7 mg NO_3-N/L for optimal health and growth of trout. Several other studies have indicated that NO_3-N can be chronically toxic to salmonid eggs and fry at concentrations <200 mg/L with sublethal effects occurring at <25 mg/L (Kincheloe et al., 1979; McGurk et al., 2006).

Although some information is available regarding the effect of NO_3-N to rainbow trout, chronic toxicity research is lacking. Chronic toxicity studies are a sensitive indicator of the sublethal effects to species and help to define the lowest concentration of a water quality parameter that has a significant negative effect (Petrocelli, 1985). Definition of chronic NO_3-N toxicity thresholds for rainbow trout and other species cultured in WRAS is imperative because: (1) it provides a guideline for culture conditions that are conducive with optimal health, welfare, and performance of cultured species and (2) it establishes a critical water quality criterion that impacts the WRAS engineering design, including the makeup water flushing and feed loading rates, requirements for denitrification, as well as the wastewater discharge volume and energy required to heat or cool the WRAS.

Therefore, a controlled study was conducted that would aid in the establishment of a chronic nitrate nitrogen threshold for juvenile rainbow trout by evaluating the potential effects of 80–100 mg/L NO_3-N on trout performance, health, and welfare. The research described herein was complementary to Davidson et al. (2011a) which identified NO_3-N as a potential cause of rainbow trout health and welfare problems in low exchange WRAS.

2. Methods

2.1. Rainbow trout

All rainbow trout used for the study were hatched within a recirculating hatching system and then cultured within 0.5 m^3 circular tanks within a flow-through system in which NO_3-N concentrations averaged <3 mg/L. Rainbow trout (103 ± 1 mm; 16.4 ± 0.3 g) from each 0.5 m^3 flow-through tank were randomized and divided equally amongst six replicated WRAS at an initial stocking density of 6 kg/m^3 (2050 fish/tank). The trout were 108 days old (post-hatch) when the study began.

2.2. Experimental treatments

Six replicated WRAS (9.5 m^3) were used (Fig. 1) during a 3-month study. Rainbow trout within two randomly selected sets of 3 WRAS were exposed to the following treatments: (1) "high" NO_3-N (target 80–100 mg/L) and (2) "low" NO_3-N (target 20–40 mg/L), representing the control. Nitrate nitrogen concentrations for the high treatment were controlled by continuously dosing a sodium nitrate stock solution into the LHO sump using a peristaltic pump, in addition to the natural accumulation resulting as an end product of nitrification. Nitrate nitrogen concentrations within the control systems were created only as an end product of the nitrification process and controlled by water exchange. All fluidized sand biofilters were fully acclimated and capable of complete nitrification when the study began. In addition, a sodium sulfate solution was continuously dosed to the low NO_3-N systems using a peristaltic pump in order to balance the sodium concentration and conductivity between treatments.

2.3. System description and operation

The replicated WRAS used during the present study were previously described in detail (Davidson et al., 2009). To summarize, each WRAS recirculated 380 L/min (100 gpm) of water through a 5.3 m^3 dual drain culture tank, a radial flow settler, a microscreen drum filter with 60 μm screens, a fluidized sand biofilter, a geothermal heat exchanger, a carbon dioxide stripping column, and a low head oxygenator (LHO) (Fig. 1). A constant 24-h photoperiod was provided throughout the study.

Ozone was applied to all WRAS seven weeks into the study in order to reduce the color of the water so that fish could be more easily observed and to facilitate measurement of behavioral metrics. Three ozone generators were used (Model G22, Pacific Ozone Technology, Benicia, CA, USA). Approximately 1–6% of the >99% pure oxygen feed gas passing through the Corona discharge cell of each generator was converted to ozone and injected equally within each LHO. Ozone was monitored and controlled via oxidation reduction potential (ORP), measured in each culture tank just in front of the inlet flow structure with a differential ORP digital sensor equipped with a platinum electrode (Model DRD1R5, Hach Company, Loveland, CO, USA) and displayed by an SC100 Universal Controller (Hach Company, Loveland, CO, USA).

2.4. Water exchange and feed loading rates

Makeup water flow rates were maintained equally for all WRAS throughout the study. To begin, 1.3 L/min of makeup water was continuously added to each pump sump, equivalent to 0.34% of the total recycle flow and a 5-day system hydraulic retention time. Makeup water flow rates were increased to all WRAS on three occasions as follows: 2.6, 3.8, and 5.7 L/min or 0.69, 1.00, and 1.51% of the total recycle flow; in order to maintain maximum NO_3-N concentrations for the control treatment at ≤40 mg/L and to prevent the accumulation of other water quality and ionic concentrations. The system hydraulic retention times resulting from adjustments to makeup water rates were 2.5, 1.7, and 1.2 days, respectively.

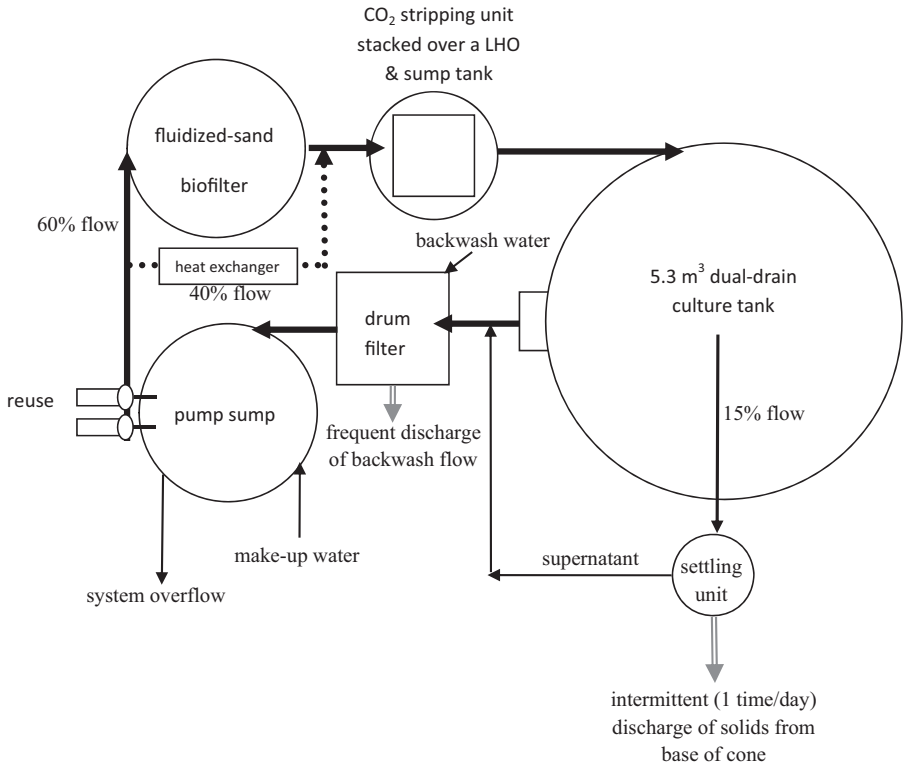

Fig. 1. Design schematic of an individual 9.5 m^3 experimental water recirculating aquaculture system. Arrows indicate direction of water flow through unit processes.

2.5. Feeding methods and feed loading

A standard slow-sinking trout diet (Zeigler Brothers, Inc., Gardners, PA, USA) with a protein: fat ratio of 42:16 was used throughout the study. Fish were fed equal rations with feeding events occurring every other hour, around the clock, using automated feeders (T-drum 2000CE, Arvotec, Huutokoski, Finland). Feeding was estimated based on standardized feeding charts, as well as observations of feeding activity and wasted feed. The mean feed loading rate amongst all WRAS over the study duration was 0.72 kg daily feed per cubic meter daily makeup water.

2.6. Water quality sampling and analysis

Water samples were collected weekly from the side drain of each tank and tested on-site. Specific parameters, methodologies, and frequencies of testing are outlined in Table 1. All water quality parameters measured on-site were analyzed according to methods described in APHA, 2005 and Hach (2003). Water samples for dissolved metals analysis were collected monthly and analyzed by the Cornell University Nutrient Analysis Lab (Ithaca, NY, USA).

2.7. Fish sampling protocols

Lengths and weights of a random sample of 70–115 fish (exact number dependent upon the calculated sample size requirement, Kitchens, 1998), were measured monthly, including samples at the beginning and end of the study. Fin erosion was assessed qualitatively on a 4-point scale (severe, moderate, low, or no damage) for all sampled fish during each monthly length/weight event. The prevalence of spinal deformities (i.e., lordosis, kyphosis, and/or scoliosis) was also assessed monthly for all sampled fish through visual observation. Mortalities were removed and recorded daily in order to track cumulative survival. Thermal growth coefficients (TGC) and feed conversion ratios (FCR) were calculated and compared between treatments as follows:

$$\mathrm{TGC} = \frac{\text{End Weight}^{(1/3)} - \text{Start Weight}^{(1/3)}}{(\text{Days Between} \times \text{Avg. Temp.}) \times 1000}$$

where weight is in grams and temperature is in °C.

$$\mathrm{FCR} = \frac{\text{Cumulative Feed Delivered}}{\text{Fish Biomass Gain}}$$

At the end of the study period, five fish from each WRAS were randomly sampled via dip-net collection, sedated with 75 mg/L MS-222 (Western Chemical Inc., Ferndale, WA, USA), and bled via caudal venipuncture using 21.5-guage, 1.5 in. needles and 1-ml syringes. Whole blood samples were then analyzed on-site using an i-Stat 1 portable analyzer (Abbott Laboratories, Abbott Park, IL, USA) with CG4+ and CHEM8+ cartridges. Parameters assessed with the CG4+ cartridge included pH, pCO_2, pO_2, HCO_3, total CO_2, O_2 saturation, and lactate, while CHEM8+ cartridges provided data for whole blood sodium, potassium, chloride, calcium, glucose, hematocrit, and hemoglobin.

For histopathological evaluation, samples of gill, integument, anterior and posterior kidney, liver, heart, and spleen were collected from five euthanized (200 mg/L MS-222) fish per WRAS at the end of the study and preserved in histological grade 10% formalin solution (Fisher Scientific, Pittsburgh, PA, USA) for one week prior to processing and histopathological evaluation. The evaluating pathologist was blinded to the treatment group origins of all sampled specimens. A zero-to-five 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.

Table 1
Water quality parameters sampled and descriptions of methodologies and frequency of testing for each.

Parameter	Method of analysis	Frequency of testing
Biochemical oxygen demand	Standard Methods 5210B – 5 day test (no prefiltration of sample)	Once weekly
Conductivity	YSI 30 Salinity/Conductivity/Temperature meter	4–5 times weekly
Dissolved carbon dioxide	Hach Method 8223 – Burret Titration	Once weekly
Dissolved oxygen	Hach SC100 Universal Controller & LDO® Probe	Recorded daily
Dissolved metals	Inductively Coupled Plasma Atomic Emission Spectrometry technique	Near max feeding (3 events)
Nitrite nitrogen	Hach Method 8507 – Diazotization	Once weekly
Nitrate nitrogen	Hach Method 8171 – Cadmium Reduction	4–5 times weekly
pH	Hach Model HQ40D with digital pH sensor	Once weekly
Sulfate	Hach Method 8051–Turbidimetric Method	4–5 times weekly
Temperature	Hach SC100 Universal Controller & Differential ORP Sensor	Recorded daily
Total alkalinity	Standard Methods 2320 – Sulfuric Acid Titration	4–5 times weekly
Total ammonia nitrogen	Hach Method 8038 – Nessler	Once weekly
Total suspended solids	Standard Methods 2540D – Dried at 103–105 °C	Once weekly
True color	Hach Method 8025 – Platinum-Cobalt	Once weekly
Ultraviolet transmittance	Standard Methods 5910B – Ultraviolet Absorption	Once weekly
Water hardness (as $CaCO_3$)	Hach Method 8123 – Digital Titration using EDTA	Once weekly

2.8. Swimming behavior assessment

Over the course of the study, the degree of side swimming (a condition in which the fish swims tilted on its side) was assessed weekly in each WRAS by counting the number of side swimming fish as they passed a given location in the tank. During weeks 5–8, fish could not be accurately counted due to increasing turbidity of the culture water. Ozone was applied to each system during week 7 to clear the water and allow more effective observation of fish behavior. Observations of swimming behavior were resumed during week 9.

At the conclusion of the study, rainbow trout within each WRAS were crowded and approximately 50% of the population from each WRAS (1023 ± 3 fish) was randomly selected via dip-netting and transported to a separate, single-pass system with 1.5-m^3 tanks that received spring water, where side swimming fish could be more easily observed, handled, and quantified. The next day, all side swimming fish were individually netted from each 1.5 m^3 tank and counted into separate tanks in order to assess the percentage of side swimming fish present in each WRAS at the conclusion of the study.

Over the first 4 weeks of the study, general observations were made regarding swimming speed. Swimming speed was not measured from weeks 1–4 due to fish orientation deep in the water column and/or a lesser number of fish swimming faster than the rotational velocity of the culture water. From weeks 5–8, observations of swimming behavior were limited due to the turbidity of the culture water (previously explained relative to side swimming observations). By week 9 the water was clear enough to begin an assessment of fish swimming speed, which was carried out weekly thereafter until the conclusion of the study. Swimming speeds of 15 fish from each WRAS were measured weekly. Culture tanks were gridded into four sections using two lengths of PVC pipe that spanned the tank with markings distanced one inch apart. Rainbow trout were observed from above the tank using a ladder. Fish were allowed 2 min to acclimate to the disturbances caused by setup, prior to taking measurements. Individual fish were tracked using a stopwatch as they intersected a PVC grid that encompassed one quarter of the culture tank. In addition to speed, which was timed with a stopwatch, notation was made relative to the fish location, i.e. distance from the tank wall, as it intersected the grid. Incremental 1-in. markings on the PVC pipe facilitated this measurement, which was then used to estimate the arc or distance of travel, needed to determine swimming speed. The water rotational velocity was added to the calculated fish swimming velocity to determine overall fish swimming speed.

Table 2
Summary of rainbow trout growth performance metrics (mean ± standard error) compared between high and low NO_3-N treatments ($n = 3$).

Mean growth metrics	High NO_3-N	Low NO_3-N	*P*-value
Final length (mm)	216 ± 1	221 ± 2	0.058
Final weight (g)	181 ± 5	189 ± 5	0.335
Thermal growth coefficient	2.32 ± 0.05	2.34 ± 0.04	0.805
Condition factor	1.70 ± 0.03	1.66 ± 0.01	0.388
Feed conversion ratio	1.35 ± 0.05	1.29 ± 0.03	0.407
Final biomass (kg)[a]	332 ± 6	364 ± 9	0.031
Fish density (kg/m^3)[a]	64 ± 1	70 ± 2	0.035

[a] Indicates significant difference between treatments.

2.9. Statistical analysis

All parameters that were sampled during multiple events over time from the same location, such as water quality parameters and growth rates were analyzed using a Mixed Models approach known as Restricted Maximum Likelihood (REML), which allows the assignment of Tank as a random effect, thus buffering potential variation arising from individual culture system effects (Ling and Cotter, 2003). Time was included as a random covariate for these analyses. Normality was assessed using a Shapiro–Wilk test. Non-Gaussian data were transformed for statistical comparison. If transformation procedures did not yield normally distributed data a non-parametric Wilcoxon Mann Whitney test was employed. Survival percentage data was transformed for statistical analysis using an arcsine square-root transformation. Blood chemistry data obtained from individual fish were assessed statistically for treatment effect using analyses of covariance, with blood parameter as dependent variable, treatment as independent variable, and tank (WRAS) as forced covariate. Histopathological data for each tissue type with observable lesions were analyzed using bivariable ordered logistic regression models, with treatment (high or low NO_3-N) as the independent variable in each model and specific lesion score as the ordinal dependent variable. Blood chemistry and histopathological data were analyzed with STATA 9 software (StataCorp, College Station, TX, USA); all other data analyses were carried out using SYSTAT 13 software (2009). A probability level of 0.05 was used to determine significance.

3. Results

3.1. Growth performance metrics

Growth performance metrics and results are summarized in Table 2. Rainbow trout of the same cohort, age, and size were randomized amongst the six WRAS at the beginning of the study,

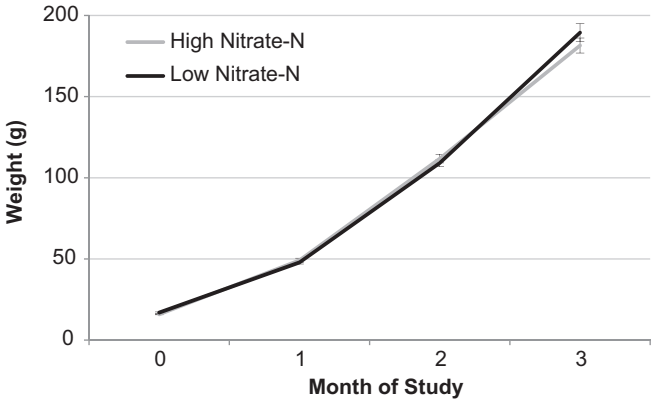

Fig. 2. Mean rainbow trout weight ± standard error (g) for the high and low NO_3-N treatments ($n = 3$).

thus trout size within all WRAS was statistically similar between treatments to begin (16.4 ± 0.3 g). At the conclusion of the study, rainbow trout cultured within the high and low NO_3-N treatments had mean lengths of 216 ± 1 and 221 ± 2 mm, respectively; and mean weights of 181 ± 5 and 189 ± 5 g, respectively (Fig. 2). There was no significant difference in mean length or weight between treatments over the study duration (Table 2). In addition, there was no statistical difference in thermal growth coefficient (TGC) between treatments, which was 2.32 ± 0.05 and 2.34 ± 0.04 for the high and low NO_3-N treatments, respectively (Table 2). Condition factor was also similar between treatments over the study duration. Mean condition factor at the conclusion of the study for the high and low NO_3-N treatments was 1.70 ± 0.03 and 1.66 ± 0.01, respectively. Mean feed conversion ratios (FCR) during the study period for the high and low NO_3-N treatments were 1.35 ± 0.05 and 1.29 ± 0.03, respectively. There was no significant difference in FCR between treatments. At the conclusion of the study no statistical differences existed for any of the aforementioned rainbow trout performance metrics (Table 2).

3.2. Survival

Rainbow trout mortality began to increase for the high NO_3-N treatment only 1-week after treatments were initiated (Fig. 3). Thereafter, cumulative survival was consistently lower for the high NO_3-N treatment for the remainder of the study (Fig. 3). Over the final 2–3 weeks of the study rainbow trout mortality increased for both groups but was more severe within the high NO_3-N treatment (Fig. 3). At the conclusion of the study, cumulative rainbow

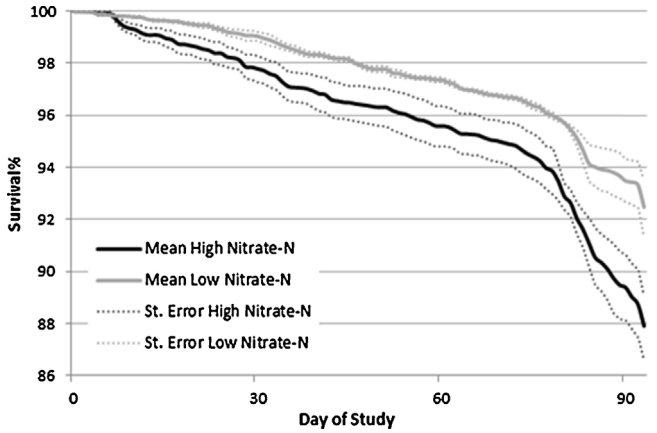

Fig. 3. Cumulative survival percentage (mean ± standard error) for rainbow trout from the high and low NO_3-N treatments ($n = 3$).

Table 3
Blood chemistry results (mean ± standard error) from fish ($n = 5$) sampled from the high and low NO_3-N treatments at the conclusion of the study.

Parameter	High NO_3-N	Low NO_3-N	*P*-value
Bicarbonate (mmol/L)	20.94 ± 0.346	21.35 ± 0.912	0.683
Calcium (mmol/L)	1.493 ± 0.009	1.522 ± 0.018	0.703
Chloride (mmol/L)	ND	124.4 ± 0.653	NA
Glucose (mg/dL)	96.00 ± 10.27	80.70 ± 4.055	0.598
Hematocrit (%PCV)	38.67 ± 0.987	36.30 ± 1.407	0.753
Hemoglobin (g/dL)	13.14 ± 0.334	12.34 ± 0.479	0.756
Lactate (mmol/L)	5.122 ± 0.637	4.900 ± 0.814	0.513
pCO_2 (mm Hg)	79.67 ± 4.817	81.59 ± 3.832	0.869
pH (mg/L)	7.058 ± 0.036	7.027 ± 0.030	0.647
pO_2 (mm Hg)	5.461 ± 0.462	5.917 ± 0.668	0.672
Potassium (mmol/L)	6.033 ± 0.317	5.130 ± 0.450	0.731
Sodium (mmol/L)	145.8 ± 0.661	144.5 ± 0.687	0.285
Total CO_2 (mmol/L)	23.31 ± 0.382	23.83 ± 0.911	0.631
Urea nitrogen (mg/dL)	13.00 ± 1.135	10.70 ± 0.857	0.079

trout survival for the high and low NO_3-N treatments was 87.9 ± 1.1 and 92.5 ± 1.1%. Statistical comparison of final survival percentages bordered significance ($P = 0.050$).

3.3. Fish biomass and density

Statistical evaluation of growth did not yield differences between treatments and a potential difference in survival trended toward significance; however, the combined effect of slightly slower growth and decreased survival for the high NO_3-N treatment resulted in significantly lower fish biomass and density. Mean rainbow trout biomass to conclude the study for the high and low NO_3-N treatments was 332 ± 6 and 364 ± 9 kg per WRAS, respectively ($P = 0.031$). Mean fish density at the conclusion of the study was 64 ± 1 kg/m^3 within the high NO_3-N systems and 70 ± 2 kg/m^3 within the low NO_3-N systems ($P = 0.035$).

3.4. Fin erosion and spinal deformities

There was no significant difference in the degree of fin erosion between treatments over the duration of the study for the following fins: left pectoral, right pectoral, left pelvic, right pelvic, and dorsal. At the conclusion of the study, the caudal fin of trout cultured within the low nitrate treatment was found to have significant greater fin erosion compared to trout cultured in the high NO_3-N treatment ($P = 0.021$). Very few spinal deformities were observed (<1% during each sampling event) for either treatment ($P > 0.05$).

3.5. Blood chemistry

There were no significant differences between treatments for a suite of 14 blood chemistry parameters analyzed from samples collected at the conclusion of the study (Table 3). However, chloride concentrations for rainbow trout from the high NO_3-N treatment were nondetectable for all 15 fish sampled; while blood from the low NO_3-N treatment fish contained 124.4 ± 0.653 mmol/L chloride. Due to the lack of numerical data for chloride, a statistical comparison was not possible. In addition, blood urea nitrogen concentrations for the high and low NO_3-N treated fish were 13.00 ± 1.135 and 10.70 ± 0.857 mg/dL, respectively ($P = 0.079$). A significant difference was not detected between treatments for blood urea nitrogen, but it was the only measured blood chemistry parameter that trended toward significance.

3.6. Histopathology

Among all tissue types examined, consistent lesions were only noted in gill and kidney tissue, and neither treatment group

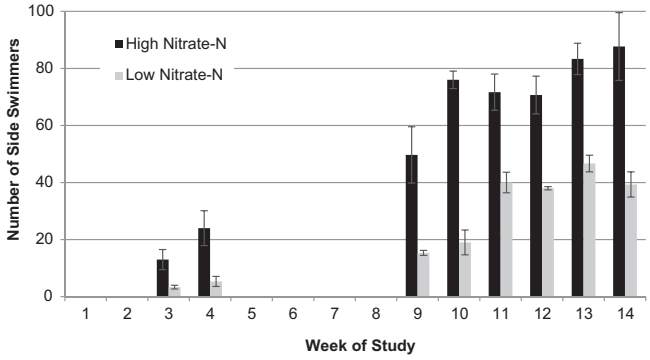

Fig. 4. Relative number of side swimming rainbow trout observed passing a given culture tank location for the high and low NO_3-N treatments ($n = 3$). Observations were not made during weeks 5–8 due to increased turbidity of the culture water.

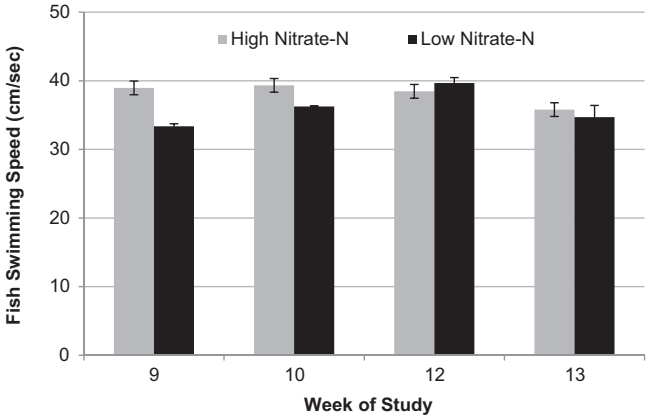

Fig. 5. Mean rainbow trout swimming speed ± standard error (cm/s) for the high and low NO_3-N treatments during weeks 9–13 of the study ($n = 3$). Note that week 11 data was excluded due to potential confounding caused by inconsistent hydraulics amongst systems, specifically related to position of the center vortex.

exhibited statistically higher scores (on the 0–5 point scale) for either tissue pathology. Observed gill pathology comprised of mild to moderate hyperplasia of the basal lamellar and interlamellar epithelium, with occasional separation of the lamellar epithelium from the subadjacent stroma and variable hypertrophy of the mid-zonal lamellar epithelial cells. Fish in the high NO_3-N group scored 1.53 ± 0.27 for this lesion type, while those examined from the low NO_3-N group scored 1.67 ± 0.27 for this lesion type ($P = 0.835$). Kidney lesions consisted of mild to severe nephrocalcinosis and renal interstitial fibrosis. High NO_3-N fish scored 0.60 ± 0.41 for this lesion type, while the low NO_3-N group scored 1.00 ± 0.40 for this lesion type ($P = 0.268$).

3.7. Swimming behavior

To begin the study through week 2, no side swimming fish were observed for the high or low NO_3-N treatments (Fig. 4). During week 3 a few fish began to swim oriented on their sides for each treatment, i.e. 13 ± 4 and 3 ± 1 fish for the high and low NO_3-N treatments, respectively (Fig. 4). Fish could not be observed from weeks 5–8 due to turbidity of the culture water. When observations resumed during week 9, the number of side swimming fish had increased for both treatments (Fig. 4). The prevalence of side swimming fish was significantly greater for the high NO_3-N treatment during every weekly assessment ($P < 0.05$). At the conclusion of the study, over 1000 fish from each WRAS were relocated to smaller tanks where side swimming fish could be captured and separated in order to determine a percentage of the population that exhibited the behavior. Results indicated that 11.5 ± 1.1% of the population exhibited the side swimming behavior from the high NO_3-N treatment and 3.8 ± 0.7% of the population exhibited side swimming behavior from the low NO_3-N treatment ($P = 0.006$).

Swimming speed observations during week 1 indicated that rainbow trout generally held position in the water column for both treatments, i.e., swam at a rate equivalent to the rotational velocity of the culture water, 17.9 ± 0.40 and 17.8 ± 0.36 cm/s for the high and low NO_3-N treatments, respectively. These initial swimming velocities equated to 1.76 ± 0.02 and 1.66 ± 0.04 bl/s ($P = 0.105$). From weeks 2–4 approximately 2/3 of the fish in all WRAS tanks began to swim faster than the rotational velocity of the culture tank. As previously mentioned, observations were inhibited from weeks 5–8 due to increased turbidity of the culture water in all WRAS. The use of ozone, beginning at week 7, cleared the water and allowed swimming speed measurements to resume by week 9. During weeks 9 and 10, significantly greater swimming speed was measured for rainbow trout cultured within the high NO_3-N treatment (Fig. 5). During the final two weeks of the study, there was no significant difference in swimming speed between treatments (Fig. 5). Although rainbow trout swimming speed was generally greater over the majority of the assessment period, the average swimming speed over the course of the study was not significantly different, i.e., 38.1 ± 0.4 and 36.0 ± 1.2 cm/s ($P = 0.200$), for the high and low NO_3-N treatments, respectively. When expressed as body lengths/s, mean rainbow trout swimming speeds (week 9–13) for the high and low NO_3-N treatments were 2.03 ± 0.07 and 1.87 ± 0.06 bl/s, respectively ($P = 0.176$).

3.8. Water quality

The majority of measured water quality parameters were successfully controlled between treatments (Table 4). Significant differences were not detected for alkalinity, biochemical oxygen demand, carbon dioxide, color, conductivity, dissolved oxygen, hardness, ORP, pH, temperature, total ammonia nitrogen, and unionized ammonia (Table 4). Of the 25 dissolved nutrients and elements that were analyzed; aluminum, arsenic, beryllium, cadmium, chromium, cobalt, iron, lead, manganese, molybdenum, nickel, selenium, titanium, and vanadium concentrations were below the minimum detection limits for both treatments (Table 5). In addition, the following dissolved nutrients and metals that existed at measureable concentrations were not significantly

Table 4
Mean culture tank water quality concentrations (mg/L, unless otherwise noted) for high and low NO_3-N treatments ($n = 3$).

Parameter (mg/L)	High NO_3-N	Low NO_3-N	*P*-value
Alkalinity	194 ± 1	195 ± 1	0.700
Biochemical oxygen demand	4.9 ± 1.0	3.5 ± 0.2	0.092
Carbon dioxide	14 ± 0	14 ± 0	1.000
Color (Pt-Co units)	25 ± 2	23 ± 0	0.099
Conductivity (μS)	1215 ± 8	1210 ± 3	0.700
Dissolved oxygen	10.1 ± 0.0	10.1 ± 0.0	1.000
Hardness (as $CaCO_3$)	308 ± 1	307 ± 1	0.750
Nitrate nitrogen[a]	91 ± 0	30 ± 0	0.000
Nitrite nitrogen[a]	0.091 ± 0.012	0.021 ± 0.002	0.000
Oxidative reduction potential (mV)	244 ± 9	257 ± 5	0.289
pH	7.59 ± 0.01	7.58 ± 0.01	0.502
Sulfate[a]	36 ± 0	262 ± 2	0.000
Temperature (°C)	15.5 ± 0.0	15.5 ± 0.0	0.794
Total ammonia nitrogen	0.40 ± 0.02	0.37 ± 0.01	0.098
Total suspended solids[a]	6.6 ± 1.1	4.3 ± 0.7	0.026
Unionized ammonia	0.0035 ± 0.0002	0.0033 ± 0.0000	0.430
Ultraviolet transmittance (%)[a]	76 ± 1	81 ± 0	0.000

[a] Indicates significant difference between treatments.

Table 5
Mean culture tank dissolved metals and nutrient concentrations (mg/L) for "high" and "low" nitrate nitrogen treatments ($n = 3$).

Parameter (mg/L)	"High" NO_3-N	"Low" NO_3-N	*P*-value
Barium	0.063 ± 0.003	0.051 ± 0.002	0.589
Boron*	0.047 ± 0.001	0.023 ± 0.001	0.049
Calcium*	114 ± 0	118 ± 0	0.034
Copper	0.023 ± 0.002	0.024 ± 0.005	0.903
Magnesium	13.6 ± 0.0	13.6 ± 0.0	0.901
Phosphorous	1.4 ± 0.0	1.5 ± 0.0	0.213
Potassium*	16.0 ± 0.1	12.3 ± 0.1	0.000
Sodium*	107 ± 1	127 ± 5	0.002
Strontium	0.92 ± 0.00	0.93 ± 0.00	0.543
Sulfur*	12 ± 0	88 ± 2	0.000
Zinc	0.031 ± 0.002	0.049 ± 0.010	0.255

Note: The following dissolved metals and nutrients were <MDL: aluminum, arsenic, beryllium, cadmium, chromium, cobalt, iron, lead, manganese, molybdenum, nickel, selenium, titanium, and vanadium.

* Indicates significant difference between treatments.

different between treatments: barium, copper, magnesium, phosphorous, strontium, and zinc (Table 5).

Several water quality parameters were statistically different due to the experimental design of the study including: nitrate nitrogen, sulfate, sulfur, and sodium (Tables 4 and 5). Mean NO_3-N concentrations for the high and low NO_3-N treatments were 91 ± 0 and 30 ± 0, respectively. Fig. 6 illustrates the NO_3-N concentrations established for each treatment during the study period. Maximum NO_3-N concentrations for the high and low NO_3-N treatments were 110 and 40 mg/L, respectively. Sulfate and dissolved sulfur were significantly greater within the low NO_3-N treatment due to the addition of sodium sulfate to balance conductivity between treatments. Dissolved sodium was also significantly greater within the low NO_3-N WRAS.

Several other water quality parameters were significantly different between treatments. These parameters included nitrite nitrogen, total suspended solids, and ultraviolet transmittance; as well as the following dissolved nutrients and metals: boron, calcium, and potassium (Tables 4 and 5). Nitrite nitrogen, total suspended solids, boron, and potassium concentrations were significantly greater within the high NO_3-N treatment. Ultraviolet transmittance was significantly lower for the high NO_3-N treatment, indicating slightly greater turbidity. Calcium concentrations were also significantly greater for the low NO_3-N treatment (Table 5).

4. Discussion

4.1. Swimming behavior

Many aspects of the fish performance results and behavioral observations from the present study mirrored those of Davidson et al. (2011a). The most notable finding from the present study was replication of the side swimming behavior, which was reported as one of the primary health and welfare effects in Davidson et al. (2011a) for rainbow trout cultured in low water exchange WRAS with mean NO_3-N concentrations of 99 ± 7 mg/L. During the present study, a significantly greater percentage of rainbow trout exhibited side swimming behavior within the high NO_3-N treatment compared to the low NO_3-N treatment. It is important to note that there were absolutely no side swimming rainbow trout in either treatment when the study commenced (Fig. 4). Therefore, the side swimming behavior was likely instigated by conditions established within the experimental tanks.

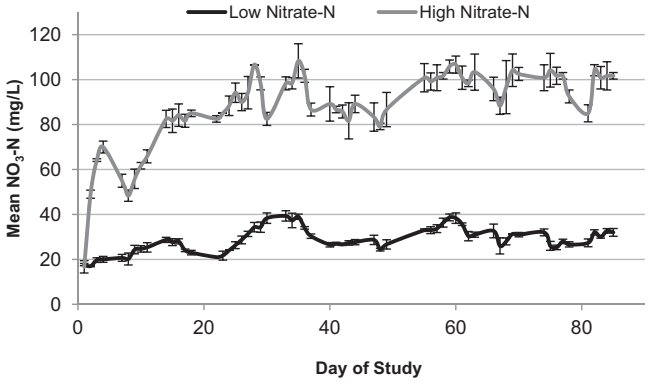

Fig. 6. Mean nitrate nitrogen concentrations ± standard error (mg/L) for the high and low NO_3-N treatments measured 4–5 times per week ($n = 3$).

In addition to side swimming behavior, Davidson et al. (2011a) measured rapid swimming speeds in rainbow trout cultured in tanks with mean NO_3-N concentrations of approximately 100 mg/L. Rainbow trout cultured under these conditions swam faster than the rotational velocity of the culture tank, while trout cultured within WRAS with a ten-fold greater flushing rate and NO_3-N concentrations of 13 ± 0 mg/L generally held position with the rotational velocity of the culture tank (Davidson et al., 2011a). During the present study, there was not a significant difference between treatments in overall swimming speed calculated as a grand mean from weeks 9–13; however, swimming speed was significantly greater in the high NO_3-N WRAS during weeks 9 and 10 and was generally greater for the majority of swimming speed assessments. Davison (1997) concluded that swimming speeds ≤1.5 bl/s were optimal for growth and feed conversion and suggested that sustained swimming at speeds >1.5 bl/s could have negative impacts on fish. During the first week of the study, rainbow trout swimming speeds were 1.76 ± 0.02 and 1.66 ± 0.04 bl/s for the high and low NO_3-N treatments, respectively. These swimming speeds were slightly greater but close to the recommendations set forth by Davison (1997). From weeks 9–13 average swimming speeds increased to 2.03 ± 0.07 and 1.87 ± 0.06 bl/s for the high and low NO_3-N, treatments respectively, and therefore exceeded the recommendation of ≤1.5 bl/s (Davison, 1997). Jain et al. (1997) determined that the "fatigue swimming velocity" for rainbow trout was 2.10 ± 0.06 bl/s.

Although, the background literature suggests that rainbow trout from both treatments were swimming at potentially exhaustive rates, it is clear that the fish elected to swim at increased speeds possibly as an effort to balance metabolic and/or osmoregulatory function. To maintain a constant position against the rotational current, the fish were forced to swim no faster than the mean rotational velocity of the culture water, which was 18.4 ± 0.2 and 18.3 ± 0.3 cm/s for the high and low NO_3-N treatments, respectively; but average swimming speeds for both treatments exceeded 36 cm/s (approximately two times faster than the water rotational velocity). Most behavioral responses, such as changes to fish swimming behavior, are based on underlying physiological and biochemical factors (Rand, 1985). Gallaugher et al. (2001) reported that high intensity exercise training potentially diminished osomoregulatory compromise in Chinook salmon (*Oncorhynchus tshawytscha*) and allowed the fish to "multitask physiological functions while swimming."

Several swimming behavior measurements from the present study differed from those described by Davidson et al. (2011a); specifically, the increased prevalence of side swimming as well as the rapid swimming speeds measured in the low NO_3-N treatment. The reason for the occurrence of these behaviors within the low NO_3-N treatment is unclear, but the authors offer several possible explanations: (1) The control NO_3-N concentration (30 mg/L) possibly caused a mild toxicity to a small percentage of the population. The control concentration used in the present study was more than two times greater than that reported in Davidson et al. (2011a), i.e., 13 ± 0 mg/L NO_3-N, for which very few side swimming

trout were observed. (2) Rainbow trout that were evaluated during Study 1 of the Davidson et al. (2011a) were 151 ± 3 g to begin; while trout used during the present study were 16.4 ± 0.3 g. Therefore, the younger rainbow trout used during the present study could have been more susceptible to lower NO_3-N concentrations. Sprague (1985) reported that the most sensitive life stages of fish to toxicants are the embryo-larval and early juvenile life stages. Camargo et al. (2005) reported that nitrate toxicity generally decreases in aquatic species with increasing body size, and noted that establishment of acute, chronic, and sublethal NO_3-N levels would certainly depend upon life stage. (3) Other water quality concentrations perhaps interacted to cause a mild chronic effect to rainbow trout in the low NO_3-N treatment systems; or sodium sulfate, the compound used to control conductivity within the low NO_3-N treatment could have caused a mild chronic reaction. However, background literature suggests that sulfate concentrations were safe for rainbow trout (Heinen, 1996; Davies, 2007; Davies and Hall, 2007; Elphick et al., 2011).

4.2. Blood chemistry and histopathology

The majority of measured blood parameters were not significantly different between high and low NO_3-N treatment groups (Table 3), and the values obtained were generally comparable to previously conducted on-site research (e.g. Good et al., 2009) using the i-Stat 1. The use of point-of-care instruments, such as the i-Stat 1, is becoming a more frequent approach in published studies evaluating animal blood parameters; however, the accuracy of results obtained from such instruments, compared to values generated through conventional laboratory methodologies, has been questioned (DiMaggio et al., 2010). As published reference ranges for blood chemistry parameters in fish (e.g. Wedemeyer and Chatterton, 1970; Stoskopf, 1993; Wedemeyer, 1996) have been derived from data obtained through conventional laboratory methodologies, it is often difficult to directly compare results from point-of-care instruments to these published values. Employing instruments such as the i-Stat 1 in aquatic research studies, however, can still be extremely useful, particularly when comparing results from two or more treatment groups, and other situations in which instrument precision is more important than its accuracy relative to traditional laboratory approaches.

The most striking difference between the two treatments was in chloride concentration, for which all fifteen fish sampled in the high NO_3-N group had values outside the i-Stat 1's detection range (65 and 140 mmol/L), while all 15 fish in the low NO_3-N group had expected values for whole blood chloride. Whether chloride concentrations in the high NO_3-N group were above or below instrument detection range cannot be determined. Hrubec (1996), however, reported reductions in plasma chloride in hybrid striped bass exposed to high NO_3-N administered as various salts ($NaNO_3$, $CaNO_3$, KNO_3), and furthermore the observed reduction in plasma chloride in $NaNO_3$ treated fish was profound compared to those exposed to $CaNO_3$ or KNO_3. The study by Hrubec (1996) provides evidence that $NaNO_3$ can drastically reduce plasma chloride without major effects on other blood electrolytes (as was observed during the present study), and it is therefore likely that the high NO_3-N group had whole blood chloride concentrations below the i-Stat 1 detection limits (i.e., <65 mmol/L). The reasons for NO_3-N's association with reduced chloride concentration, and in particular for the relatively severe reduction in plasma chloride associated with $NaNO_3$, remain unclear and warrant further investigation. Reduced plasma chloride is often considered a physiological consequence of stress in fish, which lose chloride ions to the water as gill epithelial cells become more permeable with increased blood pressure (Wedemeyer, 1996). However, as no other blood parameters that are typically influenced by stress (e.g. other electrolytes, glucose, and lactate) showed significant differences between treatment groups during the present study, it is unlikely that the theorized marked reduction in chloride concentration in the high NO_3-N group was due to short- or long-term stress. This is in agreement with Hrubec (1996) and Hamlin (2007), who did not observe increased plasma glucose and cortisol levels in hybrid striped bass or Siberian sturgeon (*Acipenser baeri*), respectively, when exposed to elevated NO_3-N.

Although the difference bordered significance, discussion is warranted regarding blood urea nitrogen (BUN) concentrations, which were higher ($P = 0.079$) in fish from the high NO_3-N group. In a previous study conducted on-site using the same experimental WRAS, Good et al. (2009) reported increasing BUN levels for juvenile rainbow trout (133 grams to begin) relative to decreasing system water exchange. Rainbow trout cultured within a flow through system, a high exchange WRAS, and a low exchange WRAS had BUN concentrations of <2.0, 15.9 ± 0.62, and 19.0 ± 0.80 mg/dL, respectively (Good et al., 2009). Corresponding NO_3-N levels in the culture water in the high exchange, low exchange, and flow through systems were <3, 12 ± 0, and 70 ± 4 mg/L (Good et al., 2009). Therefore, there appears to be an association between BUN concentrations in rainbow trout and the NO_3-N concentration of the culture water. Elevated blood urea nitrogen levels are thought to be related to liver and/or gill damage or dysfunction due to the capacity of these organs to produce and excrete urea, respectively (Stoskopf, 1993). Mensinger et al. (2005) reported that increasing BUN is a likely indicator of failing gill osmoregulatory function and noted that increased BUN was correlated with fish that had compromised health or were terminally ill. Among the organs evaluated through histopathology (gill, skin, heart, liver, spleen, and kidney), only gill and kidney tissue demonstrated noticeable, albeit predominantly mild damage, and furthermore no significant differences in the extent and severity of observed lesions were noted between treatments. The observation of mild gill lesions is interesting due to the association of gill dysfunction with increased BUN (Mensinger et al., 2005), as well as the potential respiratory advantages gained by increased swimming speeds. However, the severity of gill lesions was similar between treatment groups which confounds any assumption that mild gill lesions and increased BUN were in some way related. Therefore, based on the blood chemistry results of the present study, it is unknown whether organ dysfunction in the absence of significant observable pathology was related to elevated BUN.

The mechanisms of nitrate uptake in fish are not fully understood. Camargo et al. (2005) reported that uptake of nitrate in fish is passive and that gills have a low branchial permeability for nitrate. The theory of passive uptake and low branchial permeability has been used as an explanation for the potentially low affinity of nitrate to cause toxicity compared to other nitrogenous wastes (Camargo et al., 2005). Stormer et al. (1996) suggested that nitrate ions within the blood plasma of rainbow trout fingerlings remained below the ambient concentration (14 mg/L NO_3-N) after 8 days of exposure, indicating that nitrate ions were taken up passively. However, Schram et al. (2012) found that blood plasma nitrate increased almost linearly with the concentration of the culture water in juvenile African catfish even though the ratio of plasma nitrate to waterborne nitrate was relatively low, i.e. 0.15–0.25. Therefore, the theory of passive uptake of nitrate in freshwater fish is reasonable; but nonetheless uptake of nitrate does occur, suggesting that prolonged exposure times that are common within low exchange WRAS might lead to longer term toxic effects.

Another reported hematological effect of exposure of fish to excess nitrate is the conversion of hemoglobin to methemoglobin, and the resultant inhibition of oxygen binding and transport within the blood (Camargo et al., 2005), similar to the effect of nitrite. Several papers have also suggested the in vivo conversion of

nitrate ions to the more toxic nitrite ion, but these articles were not specific to aquatic species (Walker, 1996; Panesar and Chen, 2000). In the present study, hemoglobin levels were not significantly different between the high and low NO_3-N treatments. There was no evidence of methemoglobinemia, as hemoglobin concentrations for both treatments were actually elevated and above the range reported as normal in rainbow trout, 8.9–15.9 mmol/L (Wedemeyer, 1996). Davison (1997) reported that exercise training increases hematocrit and thus hemoglobin concentration in the blood; thus the elevated hemoglobin concentrations measured for both treatments were likely related to continuous exercise and the rapid swimming speeds observed.

4.3. Growth, survival, and biomass

Despite the increased prevalence of side swimming behavior and slightly increased swimming velocity in the high NO_3-N WRAS, rainbow trout growth rates were not significantly different compared to the low NO_3-N treatment. These results were similar to Study 1 of Davidson et al. (2011a, 2011b) which also indicated statistically similar growth rates of rainbow trout amongst various NO_3-N exposures. Typically, a negative impact to fish growth would qualify as a tertiary stress response that is indicative of excessive utilization of energy reserves to physiologically compensate for an environmental stressor or toxicant (Jobling, 1995). In the present study, blood chemistry results did not provide substantial evidence of even a secondary stress response; which is qualified by hyperglycemia, osmotic imbalance leading to loss of electrolytes, and other blood chemistry indicators such as decreased chloride and sodium concentrations (Jobling, 1995; Wedemeyer, 1996). Therefore, a lack of impact to rainbow trout growth by the experimental conditions is not surprising.

Davidson et al. (2011a, 2011b) reported no difference in rainbow trout survival between WRAS operated at high and low water exchange with mean NO_3-N concentrations of 13 ± 0 and 99 ± 7 mg/L, respectively. Corresponding survival rates during the Davidson et al. (2011a, 2011b) study were 93.1 ± 0.5 and 93.3 ± 1.6%, respectively. However, during the present study, cumulative rainbow trout survival appeared to be negatively impacted by the high NO_3-N treatment at mean concentrations of 91 ± 0 mg/L (Fig. 3). Statistical analysis was not completely conclusive because the resultant *P*-value was 0.05, but a clear separation in survival between treatments was evident as the study progressed (Fig. 3). The resulting survival percentage for the low NO_3-N treatment, 92.5 ± 1.1% was similar to that of Davidson et al. (2011a, 2011b) for trout cultured at 13 mg/L NO_3-N. Decreased survival was not noted for trout cultured at approximately 100 mg/L during Davidson et al. (2011a, 2011b), but fish age/size varied between studies, which could have caused a slightly different response (Sprague, 1985; Camargo et al., 2005). Sprague (1985) noted that when toxicity studies are repeated, results may not correspond precisely due to individual variation of resistance within a group of organisms. In addition, differences in toxic responses between cohorts of the same species to similar concentrations of NO_3-N have been reported (Sprague, 1985; Hamlin, 2007, Pedersen et al., 2012). For example, Pedersen et al. (2012) did not observe negative impacts to rainbow trout survival or swimming behavior when exposing trout to NO_3-N concentrations of approximately 50–200 mg/L. The reason(s) for conflicting results between the present study and Pedersen et al. (2012) are unclear. Many differences existed between respective studies including: rainbow trout genetics (North American vs. Danish strain), initial rainbow trout size (16 vs. 150 g), feeding regime (24 vs. 6 h), feed composition–protein/fat ratio (42/16 vs. 44/30), tank hydraulics (continuous rotational velocity vs. relatively low velocity), and fish exercise (forced continuous swimming at maximum speeds vs. relatively low exercise training). Variables such as tank size and shape, hydraulics, and degree of fish exercise (Jobling, 1995) could partly account for differences in swimming behavior observed in each study.

The combined effect of slightly (but not significantly) lower mean weight and reduced survival for rainbow trout cultured within the high NO_3-N treatment resulted in significantly reduced end biomass. Reduced biomass, no matter the mechanism, is a negative constraint to a private venture fish farmer and equates to decreased profitability. Thus, although the performance effects of the high NO_3-N treatment were not dramatic, they would likely be significant enough to impact outcomes at a fish farm. With knowledge of the results of the present study, a commercial trout farmer would likely choose to operate his WRAS with either a denitrification process or enough water exchange to limit the accumulation of nitrate nitrogen below levels used in the high NO_3-N treatment.

4.4. Water quality

In order to diminish the effects of other potential accumulating toxicants, WRAS were operated at feed loading rates that were approximately four times lower than the Davidson et al. (2011b) study, i.e. 1.3 kg feed/m^3 daily makeup flow (present study) vs. 4.1 kg feed/m^3 daily makeup flow (Davidson et al., 2011b). Thus, concentrations of parameters such as dissolved copper and potassium, which could not be ruled out as potentially toxic by Davidson et al. (2011a), were substantially diluted in the present study and therefore unlikely to cause negative impacts to rainbow trout health and welfare. Although the majority of water quality parameters were controlled between treatments, the authors found it impossible to control for every measured water quality parameter. For example, by attempting to balance conductivity and alkalinity between treatments through the addition of sodium sulfate and sodium bicarbonate, the concentrations of other water quality parameters such as sulfate, dissolved sulfur, and dissolved sodium became statistically different between the high and low NO_3-N treatments. In addition, several other water quality parameters existed at significantly different levels between treatments most likely due to trace amounts present within chemical additives or indirect impacts of the treatment on the bacterial ecology of the WRAS.

For example, nitrite nitrogen was significantly greater within the high NO_3-N treatment, i.e., 0.091 ± 0.012 mg/L compared to 0.021 ± 0.002 mg/L within the low NO_3-N treatment, most likely due to passive denitrification and back conversion of nitrate to nitrite. Although the nitrite nitrogen concentrations were significantly greater within the high NO_3-N systems, mean concentrations were below levels that have been implicated as causing toxicity. Wedemeyer (1996) reported that nitrite can be toxic to rainbow trout at levels >0.2–0.4 mg/L. On-site observations of nitrite nitrogen toxicity to rainbow trout during biofilter acclimation have indicated that rainbow trout do not exhibit brown blood disease until concentrations reach 0.8 mg/L or greater. Buffered toxicity of nitrite is associated with calcium and chloride concentrations of the culture water, as well as pH levels (Wedemeyer and Yasutake, 1978; Wedemeyer, 1996). Chloride and calcium concentrations of the high alkalinity spring water used on-site as makeup are substantial and pH is controlled via automation, thus water quality buffering is enhanced. During the present study, blood hemoglobin concentrations for both treatments were measured at levels above the normal range for rainbow trout; therefore, there was no evidence of a toxic effect of nitrite.

In addition, total suspended solids concentrations were significantly greater within the high NO_3-N treatment, i.e. 6.6 ± 1.1 versus 4.3 ± 0.7 mg/L in the low NO_3-N treatment. Ultraviolet transmittance (UVT) was significantly lower within the high NO_3-N

treatment, reflecting the decreased water clarity related to TSS. Davidson et al. (2009) provided an overview regarding recommended limits for suspended solids, but ultimately little specific information is available. The levels measured during the present study were within a similar range as other on-site studies in which no negative impacts were observed (Davidson et al., 2009, 2011a, 2011b); and the discrepancy between treatments was minimal (Table 4). Therefore, negative impacts to fish performance, health, and welfare related to TSS were unlikely.

Dissolved potassium was also found to be significantly greater within the high NO_3-N treatment. Potassium concentrations for both the high and low NO_3-N treatments were limited because the WRAS were operated at increased flushing rates and substantially lower feed loading rates that were 3–4 times lower than previous studies (Davidson et al., 2011a, 2011b). Potassium concentrations measured during the present study were similar to those within WRAS in which no ill effects were observed during previous studies (Davidson et al., 2011a, 2011b). In addition the discrepancy in dissolved potassium concentration between treatments was less than 4 mg/L (Table 5) and thus would not be expected to illicit measurable differences in performance, health, and welfare. Thus, dissolved potassium was also an unlikely contributor to the chronic effects to rainbow trout observed during the present study.

The only other parameter that existed at a significantly greater concentration within the high NO_3-N treatment was boron. Lowengart (2001) cited normal rainbow trout survival and reproduction in natural waters containing up to 1 mg/L of boron. Boron concentrations within the high NO_3-N treatment were much lower, 0.047 ± 0.001 mg/L, and therefore should not have been problematic.

The concentrations of several water quality parameters (dissolved sulfur, sulfate, and sodium) were found to exist at significantly greater concentrations within the low NO_3-N treatment. Discussion of these parameters and their potential toxicity is warranted due to the unexplained incidence of side swimming and rapid swimming speed in the control treatment. Increased concentrations of dissolved sulfur, sulfate, and sodium were related to dosing sodium sulfate within the low NO_3-N WRAS to control ionic conductivity between treatments. Davies and Hall (2007) determined an LC_{50} for sodium sulfate for lower freshwater aquatic organisms, *Daphnia magna* and *Hyella azteca*, of 5269 mg/L at 250 mg/L hardness as $CaCO_3$ and 3203 mg/L at 100 mg/L hardness as $CaCO_3$, respectively. Each of these studies linked increasing water hardness to a corresponding increase in LC_{50} concentration for sulfate. Elphick et al. (2011) determined a 10-day EC_{10} sulfate concentration of 356 mg/L in soft water (15 mg/L hardness as $CaCO_3$) for early life stage rainbow trout and also linked increasing hardness levels to decreased sulfate toxicity. An EC_{10} is the effective concentration that will have a negative effect (not necessarily a lethal effect) on 10% of the population. Based on the aforementioned literature sources, the hardness levels measured during the present study (>300 mg/L; Table 4) were substantial and likely provided a buffering effect to sulfate and other ionic toxicities. In addition, Heinen (1996) suggested that rainbow trout survival would not be impacted at sulfate concentrations from 850 to 950 mg/L. The average sulfate concentration measured within the low NO_3-N treatment was 262 ± 2 mg/L. Based on current literature; it is unlikely that the sulfate concentrations that accumulated within the low NO_3-N treatment caused any adverse effect to rainbow trout.

Sodium and dissolved sulfur were also detected at significantly greater concentrations within the low NO_3-N WRAS. Increased concentrations of sodium and sulfur were related to addition of sodium sulfate to control the ionic conductivity between treatments. These parameters are generally considered as being innocuous to salmonid species. In fact, increasing sodium concentration has been cited as a means to reduce the toxicity of some water quality parameters such as unionized ammonia (Colt, 2006). In general, very little research is available that evaluates the potential toxicity of sodium or sulfur in only the dissolved elemental form. While it was unlikely that increased sodium and sulfur concentrations elicited any adverse effects to fish within the low NO_3-N treatment, further investigation of these elements as potential toxicants to rainbow trout would be useful.

Lastly, calcium existed at a significantly greater concentration within the low NO_3-N treatment, 118 ± 0 mg/L vs. 114 ± 0 mg/L within the high NO_3-N treatment (Table 5). Recommended limits for calcium for rainbow trout culture range from 4 to 160 mg/L (Heinen, 1996; Piper et al., 1982). Thus, the concentrations measured during the present study were within recommended safe limits. In addition, the difference between treatments was only 4 mg/L.

5. Conclusion

Although most water quality variables were controlled during the present study, unexpected differences between treatments for the concentrations of several water quality parameters inhibited definitive conclusions regarding the effect of nitrate nitrogen. Nonetheless, toxicity of the potentially confounding parameters was unlikely, as supported by data from past on-site studies and other literature. Therefore, results from the present study provide strong evidence that NO_3-N concentrations of 80–100 mg/L were at least partly responsible for the measured chronic effects to rainbow trout under the described rearing conditions.

As recirculating aquaculture systems are designed and implemented for various species, establishment of water quality thresholds should be based on known effects to cultured species. Development of such water quality limits is challenging because these thresholds reflect concentrations that are at the bottom range of toxicity where only mild effects to cultured species begin to occur. The present study likely elucidated the mild/chronic effects of relatively low NO_3-N to rainbow trout such as changes in swimming behavior, as well as slightly decreased survival and reduced total biomass. Based on these findings, the authors currently recommend 75 mg/L NO_3-N as the upper design limit for water recirculating aquaculture systems used for rainbow trout culture. Additional research to evaluate NO_3-N concentrations <75 mg/L and >100 mg/L would be helpful in fine tuning the NO_3-N design threshold for recirculating aquaculture systems used for rainbow trout production at various life stages.

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