Evaluating the suitability of RAS culture environment for rainbow trout and Atlantic salmon: A ten-year progression of applied research and technological advancements to optimize water quality and fish performance

John Davidson

University of Bergen - Dr. Philos Thesis

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Dedicated to my daughter Kamryn,

Work hard and strive for excellence. Follow your passions in life. The sky is the limit!

– Love, Dad

Acknowledgements

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Important abbreviations

Alk – alkalinity
APHA – American Public Health Association
B – boron
Ca – calcium
$cBOD^5$ - carbonaceous biochemical oxygen demand
CO_2 – carbon dioxide
Cu – copper
DOC – dissolved organic carbon
FAO – Food and Agriculture Organization
FCR – feed conversion ratio
Fe – iron
FI – Freshwater Institute
FLR – feed loading rate
HRT – hydraulic retention time
K - potassium
LCA – life cycle assessment
LHO – low head oxygenator
MBR – membrane biological reactor
Mg – magnesium
Na – sodium
NOAA – National Oceanic and Atmospheric Administration
NO_2-N – nitrite-nitrogen
NO_3-N – nitrate-nitrogen
O_2 – oxygen (dissolved)
ORP – oxidation reduction potential
PAA – peracetic acid
P - phosphorus
RAS – recirculating aquaculture systems
S – sulfur
Sr – strontium
TAN – total ammonia nitrogen
TC – total color
TGC – thermal growth coefficient
THBC – total heterotrophic bacteria count
TSS – total suspended solids
USDA – United States Department of Agriculture
USFDA – United States Food and Drug Administration
UVT – ultraviolet transmittance
Zn – zinc

Summary and objectives

Over the last several decades, recirculating aquaculture systems (RAS) have become a viable technology for the production of high-value food-fish. In Norway, for example, many Atlantic salmon smolt farms are now using RAS, and there is increased interest and investment in land-based facilities for the production of larger smolts, post-smolts, and, in some cases, market-size Atlantic salmon. Similar trends are taking shape in other countries, including the United States where multi-million-dollar land-based salmon and trout facilities are being planned and constructed with several already in operation.

RAS continuously recycle water through specialized unit processes that recondition the flow to support intensive fish production. Core advantages of RAS include substantial water savings, diminished waste discharge, and increased flexibility for siting facilities near major seafood markets; however, a critical tradeoff is the accumulation of dissolved nutrients, metals, and compounds that can negatively affect fish health and performance in the absence of proper water treatment and system management techniques. Therefore, research that prioritizes assessment of technologies and operational metrics that optimize the RAS environment has been and will continue to be essential for sustainable industry growth.

During my 21-year career as a researcher at The Conservation Fund's Freshwater Institute, I have focused largely on evaluating the suitability of environmental conditions for salmonid production in RAS. Early research sought to identify accumulating water quality variables of concern, followed by studies designed to establish safe water quality thresholds for salmonids, namely nitrate. Assessment of specialized technologies for water quality control was intertwined with these objectives and is now at the forefront of today's research. As the use of RAS for intensive salmonid production is still a relatively new frontier, novel questions continue to arise and evolve with increasing RAS scale, adoption of new technologies, and the declining availability of clean water resources.

My thesis will track the evolution of research that I have contributed to as author and researcher within the focal area of RAS culture environment with special attention to seven peer-reviewed articles. Each manuscript resulted in novel information regarding the RAS environment for salmonids while raising new questions and providing direction for important follow-up studies. This manuscript provides a history of related research, concluding with up-to-date studies that blend optimization of the RAS culture environment with use of advanced water treatment technologies such as ozone and membrane biological reactor systems. My research synopsis will also focus on the relevance and practicality of these studies to the salmonid aquaculture industry, particularly in the United States, and seeks to extract additional value when considering the results with broader perspective related to water use and technology selection for commercial scale operations.

List of associated publications

This thesis is based on the following published peer-reviewed manuscripts which I contributed to as lead author and researcher. Articles **1-6** were published in *Aquacultural Engineering* and the final article (**7)** was recently published in the journal *Aquaculture*. Herein mention of respective articles will be done with standard citation along with bold numeric text.

1 – Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2011a. Abnormal swimming behavior and increased deformities in rainbow trout *Oncorhynchus mykiss* cultured in low exchange recirculating aquaculture systems. *Aquacultural Engineering* 45, 109-117. https://doi.org/10.1016/j.aquaeng.2011.08.005

2 - Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2011b. The effects of ozone and water exchange rates on water quality and rainbow trout *Oncorhynchus mykiss* performance in replicated water recirculating systems. *Aquacultural Engineering* 44, 80-96. https://doi.org/10.1016/j.aquaeng.2011.04.001

3 - Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2014. 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. *Aquacultural Engineering* 59, 30-40. https://doi.org/10.1016/j.aquaeng.2014.01.003

4 - Davidson, J., Good, C., Russell, C., Summerfelt, S.T., 2017. Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon *Salmo salar* in freshwater recirculation aquaculture systems. *Aquacultural Engineering* 79, 1-8. https://doi.org/10.1016/j.aquaeng.2017.08.003

5 - Davidson, J., Summerfelt, S., Straus, D., Good, C., 2019a. Evaluating the effects of prolonged peracetic acid dosing on water quality and rainbow trout *Oncorhynchus mykiss* performance in recirculation aquaculture systems. *Aquacultural Engineering* 84, 117-127. https://doi.org/10.1016/j.aquaeng.2018.12.009

6 - Davidson, J., Summerfelt, S., Vinci, B., Schrader, K., Good, C., 2019b. Integrating activated sludge membrane biological reactors with freshwater RAS: Preliminary evaluation of water use, water quality, and rainbow trout *Oncorhynchus mykiss* performance. *Aquacultural Engineering* 87, 102022. https://doi.org/10.1016/j.aquaeng.2019.102022

7 - Davidson, J., Summerfelt, S., Espmark, A.M.O., Mota, V., Marancik, D., Early, R., Snead, A. Good, C. 2021. Effects of ozone on post-smolt Atlantic salmon *Salmo salar* performance, health, and maturation in freshwater recirculation aquaculture systems. *Aquaculture* 533, 736208. https://doi.org/10.1016/j.aquaculture.2020.736208

1. Introduction

1.1. Recirculating aquaculture systems – background, advantages, & drawbacks

Recirculating aquaculture systems (RAS) are specialized fish production technologies that recycle and recondition water via mechanical and biological processes (Timmons et al., 2018). RAS typically include solids removal devices (Cripps and Bergheim, 2000; Davidson and Summerfelt, 2005), water recirculation pumps, biofilters that facilitate nitrification (Summerfelt, 2006; Guerdat et al., 2010; Pedersen et al., 2015a), gas conditioning processes for carbon dioxide removal and oxygen addition (Boyd and Watten, 1989; Summerfelt et al., 2000), technologies for fine particle control (Timmons, 1994; Davidson et al., 2011; Holan et al., 2014), and culture tanks designed with hydraulics that promote fish exercise and rapid solids removal (Davidson and Summerfelt, 2004; Gorle et al., 2018; 2019) (Fig. 1). In addition, systems that are nearly "closed", i.e., those that use very little water, often incorporate denitrification unit processes that minimize accumulating nitrate concentrations (van Rijn et al., 2006). Heat exchangers and chillers may also be included in the recycle loop for temperature control (Saidu et al., 2012), and certain RAS designs utilize disinfection technologies such as ultraviolet irradiation and high-dose ozone (Summerfelt, 2003) or application of disinfectants such as peracetic acid (Straus and Meinelt, 2019). Selection of water treatment processes for RAS varies around the world and is dictated by requirements of the cultured species, water availability and intended water use, salinity (freshwater vs. saltwater), water chemistry (hard vs. soft), and local understanding and familiarity of system components. Regardless of design or unit process selection, the primary reason for utilizing RAS technology is to create a controlled environment that optimizes fish performance while maximizing the potential of a finite water resource.

Other advantages are also inherent of RAS (Intrafish, 2018; Timmons et al., 2018), including: i) flexibility for siting facilities near major seafood markets, ii) exclusion of pathogens via indoor biosecurity measures, iii) reduced opportunity for fish escapement and interaction with wild populations, and iv) discharge of small, concentrated effluents that can be effectively treated and repurposed for value-added opportunities such as aquaponics, composting, or biogas production (Bao et al., 2019). In addition, the COVID-19 pandemic (FAO, 2020a) and recent trade tensions between the United States and China (FAO, 2020b) are changing paradigms related to international seafood trade and food security. These events appear to be driving an increased focus on local, domestic supply of aquaculture products which aligns well with RAS due to the propensity for locating these facilities near markets where water resources are scarce and where traditional aquaculture methods are not possible. Accordingly, increased domestic reliance on RAS would result in diminished "food miles", lower costs for shipping aquaculture products, and reduced carbon emissions related to transportation (Liu et al., 2016).

Despite these advantages, a commercial RAS sector has been relatively slow to develop. In fairness, RAS is a relatively new technology for the commercial production of market-size salmonids; however, these systems have been utilized for smaller scale fish production for decades. In fact, RAS have been around long enough that comprehensive books describing engineering metrics and operational details have been published (Timmons et al., 2001; 2018), along with numerous research articles. With this wealth of available knowledge, why then has

RAS been slow to emerge commercially? Reasons for the slow adoption of RAS are seemingly many, ranging from inadequate system designs and mechanical failures, insufficient management and operator error, economies of scale, and inaccurate business modeling and marketing (Badiola et al., 2012; author's personal experience). In addition, an overarching drawback of RAS is the substantial capital investment required to construct systems that include massive tanks, expensive mechanical equipment, monitoring systems, lighting, and other infrastructure components within a surrounding building structure. Liu et al. (2016) reported that the estimated operating costs of RAS and traditional salmon farms are comparable, but the projected capital investment for RAS is approximately 80% greater. Further, Badiola et al. (2018) described the high energy requirement of RAS as a negative attribute of these fish production systems, highlighting increased operational costs and use of fossil fuels (depending on renewable energy resource availability) as disadvantages. Additionally, several life cycle assessment (LCA) studies modeling the environmental impacts of RAS used to produce rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) have suggested detrimental effects including global warming, acidification, and land space use (Samuel-Fitwi et al., 2013; Liu et al., 2016; Song et al., 2019). Albeit, positive LCA attributes have also been described such as reduced carbon footprint related to shipping (Liu et al., 2016), limited water use, and decreased expectation for eutrophication (Samuel-Fitwi et al., 2013).

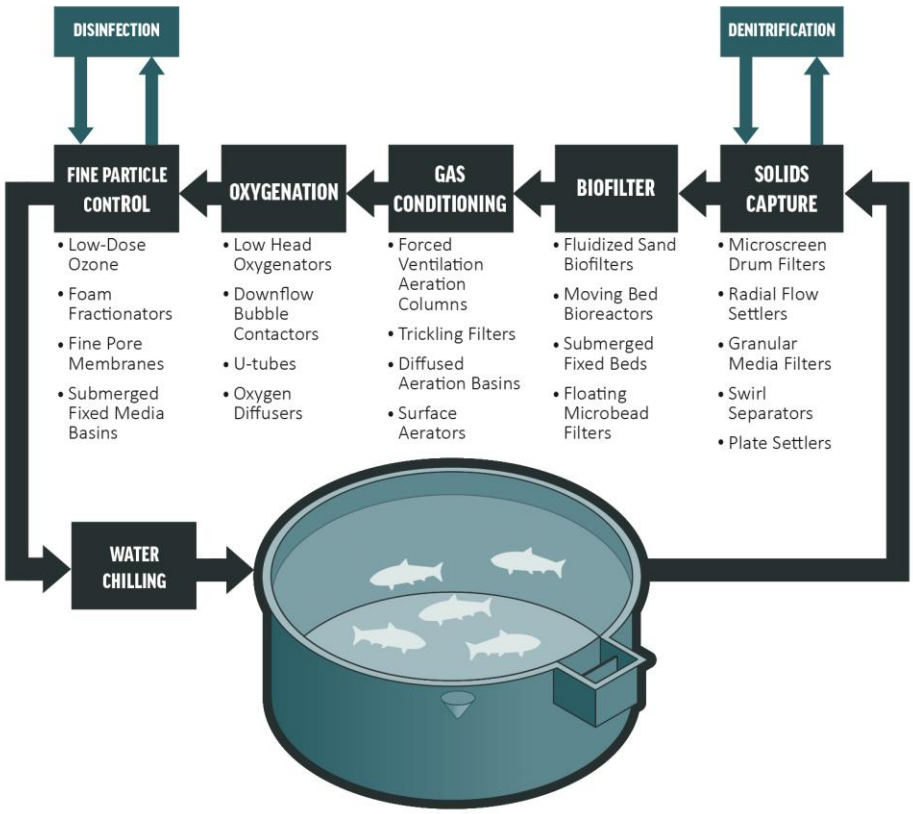

Fig. 1. General RAS design and system unit processes including components that are utilized in commercial industry.

1.2. Factors driving adoption of Atlantic salmon RAS

Recently, the advantages of RAS appear to be outweighing the drawbacks, as evidenced by increased commercial fish production with this technology, particularly for Atlantic salmon farming. Bergheim et al. (2009) described early trends for the transition of salmon smolt production from flow-through to RAS in the Faeroe Islands, referencing favorable attributes such as water savings, temperature and alkalinity control, and improved fish survival in sea cages. A few years later, Dalsgaard et al. (2013) reported that salmon smolt farms in Norway were expanding the use of RAS due to freshwater resource limitations, inlet water quality concerns including carbon dioxide and pathogen levels, and fish growth advantages related to water temperature control (Kristensen, 2009). Further, traditional Atlantic salmon farming companies are beginning to use RAS to produce larger smolts in order to reduce production time at sea and minimize biological risks such as disease, sea lice, and related mortalities (Dalsgaard et al., 2013; EY Global, 2019; Ytrestøyl et al., 2020).

While adoption of RAS by the existing Atlantic salmon industry has primarily focused on smolt production, several developments have leveled the playing field and economic outlook for full life-cycle Atlantic salmon production in RAS (Intrafish, 2018) including: increasing expenses for new ocean farming licenses (Färe et al., 2009; Liu et al., 2016), costs for disease treatment and prevention (Pettersen et al., 2015; Iversen et al., 2020), and regulations and associated expenses for sea lice control (Liu and Bjelland, 2014; Abolofia et al., 2017). For example, to manage sustainable industry growth, Norway, the world's leading producer and exporter of farmed Atlantic salmon, recently established a "traffic-light" system using sea lice risk as the guiding metric for farm site expansion (Bailey and Eggereide, 2020). Each of the previously described factors, among others (e.g. feed, labor, smolt production), have contributed to steady production cost increases for conventional salmon farming (Fig. 2; Iversen et al., 2019; 2020). In addition, generally slowed growth in salmon farming output may be providing directive for alternative production methods. For instance, the world supply of farmed-raised Atlantic salmon increased by 478% from 1995 to the present, but the annual increase over the last decade was 7%, and projected increases from 2019-2023 are only 3% per year (Fig. 3; Mowi, 2020). When considering the example of U.S. per capita salmon consumption, which more than tripled from 1990 to 2017 (Shamshak et al., 2019) along with a recent 6% increase in U.S. salmon imports (NOAA, 2019; FAO, 2020c), current production capacity may not be enough to effectively meet consumer demand. The sum of these factors has culminated in increased investment in a prospective land-based salmon farming industry.

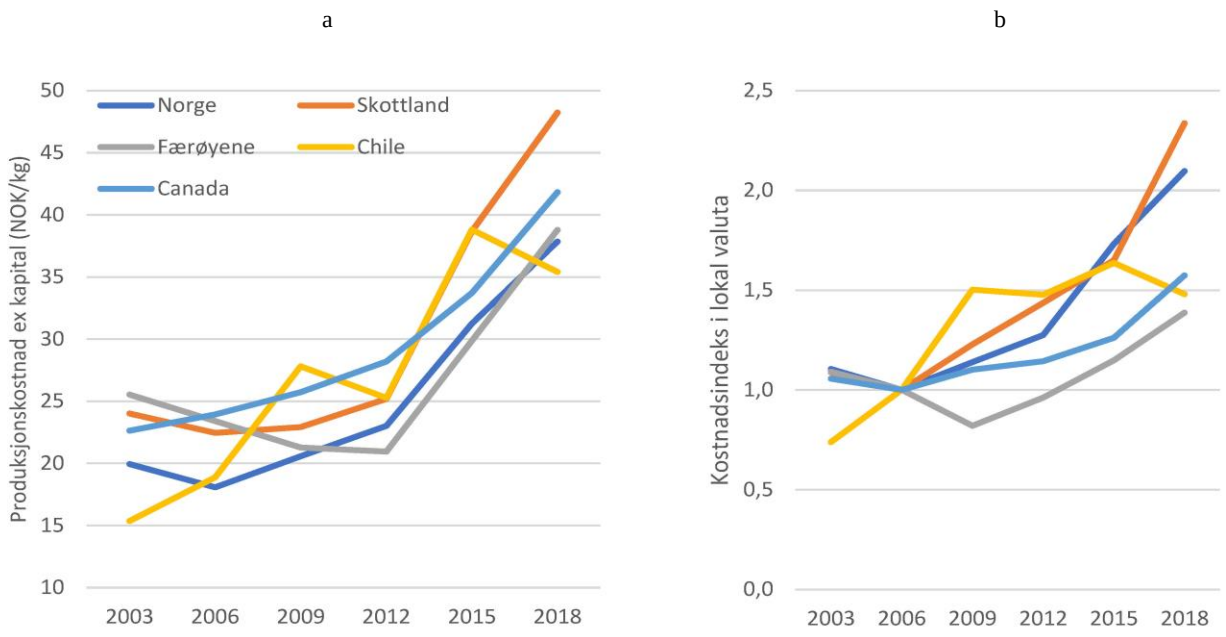

Fig. 2. 2003-2018 Atlantic salmon production costs for Norway, and competitor countries: Faeroe Islands, Canada, Scotland, and Chile for slaughtered (a) and packaged fish (b). Courtesy Iversen et al. (2019).

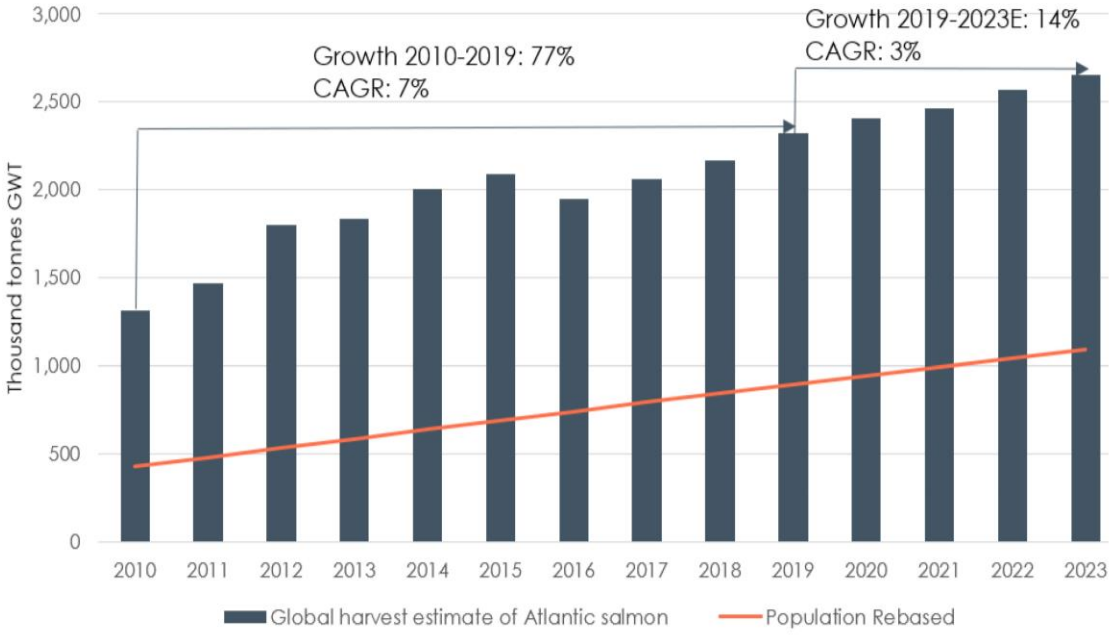

Fig. 3. Increase in global Atlantic salmon production from 2010 to present and expected increases extending to 2023. (Mowi, 2020).

1.3. Commercial RAS for market-size salmonids

The aforementioned factors, along with relatively high farmed Atlantic salmon prices, (NASDAQ, 2020) have opened the door for investment and construction of commercial land-based RAS facilities. EY Global (2019) reported planned production capacity for RAS-produced salmon of 152,100 tons in 2020 with predicted expansion to 973,200 tons by 2022 and beyond. Several notable facilities are already operating in Europe and Canada including Langsand Laks and Danish Salmon (Denmark), Fredrikstad (a subsidiary of Nordic Aquafarms – Norway), Swiss Alpine (Switzerland), Sustainable Blue (Nova Scotia), and Kuterra (British Columbia), while other prospective companies have plans for large-scale projects, including Pure Salmon who announced plans for global production of 260,000 mT of Atlantic salmon (EY Global, 2019). In addition, a handful of commercial RAS farms are already raising market-size Atlantic salmon in the U.S. and have begun to sell product including Atlantic Sapphire (Florida), Superior Fresh (Wisconsin), and Aquabounty (Indiana). Of these facilities, Atlantic Sapphire is poised to become the largest land-based Atlantic salmon producer in the world, with Phase I plans to grow 8,500 mT/yr (2020), Phase II plans to increase production to 30,000 mT/yr, and originally announced plans to grow a maximum of 90,000 mT/yr of Atlantic salmon (Intrafish, 2018). Recently, however, Atlantic Sapphire increased their long-term (2030) annual target volume to 220,000 mT (EY Global, 2019). In addition, other salmonid species are cultured in RAS in the U.S.; for example, Hudson Valley Fisheries (New York) is raising 1,200 mT of steelhead trout, and Local Coho (New York) recently began to produce Coho salmon. Additionally, Riverence, the largest U.S. producer of rainbow trout/steelhead, utilizes some degree of water reuse at its aquaculture facilities in Idaho and Washington State. A host of other RAS-based companies intending to raise salmonids in the U.S. are in planning or early construction phases including Whole Oceans (Maine), Nordic Aquafarms (Maine and California), Indoor Seas (Texas), AquaCon (Maryland), and Pure Salmon (Virginia).

The long list of existing and prospective RAS facilities in the U.S. is not by coincidence. The U.S. ranks only 17th in the world in aquaculture production and imports approximately 90% of the seafood products that are consumed domestically (NOAA, 2020). In 2018 alone, the U.S. imported 2.7 million mT of seafood valued at 22.4 billion U.S. dollars (NOAA, 2019), and salmon imports recently totaled 426,500 mT, reflecting a yearly volume increase of 5.82% and a trend for increased salmon consumption (NOAA, 2019; FAO, 2020c). In fact, salmon recently become the second most consumed seafood in the U.S. (excluding shellfish), surpassing tilapia and lagging only behind shrimp (Shamshak et al., 2019) (Fig.4). Nevertheless, very little salmon is produced domestically. Apart from the previously mentioned RAS start-ups, commercial Atlantic salmon production in the U.S. has historically been limited to conventional net cage farming along the coasts of Maine and Washington state. Limited production of salmon in the U.S. is contrary to the relatively large offshore footprint that is potentially suitable for production (Kapetsky et al., 2013), but further expansion of the traditional salmon industry has been restricted by federal policy and regulations stemming from public perception of environmental impacts (Kite-Powell et al., 2013; Rust et al., 2014). However, an Executive Order entitled, “Promoting American Seafood Competitiveness and Economic Growth” was recently enacted to

boost domestic aquaculture production and to reduce U.S. reliance on imported seafood products (United States Executive Office of the President, 2020). Although this order appears to be largely focused on increasing seafood production via offshore methods, commercial-scale RAS are poised to be at the forefront of U.S. aquaculture industry expansion as evidenced by the growing list of salmonid RAS facilities and proposed startups.

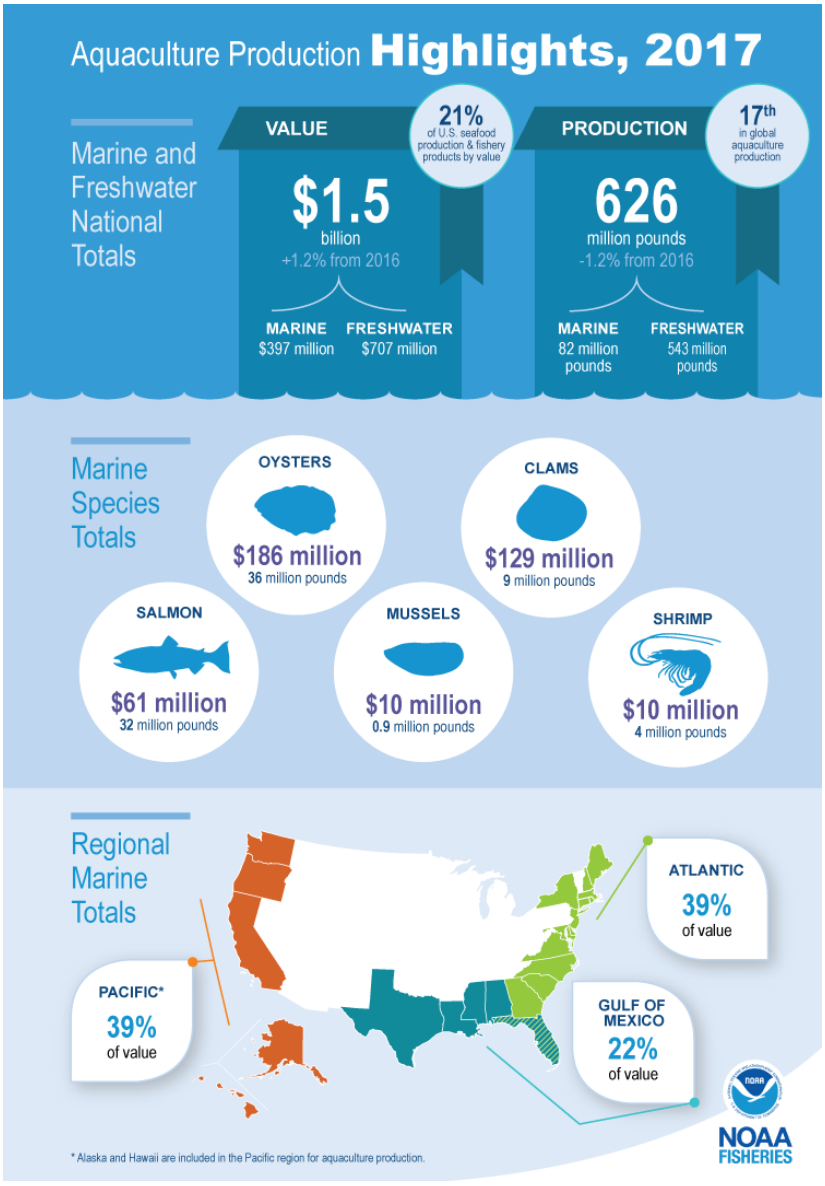

Fig. 4. United States aquaculture production, including salmon, in 2017. Courtesy National Oceanic and Atmospheric Administration.

1.4. Water use and availability for RAS

While the previously described advantages and industry trends are driving adoption of RAS, other factors are influencing the necessity to operate these systems with less water use. Major drivers include: i) increasing scale and expanding production goals of planned RAS facilities (Intrafish, 2018), ii) impetus for locating RAS close to major markets, in some cases far inland, away from the coast, and iii) the general decline and demand for clean water resources (Kummu et al., 2016). It is important to qualify that RAS already provide tremendous water savings compared to traditional land-based aquaculture methods. For example, catfish (*Ictalurus punctatus*) grown in outdoor ponds and rainbow trout cultured in flow-through raceways require approximately 4,000 and 210,000 L of new water/ kg of fish produced, respectively (Timmons et al., 2018), but Liu et al. (2016) estimated that a 3,300 mT land-based RAS salmon farm would only require 803 L of water/ kg of feed. At my place of employment at the Conservation Fund Freshwater Institute (*FI*) (Shepherdstown, WV, USA), a freshwater spring originating from an underground karst aquifer consistently provides 3.8 m^3/min (3,800 L/min) of clean water for research activities that result in the annual production of approximately 15 mT of market-size salmonids. Less than half of this available water is typically required at *FI* due to the use of RAS and partial reuse systems.

Although RAS provide significant water savings, commercial production goals are rapidly increasing, equating to a requirement for more water. Planned salmonid production of 5,000 mT/yr now tends to be near the lower limit for newly announced operations and estimates of $\geq$10,000 mT/yr are not uncommon (Intrafish, 2018; EY Global, 2019). This increased scale of production logically equates to the necessity for greater water use and required water availability. Investors and prospective RAS companies are not naïve to these dynamics and are purposely locating facilities near the coastline to access abundant water resources. An alternate approach, however, is integration of denitrification technologies within the water recycle loop, which allows RAS to be operated with a reduced water requirement (van Rijn et al., 2006; Davidson et al., 2019b - **Study 6**). This scenario may apply more specifically to facilities wishing to locate inland near major seafood markets where clean freshwater resources are available but limited by volume. In this light, the concept of utilizing advanced RAS technologies that require less water may be appealing for future U.S. industry expansion, particularly when considering Figure 4 data which demonstrates a void in inland aquaculture production. Lastly, increasing population pressure, changing water consumption behavior, climate change, and increasing competition for water resources will influence water availability (Kummu et al., 2016) for RAS in the near future. The research studies described in this thesis were developed within this framework, with expected necessity for reduced water use in RAS, and with focus on how this dynamic will impact the fish culture environment and the technology that is required to maintain suitable conditions for rainbow trout and Atlantic salmon production.

1.5. Research and development – Characterizing the RAS culture environment

Continued expansion and future success of the salmonid RAS sector will also depend on research designed to overcome new and unique challenges. The Conservation Fund Freshwater Institute (*FI*) has been at the forefront of research and development of sustainable aquaculture technologies centered around RAS through a longstanding partnership with the United States Department of Agriculture's Agricultural Research Service and through collaborations with other domestic and international partners including CtrlAQUA, Norway. Over this period, *FI* has become a leader in RAS engineering and technological development and a recognized research hub that delivers practical science in support of the aquaculture industry.

In anticipation of the previously described industry trends and water availability dynamics, a specific research focus at *FI* has been characterization and optimization of the RAS culture environment for salmonids, namely rainbow trout and Atlantic salmon. Although the "controlled" environment provided by RAS is typically recognized as a technology advantage, in some respects, RAS provide a new and uncharted habitat for fish production. For example, under low-water-exchange operating conditions, a range of dissolved water quality constituents can reach levels that are atypical of conventional aquaculture systems. In some instances, detrimental biological effects of increasing water chemistry concentrations in RAS have been reported (Deviller et al., 2005; Davidson et al., 2009; Martins et al., 2009a; 2009b). Specifically, Deviller et al. (2005) attributed a 15% growth reduction in European sea bass *Dicentrarchus labrax* cultured in RAS to an unknown "growth-inhibiting substance" and implied that metals accumulation may have been the cause. In addition, Martins et al. (2009a) concluded that ortho-phosphate-P, nitrate, and certain heavy metals (arsenic and copper) accumulated to levels that likely impaired the embryonic and larval development of common carp *Cyprinus carpio* in RAS. Further, several studies have demonstrated reduced growth of tilapia *Oreochromis niloticus* in RAS operated with increasing feed loading rates that supported the accumulation of toxic substances (Martins et al., 2009b; Mota et al., 2015). These trials evaluated the effect of RAS culture environment on warmwater species, but similar research focused on cool and coldwater finfish, including salmonids, was formerly lacking.

Simultaneous to RAS characterization for warmwater species in Europe, research began at *FI* to evaluate the effect of the RAS culture environment on salmonids (Davidson et al., 2009). Anecdotal evidence from onsite demonstration trials indicated that rainbow trout health and survival declined in accordance with reduced RAS dilution rates. These observations were the impetus for early research evaluating water quality and rainbow trout performance in replicated RAS operated with "high" or "low" make-up water flushing rates equating to a 10-fold dilution difference (Davidson et al., 2009). During this study, the majority of measured water chemistry parameters were significantly greater in RAS operated with low flushing; however, this largely different culture environment did not negatively impact rainbow trout growth or survival. In short, the findings of Davidson et al. (2009) suggested that the fish culture environment produced when operating RAS with relatively low water exchange was safe for rainbow trout production in similarly designed and operated freshwater RAS.

Subsequent research (Davidson et al., 2011a; 2011b - **Study 1** & **2**) sought to push the boundaries of RAS operation by further reducing water exchange rates, while characterizing

associated RAS water quality and rainbow trout performance. These studies also incorporated the use of ozone, which will be discussed in greater detail in Section 1.6. During these trials, observations indicative of chronic toxicity, including rapid swimming speeds and increased incidence of "side-swimming" rainbow trout, were observed in RAS with reduced water exchange. Further, when RAS were operated with nearly zero flushing, additional health and welfare concerns were observed including an increased prevalence of spinal deformities, elevated mortality, and unusual swimming behaviors. Causative water quality constituents could not be conclusively identified, but accumulating nitrate and dissolved potassium correlated with adverse rainbow trout health and welfare responses. A detailed literature review pointed to nitrate as the most likely culprit of the chronic toxicity effects.

This research opened the door for subsequent trials evaluating the effect of accumulating nitrate concentrations on rainbow trout reared in RAS. Davidson et al. (2014 - **Study 3**) found that rainbow trout exposed to average nitrate-nitrogen (NO_3-N) concentrations of 90 mg/L demonstrated similar health and welfare issues as those observed during previous studies (Davidson et al., 2011a; 2011b - **Study 1** & **2**). Soon after, coinciding industry momentum for producing Atlantic salmon smolts (Bergheim et al., 2009), larger post-smolts (Dalsgaard et al., 2013), and market-size salmon (Summerfelt and Christianson, 2014) in RAS spurred a similar study to evaluate nitrate thresholds for Atlantic salmon production (Davidson et al., 2017 - **Study 4**). Interestingly, this research demonstrated that Atlantic salmon post-smolts were not negatively affected by mean NO_3-N concentrations up to 100 mg/L under the described conditions, suggesting that nitrate tolerance is variable among salmonid species. Ultimately, the cumulative findings from these trials led to an improved understanding of water quality thresholds and operational metrics required to maintain suitable conditions for rainbow trout and Atlantic salmon in RAS. Additional detail about these studies is included in the Results/Discussion section with increased focus on specific findings and outcomes.

1.6. Effects of oxidants on RAS water quality and salmonid performance

As our understanding of the RAS culture environment evolved, assessment of water treatment technologies and approaches became intertwined with objectives to optimize culture conditions for salmonids. As mentioned, the use of ozone (O_3), a powerful oxidizing agent originally used for potable and wastewater treatment (Rice et al., 1981; Maier, 1984; Langlais et al., 1991), was evaluated to understand its effect on water quality and salmonid performance in freshwater RAS. Prior research indicated that ozone can reduce and improve a range of water quality constituents in aquaculture systems including carbonaceous biochemical oxygen demand, nitrite, turbidity, total suspended solids, and color created by dissolved organic matter (e.g., Rosenthal and Otte, 1980; Rosenthal and Kruner, 1985; Bullock et al., 1997; Summerfelt and Hochheimer, 1997; Summerfelt et al., 1997). However, research evaluating the use of ozone in low- and near-zero-exchange RAS for salmonid production was formerly lacking. As such, a series of trials was carried out to evaluate the effects of low-dose ozone on water quality and rainbow trout health and performance in freshwater RAS operated with high feed loading and reduced flushing. Davidson et al. (2011b - **Study 2**) found that the use of ozone to achieve an

oxidation reduction potential (ORP - indirect measure of ozone residual) of 250-300 mV resulted in significantly improved water quality and faster rainbow trout growth compared to RAS operated without ozone but with similar water flushing rates. A second study described in the same article found that ozone produced culture conditions in low exchange RAS that were comparable to RAS with a 10-fold greater dilution rate (Davidson et al., 2001b - **Study 2**). Further, a recent study (Davidson et al., 2021 - **Study 7**) evaluating the effects of ozone on post-smolt Atlantic salmon performance in freshwater RAS also demonstrated a positive growth effect which overlapped with culture environment improvements similar to those observed by Davidson et al. (2011b - **Study 2**).

Notwithstanding the positive outcomes of ozone use, evaluation and adoption of RAS water treatment processes must take into consideration disadvantages, costs, trade-offs, and risks. For example, although ozone addition resulted in water quality improvements and improved fish growth, ozone also has inherent drawbacks including: health and safety concerns for fish and humans (Gearhart and Summerfelt, 2007), operational complexity, notable capital and operating costs (Summerfelt and Hochheimer, 1997; Gonclaves and Gagnon, 2011), and the propensity to react with naturally occurring bromides in saline aquaculture systems to form toxic byproducts (Tango and Gagnon, 2003). Peracetic acid (PAA), on the other hand, is a relatively new compound for aquaculture use that has been favorably compared to ozone (Pedersen et al., 2015b). Peracetic acid is an antimicrobial agent that is used in a variety of industrial applications including agriculture (USDA, 2016), food processing (Warburton, 2014), medical/dental (USFDA, 2015), and wastewater treatment (Kitis, 2004) that consists of an equilibrium mixture of acetic acid and hydrogen peroxide. Peracetic acid rapidly degrades in aquaculture systems (Pedersen et al., 2009, 2013; Liu et al., 2019) and does not form toxic byproducts that can harm fish or create pollution discharge. Considering these advantages, semi-continuous PAA dosing was evaluated to determine if this alternate oxidizer provides water quality and fish performance benefits similar to ozone, but with a safer, simplistic, and more cost-effective application approach (Davidson et al., 2019a - **Study 5**).

1.7. Integrating denitrification technologies – Membrane biological reactors

Several of the highlighted trials suggested that nitrate is a limiting factor to fish production in RAS, as it is not reduced by ozone or other unit processes in a RAS design that excludes denitrification technologies (Davidson et al., 2011a; 2014 – **Study 1 & 3**). Instead, each of these studies relied on dilution of accumulating water quality concentrations, namely nitrate, to maintain safe culture conditions for rainbow trout and Atlantic salmon. In this scenario, maximum achievable fish production is dictated by the amount of supply water available at a facility. However, when considering the increasing scale and expanding production goals of commercial RAS, it is apparent that these large operations will require access to sizeable water volumes that may not be prevalent across all geographies. With this in mind, integrating denitrification technology within the water recycle loop of RAS was considered as the next logical step. Under anoxic conditions and in the presence of a sufficient carbon source,

facultative anaerobic bacteria drive the typical denitrification process which reduces nitrate to dinitrogen gas (van Rijn, 2006).

$$\text{Denitrification} = NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N^2O \rightarrow N^2$$

Effective denitrification lowers the water requirement of RAS, resulting in greater flexibility to site facilities where water resources are scare while maximizing the fish production capacity of a given water supply. As such, Davidson et al. (2019b - **Study 6**) evaluated RAS-integration of activated sludge membrane biological reactors (MBRs), an advanced water treatment technology capable of denitrification. Like many water treatment technologies that are used in RAS, MBRs were adopted from the wastewater treatment industry where they are used to remove nutrients, organics, and solids from concentrated effluents (Van der Bruggen et al., 2003; Hai and Yamamoto, 2011). MBRs utilize a series of fine-pore (< 0.2 µm) membranes that create a low-solids, semi-purified filtrate, while aerobic and anoxic microbial processes within an activated sludge facilitate nitrification and denitrification, respectively (Hai and Yamamoto, 2011; Ozgun et al., 2013). MBR treatment of a solids-rich aquaculture effluent was first evaluated at *FI* without returning the filtrate to RAS (Sharrer et al., 2007; 2010). During these studies, MBRs produced a clean permeate containing < 3 mg/L total nitrogen, <0.1 mg/L total phosphorus, <1 mg/L carbonaceous biochemical oxygen demand and total suspended solids, and significantly reduced heavy metals concentrations, indicating that MBR integration directly within RAS might be feasible. As such, a study was designed, where single-vessel MBRs (Fig. 5) were integrated within the water recycle loop of three RAS, while three control RAS were operated without MBRs and typical water exchange rates. MBR reactor vessels captured RAS backwash water that otherwise would have been pumped to a separate wastewater treatment area, creating an activated sludge containing nitrifying and denitrifying microbial populations. Simultaneously, fine-pore membranes within the MBR vessel, produced a relatively clean permeate that was returned to the RAS, resulting in a nearly closed system with limited water exchange. Rainbow trout performance, RAS water quality, and water use metrics were evaluated to understand the feasibility of this new approach.

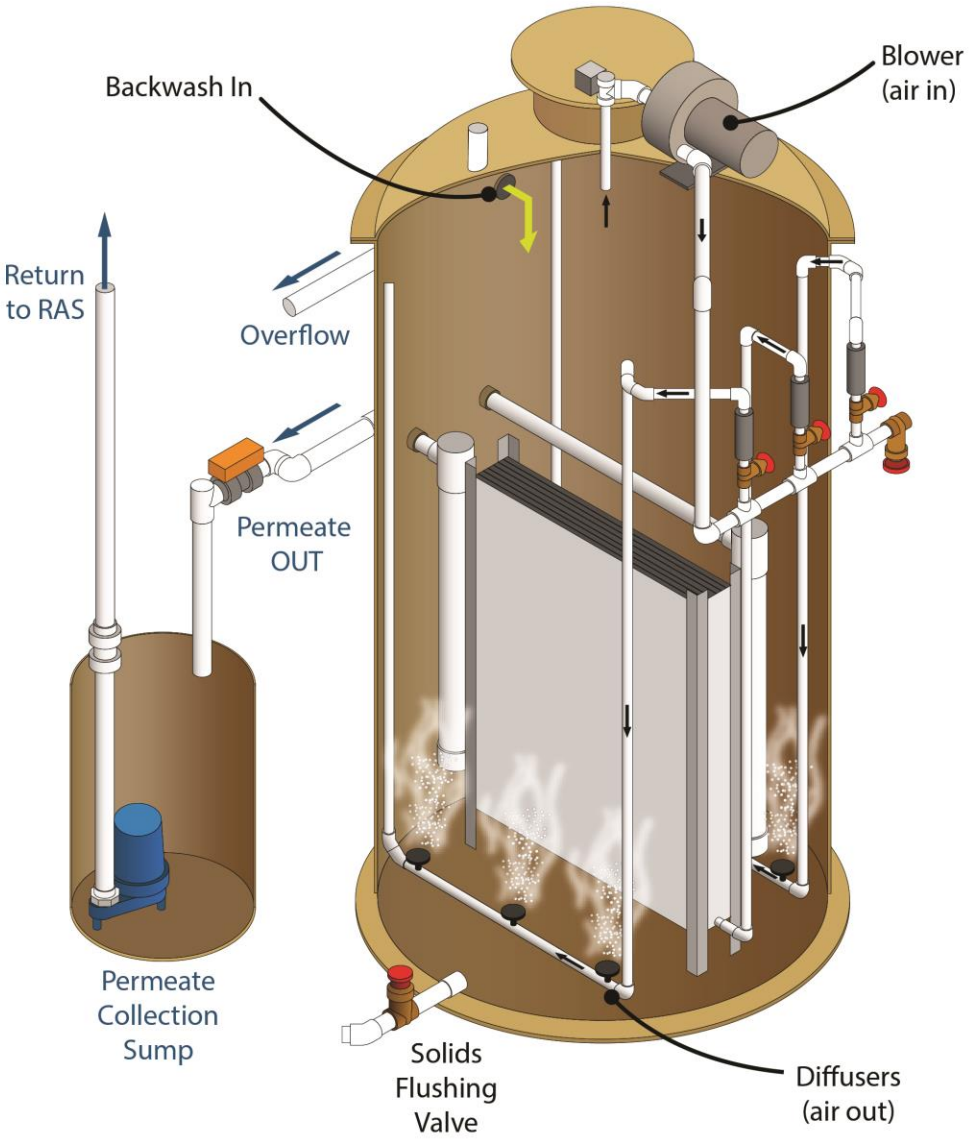

Fig. 5. Cross-sectional schematic of individual MBR showing membrane module positioning, air delivery system, and inlet and outlet water flow locations and direction (Davidson et al., 2019b - **Study 6**).

1.8. Thesis novelty and value-added analysis

Each of the studies (**1-7**) highlighted in this manuscript provided insight regarding the suitability of rainbow trout and Atlantic salmon production in RAS while operating systems with various water exchange rates and while integrating specific water treatment processes (ozone, PAA dosing, and membrane biological reactors). This body of research also provided a depth of new information regarding appropriate culture conditions for salmonid production in RAS. All of the supporting papers have been published in peer-review journals, concluding with recent acceptance and publication of the last article (Davidson et al., 2021 – **Study 7**) in the journal *Aquaculture*. As such, most of the information contained in this document has been publicly disseminated and remains openly available. Nevertheless, this thesis provides new perspective by describing more than a decade worth of related research along with the pragmatic approach that was used in its development. The thesis format is also novel in that many important research outcomes pertaining to rainbow trout and Atlantic salmon production in RAS are described comprehensively in one location. In addition, this holistic approach led to extraction of new information regarding water use and flushing requirements. For example, a fairly robust understanding of how much water is required to safely produce a given biomass of rainbow trout and Atlantic salmon in similarly designed RAS was revealed during each of the highlighted research trials. Finite definition of water flow rates required to maintain optimal culture conditions provides important information to stakeholders, fish production managers, and prospective RAS companies. For example, this research data may aid in identification of sites that offer sufficient water volumes and flows to meet expected fish production goals (mT). Additionally, insight regarding selection of appropriate RAS technology based on water availability, i.e. RAS that flush more water to dilute nitrate, or tightly operated RAS with denitrification technologies was gleaned and is described in the Results/Discussion section.

2. Methods

A summary of experimental methods used for **Studies 1-7** is provided in the following sections. All of the highlighted articles utilized the same set of six replicated RAS. Use of identical fish production systems for each study was beneficial for research continuity and subsequent data summaries. Additionally, three RAS were assigned to each of two treatments (N=3) for every trial; therefore, similar statistical methods were employed. Methods that were common to each study or several studies are provided herein, while project-specific methodologies are described in detail in the published articles, which are included later in this document.

2.1. RAS engineering, design, and operation

Six replicated RAS (9.5 m^3 total volume) originally described in Davidson et al. (2009) were used for each trial. The base design of an individual experimental scale RAS is shown in Fig. 6. Generally, each RAS recirculated 340-379 L/min of freshwater through a 5.3 m^3 dual

drain culture tank, radial flow settler, microscreen drum filter with 60 µm screens, pump sump containing water recirculation pumps, fluidized sand biofilter, geothermal heat exchanger, gas conditioning column, and a low-head oxygenator (LHO) (Fig. 6). Hydraulic retention time of the fish culture tank was 15-16 mins. This general RAS design, with the exception of tank HRT which is typically $\geq$ 30 mins in commercial operations, has been used at *FI* for more than two decades and similar system configurations have been installed and utilized at several commercial RAS facilities in North America. Important metrics used to describe water flushing rates were calculated for each study including feed loading rate (FLR), system hydraulic retention time (HRT), water recycle rate, and liters of new water/kg feed.

The following calculations were used for each of these metrics:

FLR = daily feed (kg)/daily makeup water addition (m^3)

HRT (days) = total RAS volume/ daily makeup water volume per day

Water recycle rate (%) = ((total recycle flow – makeup water flow)/total recycle flow) * 100

L/ kg feed = daily makeup water flow (L)/ daily feed ration. Note that this metric was not specifically used in the published articles but is provided as an additional guide for RAS engineers, designers, and operators.

Specific variations to RAS design and operation were applied depending on study objectives. For example, for trials evaluating the effects of ozone on salmonid health and performance and the fish culture environment (Davidson et al., 2011a; 2011b; 2021 – **Study 1, 2,** & **7**), ozone was generated onsite from a pure oxygen feed gas using a Model G22 generator (Pacific Ozone Technology, Benicia, CA, USA) and added within the air space beneath the LHO water distribution plate. To prevent ozone residuals from reaching unsafe levels, ORP was monitored in each tank using a digital sensor (Model DRD1R5, Hach Company, Loveland, CO, USA) located near the tank inlet, and SC100 Universal Controllers (Hach Company) provided proportional-integral-derivative control of ozone generator output to maintain ORP levels at 250-320 mV. Target ORP varied depending on study.

For each trial, sodium bicarbonate was periodically added to RAS in daily batch doses to maintain alkalinity levels that support nitrification (Summerfelt et al., 2015), typically 100-200 mg/L as $CaCO_3$. Makeup water, which was accessed from an underground, freshwater spring and associated pumphouse, was added to RAS pump sumps. Methods for makeup water addition and maintenance of specific flow rates varied depending on study objectives. For certain studies, makeup water was added as a continuous, regularly calibrated flow to maintain a specified dilution rate. For other trials, makeup water addition was dictated by the combined water volume removed as drum filter backwash and radial flow settler discharge, which was sensed by a float valve and replaced with an equal volume of spring water. Cumulative makeup water addition was measured in each RAS by a magnetic drive flowmeter (Model C700, Elster AMCO Water Inc., Ocala, FL, USA) installed upstream of the float valve.

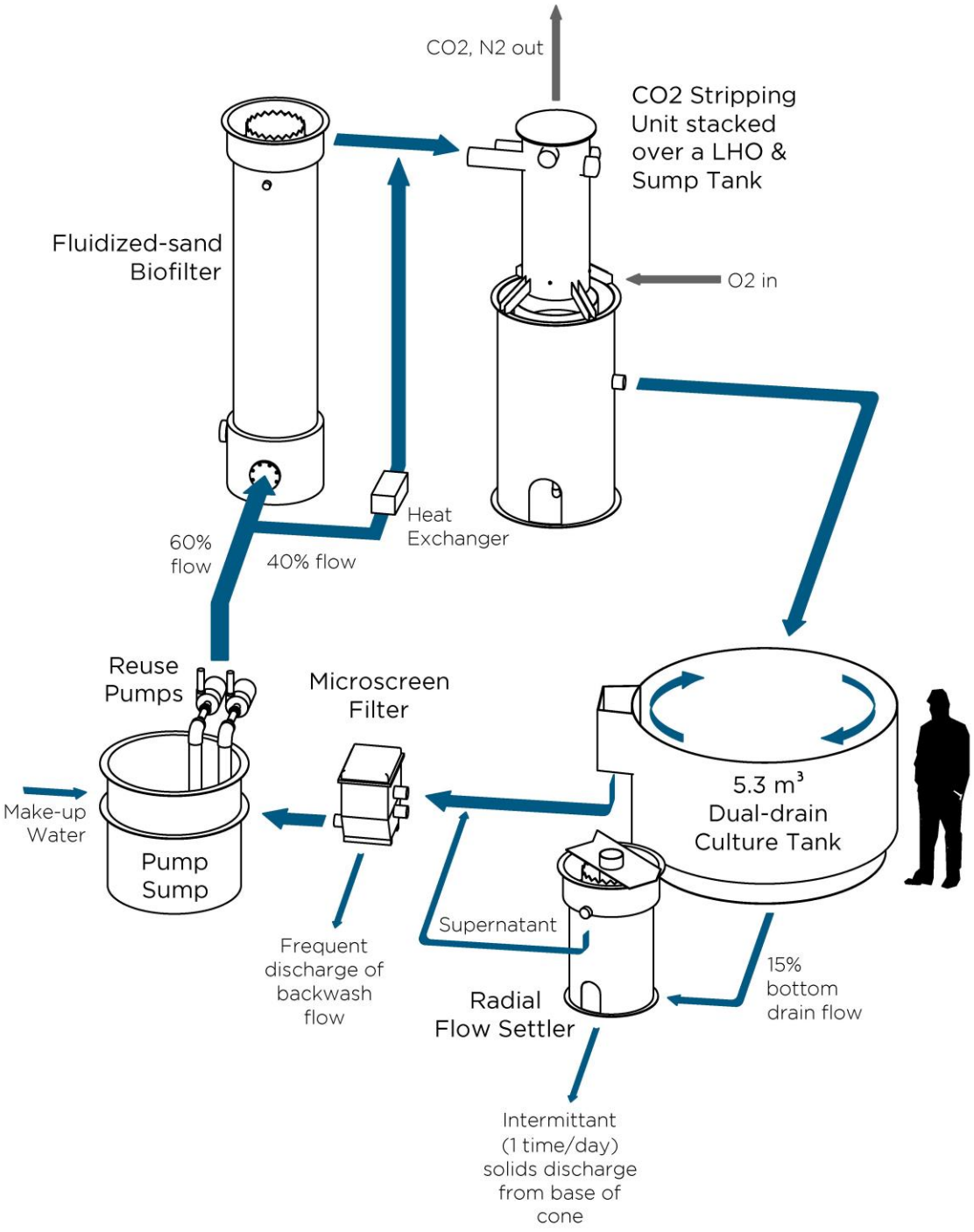

Fig. 6. General design of an individual experimental RAS used for Studies 1-7 at the Freshwater Institute, including water treatment processes and recycled water flow direction.

2.2. Fish background, feeding, and sampling regimens

Rainbow trout, commonly referred to as steelhead at larger adult sizes, and Atlantic salmon were used for various research trials. These salmonid species were selected based on suitable water temperature for culture at *FI* and their relevance for commercial production in RAS. All-female rainbow trout, obtained by crossing sex-reversed females of male phenotype with normal females (Johnstone et al., 1979), were used for select studies. Eggs were procured from commercial suppliers with the exception of Davidson et al. (2019b - **Study 6)** which utilized fingerlings from the nearby National Center for Cool and Cold Water Aquaculture (Leetown, WV, USA). Eggs were hatched onsite, and fry and fingerlings were reared in separate flow-through and partial reuse aquaculture systems prior to stocking in the six replicate RAS. Overall, the pre-study early rearing regime typically consisted of RAS incubation at 7-8 ^{o}C, flow-through fry culture at 12.5-14.0 ^{o}C, and intermediate production in a partial reuse system at 12.0-14.5 ^{o}C. Continuous 24-h light was employed throughout the production cycle for rainbow trout, while the general photoperiod approach for Atlantic salmon was provision of 24-h light until the fish reached approximately 40 grams followed by an approximate 6-week winter photoperiod (12:12 LD) to induce smoltification and subsequent return to 24-h light per industry standard practices. Specific photoperiod regimens utilized for each trial are described in the published articles.

To begin each experiment, fish were relocated from the pre-study aquaculture systems, counted, and randomly stocked to achieve equal numbers of fish per replicate RAS. A short-term acclimation period was generally provided before subjecting fish to various treatment conditions. After study commencement, fish were fed to apparent satiation using a computer operated feeding system (The Conservation Fund Freshwater Institute, Shepherdstown, WV, USA) integrated with automatic feeders (T-drum 2000CE, Arvotec, Huutokoski, Finland) that were programmed for 24-h feeding and short interval feed delivery. Feeding rates were fine-tuned separately per individual RAS based on observations of feeding activity and wasted feed. A constant 24-h photoperiod was generally provided to accommodate around-the-clock feeding and consistent water quality, and commercially available salmonid diets were used for each study.

Lengths and weight measurements of a random sample of fish were collected at monthly or bimonthly intervals depending on study. Fish sample size was calculated using equations from Bhujel (2008) and Martin et al. (1987). Mortalities were removed and recorded daily to assess cumulative survival, and thermal growth coefficient (TGC), feed conversion ratio (FCR), and fish survival (%) were calculated using the following formulae and reported as important fish performance metrics in each article.

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

where weight is in grams, length is in mm, and temperature is in ^{o}C.

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

$$\text{Survival (\%)} = ((\text{Initial Number of Fish} - \text{Cumulative Mortalities}) / \text{Initial Number of Fish}) * 100$$

2.3. Water quality sampling and analyses

To comprehensively evaluate the fish culture environment during each study, water samples were collected from the side drains (outflow) of each fish culture tank at weekly intervals. The brunt of water chemistry analyses was performed in *FI*'s Water and Environmental Chemistry Laboratory by trained personnel following methods described in APHA (2005; 2012) and HACH (2003; 2015). Detailed descriptions of analytical methods and associated equipment are described in the Methods sections of respective papers, as exemplified in Table 1. Common analytes included alkalinity (Alk), carbonaceous biochemical oxygen demand ($cBOD^5$), carbon dioxide (CO_2), total heterotrophic bacteria count (THBC), nitrate nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), phosphorus (P), total ammonia nitrogen (TAN), total suspended solids (TSS), true color (TC), and ultraviolet transmittance (UVT). Dissolved oxygen (O_2), temperature, and ORP were monitored and recorded from Hach SC100 Controller units equipped with LDO probes and differential ORP sensors (Hach Company, Loveland, CO, USA). In addition, a range of dissolved metals and elements were periodically analyzed by outside contract laboratories (Cornell Nutrient Analysis Laboratory, Ithaca, NY, USA; REI Consultants Inc. Beaver, WV, USA). Initially, a group of 25-28 dissolved metals was regularly assessed (**Study 1-5**). Later, a subset of dissolved metals was established for analysis based on consistent positive detection in RAS water. Commonly detected metals included barium, boron (B), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), potassium (K), sodium (Na), strontium (Sr), sulfur (S), and zinc (Zn) (**Study 6 & 7**).

2.4. Statistical analyses

Weekly water quality data collected over the duration of a study was generally averaged per individual RAS and a grand mean with associated standard error was then calculated per treatment (N=3). A similar data summary approach was used for fish performance metrics collected at given sampling intervals. Water quality data was analyzed using a mixed models approach that assigned water quality criterion as dependent variables; treatment, time, and treatment x time as independent fixed factors; and RAS/tank as a random effect variable nested within treatment (Ling and Cotter, 2003; Thorarensen et al., 2015). Data transformation and/or removal of outliers were carried out as needed when analyzing water chemistry data. Response variables that were measured less frequently, i.e. five or fewer events per study, including dissolved metals concentrations and fish performance metrics (e.g., weight, FCR, TGC) were typically analyzed using a Student's t-test (means comparison) for data collected at each sampling point. Normality was assessed using a Shapiro-Wilk test and non-Gaussian distributed data were analyzed using the Kruskal Wallis or Mann Whitney test. A probability level of 0.05 was generally used to determine significance. Statistical analyses were carried out using SYSTAT 13 software (2009). Minor variations to statistical approaches occurred between studies. Detailed statistical methods for each published manuscript are provided in the published articles, which are included at the end of this document.

Table 1. Example of water quality parameters evaluated, methodologies, and frequency of testing (Davidson et al., 2021 – **Study 7**).

Parameter	Method of Analysis	Frequency of Recording/Testing
Dissolved Oxygen	Hach SC100 Controller & LDO® Probe	Daily
Oxidative Reduction Potential	Hach SC100 Controller & Differential ORP Sensor	Daily
Temperature	Hach SC100 Controller & Differential ORP Sensor	Daily
Specific Conductance	YSI 30 Salinity/Conductivity/Temperature Meter	3-4 times weekly
Alkalinity	Hach Method 8203 - Sulfuric Acid Digital Titration pH endpoint Accumet #AB150	2-3 times weekly
pH	Standard Methods 4500-H^+ B – Electrode	2-3 times weekly
Biochemical Oxygen Demand	Standard Methods APHA 5210B - 5-day test (No prefiltration) YSI Model 58, YSI BOD probe #5905	Once weekly
Carbon Dioxide	Hach Method 8223 - Sodium Hydroxide Burette Titration pH endpoint Accumet #AB150	Once weekly
Dissolved Ozone	Hach Method 8311 (0.01-1.5 mg/L as O_3)	
Nitrate Nitrogen	Hach Method 8171 - Cadmium Reduction	Once weekly
Nitrite Nitrogen	Hach Method 8507 USEPA Diazotization	Once weekly
Total Ammonia Nitrogen	Hach Method 8038 USEPA Nessler	Once weekly
Total Heterotrophic Bacteria	Hach Method 8242 - Membrane Filtration, Fischer Isotemp Incubator #516D	Once weekly
Total Phosphorus	Hach Method 8190 – USEPA PhosVer3 with Acid Persulfate Digestion. DRB200 reactor and Hach Method 10127 (Molybdovanadate w/ Acid Persulfate Digestion)	Once weekly
Total Suspended Solids	Standard Methods APHA 2540D - Dried at 103-105 ^{o}C. Thelco Oven #6540, Mettler Toledo #AE240 and #PM30K	Once weekly
True Color	Hach Method 8025 - Platinum-Cobalt Standard	Once weekly
UV Transmittance	Hach Method 10054 - Organic UV Absorbing (UV-254)	Once weekly
Dissolved Metals	Inductively Coupled Plasma Atomic Emission Spectrometry	Monthly - 4 events

-Spectrophotometers DR2700 and DR6000 (Hach Company, Loveland, CO, USA) were used for analysis of dissolved ozone, nitrate nitrogen, nitrite nitrogen, total ammonia nitrogen, and total phosphorus. Spectrophotometer DR4000 (Hach Company) was used for analysis of true color and UV transmittance.

3. Results and discussion

The research articles highlighted in this thesis characterized the RAS culture environment for rainbow trout and Atlantic salmon and identified flushing, operational methods, and water treatment technologies that optimize water quality and fish performance. A synopsis of major findings was provided in the Introduction to cohesively explain study relationships and practical research progression. Important outcomes are elaborated on herein. In addition, when considering the connectivity of this body of work to the growing commercial industry, it became obvious that water flushing data gleaned from these studies was applicable to decisions related to site selection, RAS design, and establishment of realistic fish production goals; therefore water use, which was a thematic aspect of the highlighted studies, is included as a focal point of discussion.

3.1. Characterizing the RAS environment – Early research

The highlighted research built on previous knowledge regarding optimal culture conditions for salmonids in traditional aquaculture systems (e.g., Piper et al., 1982; Wedemeyer, 1996) and in RAS (Timmons et al., 2001; Colt, 2006). The springboard for new research, which I contributed to as author and researcher, was a study comparing the effects of RAS operated with "high" and "low" make-up water flushing rates on rainbow trout performance and water quality (Davidson et al., 2009). These general flushing terms (high and low) described water exchange rates equivalent to 2.6 and 0.26% of the recycle flow, which resulted in 0.67- and 6.7-day system HRT, respectively, a ten-fold difference in dilution. Objectives of this initial project were: i) to gain an improved understanding of RAS water quality resulting from reduced flushing, and ii) to determine if rainbow trout (initial weight – 133 g) health, performance, and welfare could be maintained under the described conditions. Not surprisingly, operating RAS with reduced water exchange resulted in a concentrated environment where nutrients, ions, and compounds accumulated. With the exception of controlled parameters (O_2, temperature, and CO_2), the majority of measured water chemistry concentrations were greater in RAS operated with low water exchange (Davidson et al., 2009). Accumulating water quality constituents included: $cBOD^5$, dissolved organic carbon (DOC), fine particle counts, NO_2-N, NO_3-N, TAN, THBC, TSS, TC, unionized ammonia, and dissolved concentrations of B, Cu, Mg, P, K, Na, Sr, S, and Fe. In addition, Alk and UVT were significantly reduced in low exchange RAS. Despite the largely different culture conditions created by the tested flushing regimens, rainbow trout performance was statistically similar between treatments. Mean fish weights overlapped throughout the study and TGC, FCR, and survival were similar ($P > 0.05$) for trout reared in high and low exchange RAS.

Ultimately, Davidson et al. (2009) indicated that the measured water quality concentrations, although increasingly concentrated in low exchange RAS, generally were not harmful to rainbow trout under the base study conditions. Nevertheless, an in-depth literature review was carried out to understand how these concentrations compared to toxic levels ascertained during other trials. This exercise revealed that dissolved Cu was a possible parameter of concern for rainbow trout production in low exchange RAS depending on its interaction with

other environmental metrics. For example, Cu toxicity is largely influenced by coinciding water quality parameters such as water temperature, pH, Alk, hardness, and DOC (Spear and Pierce, 1979; Sprague, 1985; Wedemeyer, 1996). Analysis of Cu toxicity thresholds suggested that relatively high DOC concentrations may have prevented acute effects of Cu to rainbow trout during Davidson et al. (2009). Further, Wedemeyer (1996) noted that Cu levels that typically cause lethal gill damage are less toxic in hard, alkaline water (pH > 7, total hardness > 200 mg/L as $CaCO_3$), conditions similar to Davidson et al. (2009). Although rainbow trout survival was > 98% within each RAS during this trial, it is important to note that regression analysis indicated that higher Cu levels correlated with mild, but increasing rainbow trout mortality (Davidson et al., 2009).

3.2. Towards an improved understanding of RAS environment

The findings of Davidson et al. (2009) set the stage for subsequent research that is central to this thesis. Because rainbow trout performed well under reduced flushing conditions, it was logical to push the boundaries further to determine if this species could be cultured with less water exchange and within a more concentrated environment. Three successive trials were carried out using replicated RAS operated with various water exchange rates and with overlapping use of ozone while rearing rainbow trout. These studies included: i) RAS operated with low water exchange rates (similar to those utilized during Davidson et al., 2009) with and without ozone; ii) low water exchange with ozone versus high water exchange without ozone, and iii) near-zero water exchange with and without ozone. Two publications resulted from this work (Davidson et al., 2011a; 2011b – **Study 1 & 2**). For purposes of maintaining a synchronous description, findings related to the effect of RAS environment on rainbow trout health and performance will be discussed first followed by an explanation of the effects of ozone.

Davidson et al. (2011a – **Study 1**) focused on the water quality conditions resulting from various RAS flushing rates and associated consequences to rainbow trout production. When RAS were operated with low water exchange (6.7-day HRT) rainbow trout exhibited symptoms indicative of chronic toxicity including increased swimming speeds and an unusual swimming behavior best described as “side-swimming.” Fish exhibiting the side-swimming condition were tilted on their sides parallel to the horizontal plane of the tank, but otherwise able to swim, feed, and maintain buoyancy in the water column. Interestingly, a significantly reduced prevalence of trout exhibiting this condition (~ four times fewer) was observed in RAS operated with a ten-fold greater dilution rate (Fig. 7, 8). Rainbow trout growth was unaffected by flushing, but cumulative survival bordered significance, where slightly greater mortality occurred in low exchange RAS.

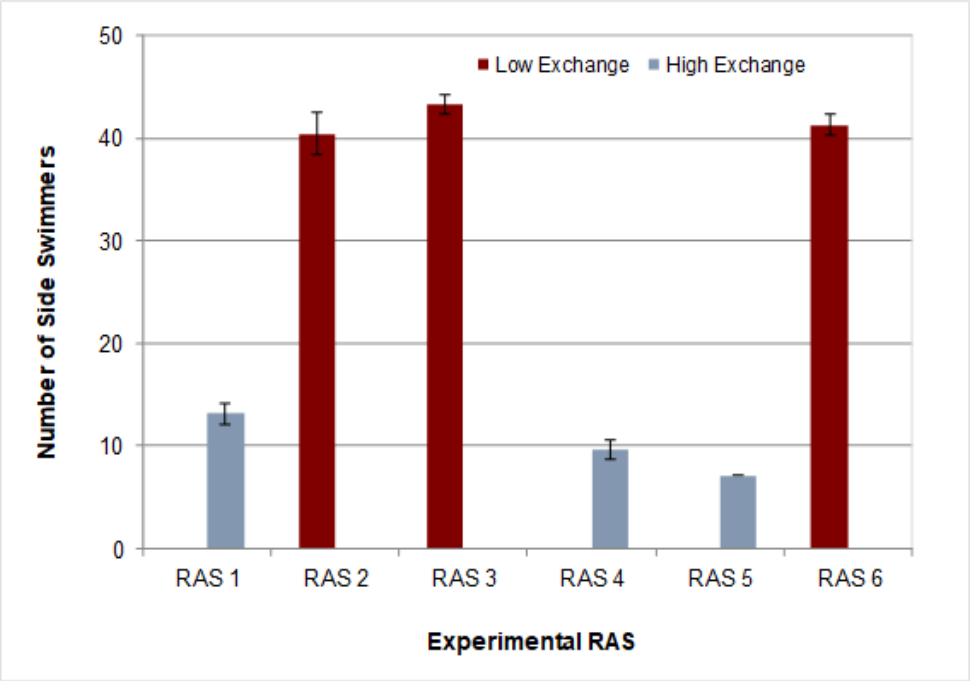

Fig. 7. Number of side-swimming rainbow trout counted in replicate RAS operated with high vs. low water exchange rates (Davidson et al., 2011a - **Study 1**).

Fig. 8. Overhead video screen captures of rainbow trout swimming in RAS operated with low water exchange (left) versus high water exchange (right). Side-swimming rainbow trout are evidenced by light reflecting off of the tilted surface of fish bodies (top) (Davidson et al., 2011a - **Study 1**).

Additional health and welfare concerns were observed during a second study described in the same article which evaluated the effects of near-zero water exchange on rainbow trout while operating RAS with and without ozone (Davidson et al., 2011a; **Study 1**). During this trial, other unusual behaviors were observed including fish swimming near the surface while exhibiting a yawning or gasping behavior. Rainbow trout cultured under these conditions demonstrated elevated mortality and an increased prevalence of spinal deformities, where negative health and welfare responses were observed in RAS with the lowest water exchange and highest feed loading rates. For example, mean rainbow trout survival in RAS operated with near-zero water exchange (> 103-day HRT) and very low water exchange (< 36-day HRT) was 85.7 ± 1.9% and 94.6 ± 0.4%, respectively. In addition, rainbow trout from RAS operated with 180-day HRT exhibited spinal deformities (Fig. 9) in 38% of the population, while trout cultured in RAS with a 5-day HRT completely lacked spinal deformities (Davidson et al. 2011a – **Study 1**). The authors hypothesized that contorted movements associated with erratic swimming behaviors instigated an increased prevalence of skeletal deformities. This assertion is supported, in part, by research indicating that various fish species including sea bass *Dicentrarchus labrax* L. (Divanach et al., 1997), common carp *Cyprinus carpio* (Backiel et al., 1984), and red sea bream *Pagrus major* (Kihara et al., 2002) exposed to consistently strong currents developed lordosis due to unusually intense muscular activity (Berillis, 2015). Increased lordosis has also been correlated with a dysfunctional swim bladder in sea bass and sea bream *Sparus auruta* (Chatain, 1994).

Fig. 9. Examples of spinal deformites observed in rainbow trout cultured in RAS operated with near-zero water exchange (Davidson et al., 2011a – **Study 1**).

At the time of these trials (Davidson et al., 2011a – **Study 1**), finite conclusions could not be drawn regarding exact water quality parameters or combinations thereof that caused the negative health and welfare responses observed in rainbow trout populations. Dissolved Cu, which had been flagged as a parameter of concern (Davidson et al., 2009), was highlighted again as a possible culprit; however, correlation analysis indicated that NO_3-N and dissolved K concentrations were closely related to the health and welfare issues (Davidson et al., 2011a – **Study 1**). Interestingly, unlike many of the water quality constituents evaluated during these trials, NO_3-N and K generally increased unabated in RAS with minimal reduction by water treatment processes, including ozone. Average NO_3-N and K levels of 99 and 25 mg/L, respectively, overlapped with increased rainbow trout swimming speeds and greater prevalence of side swimming, while mean NO_3-N and K concentrations of > 400 mg/L and 112 mg/L were recorded during the near-zero exchange study when severe spinal deformities and increased mortality were observed (Davidson et al., 2011a – **Study 1**).

3.3. Nitrate threshold for fingerling rainbow trout

The findings of Davidson et al. (2011a – **Study 1**) prompted additional literature review to evaluate the likelihood for NO_3-N and K concentrations to negatively impact rainbow trout. Several important publications suggested that typical nitrate levels in RAS are harmless to fish; however, these conclusions appeared to be based on LC50 studies, as opposed to an understanding of chronic toxicity. For example, Wedemeyer (1996) stated that nitrate is commonly considered to be nontoxic to fish, and Colt (2006) reported that the 96-h LC50 for NO_3-N is > 1,000 mg/L for freshwater fish species. Further, an earlier study by Westin (1974) determined a 96-h LC50 of 1,364 mg/L NO_3-N and a 7-day LC50 of 1,068 mg/L NO_3-N for rainbow trout fingerlings. However, anticipating a much lower concentration at which chronic impacts begin to occur, Westin (1974) predicted a maximum allowable NO_3-N concentration of approximately 57 mg/L for rainbow trout. Westin's description of health and welfare responses during acute toxicity trials was also intriguing, noting fish with "an inability to swim upright, labored respiration, little movement altered with erratic swimming, and fish yawning or gulping while swimming near the surface with their nose up as if trying to escape". These health and welfare observations are strikingly similar to those described by Davidson et al. (2011a – **Study 1**), particularly for the near-zero exchange study where mean NO_3-N levels exceeded 400 mg/L. Other toxicity studies involving various aquaculture species provide further evidence that nitrate deserves attention as a parameter of concern in RAS (Hrubec, 1996; Camargo, 2005; Hamlin, 2005; Schram et al., 2012; van Bussel et al., 2012).

Less information is available, however, regarding K toxicity, particularly with relevance to RAS. Of the limited sources describing K toxicity to fish, many studies evaluated effects of potassium salts and compounds (Trama, 1954; Kori- Siakpere, 2008; Rao, 2020). Kori-Siakpere (2008), for example, assessed the acute toxicity of potassium permanganate to African catfish *Clarius gariepinus* and observed negative responses including erratic swimming and gulping for air, similar to the behavioral observations described in Davidson et al. (2011a – **Study 1**). A few studies describing the toxicity of ionic K established a wide range of recommended thresholds

from 50 - >200 mg/L (Bell, 1990; Borvinskaya et al., 2016; personal communication Marc Laberge – ML Aquaponics, Canada). However, Rao (2020) did not observe negative effects to rainbow trout in RAS typically containing 200-250 mg/L K, and low-level trout mortality did not occur until dissolved K reached approximately 850 mg/L during the same study. The wide span of reported K toxicity levels is likely related to interaction with other water quality constituents. For example, Mount et al. (1997) reported that the toxic effect of K to fathead minnows *Pimephales promelas* is reduced in the presence of high concentrations of other cations such as Na, Ca, and Mg. Adding to the difficulty for pinpointing a toxic concentration, Borvinskaya et al. (2016) concluded that potassium toxicity primarily occurs when K concentration is greater than other cations and based on the proportion of K ions relative to the total ionic concentration of the water medium. This newly discovered information suggests a reduced likelihood of K toxicity in the Davidson et al. (2011a – **Study 1**) trial. For example, Ca and Mg levels are relatively high in the hard supply water at the Freshwater Institute, and similar levels are reflected within onsite RAS (Davidson et al., 2009; Davidson et al., 2011b – **Study 2**). Further, Ca and Na concentrations were much greater than K levels reported by Davidson et al. (2011a; 2011b – **Study 1 & 2**).

Considering this compilation of literature along with observations from Davidson et al. (2011a; 2011b – **Study 1 & 2**), a research trial was designed to first evaluate the effects of nitrate on rainbow trout reared in RAS. Davidson et al. (2014 – **Study 3**) evaluated rainbow trout health, performance, and welfare in replicated RAS with target NO_3-N concentrations of 20-40 versus 80-100 mg/L. Rainbow trout were 16.4 ± 0.3 g to begin the 3-month study. Similar flushing conditions (1.3-day HRT) were maintained between treatments, and NO_3-N levels were boosted in RAS assigned to the "high" nitrate treatment through continuous addition of sodium nitrate delivered by peristaltic pumps. Rainbow trout growth was not significantly impacted by the high NO_3-N treatment. At the conclusion of the study, rainbow trout cultured in high and low NO_3-N RAS weighed 181 ± 5 and 189 ± 5 g, respectively (Fig. 10); however, cumulative survival was slightly lower and bordered statistical significance, i.e., 87.9 ± 1.1 and 92.5 ± 1.1%, respectively (Fig. 10). In addition, a significantly greater prevalence of side swimming rainbow trout was observed in high NO_3-N RAS, and periodic observations of rapid swimming speeds were observed, mirroring observations from Davidson et al. (2011a – **Study 1**). Ultimately, Davidson et al. (2014 – **Study 3)** provided strong evidence that relatively low NO_3-N levels, 80-100 mg/L, were at least partly related to the chronic health and welfare problems observed in rainbow trout. Based on these findings, maintenance of NO_3-N levels $\leq$ 75 mg/L was recommended for rainbow trout production in freshwater RAS operated under similar conditions.

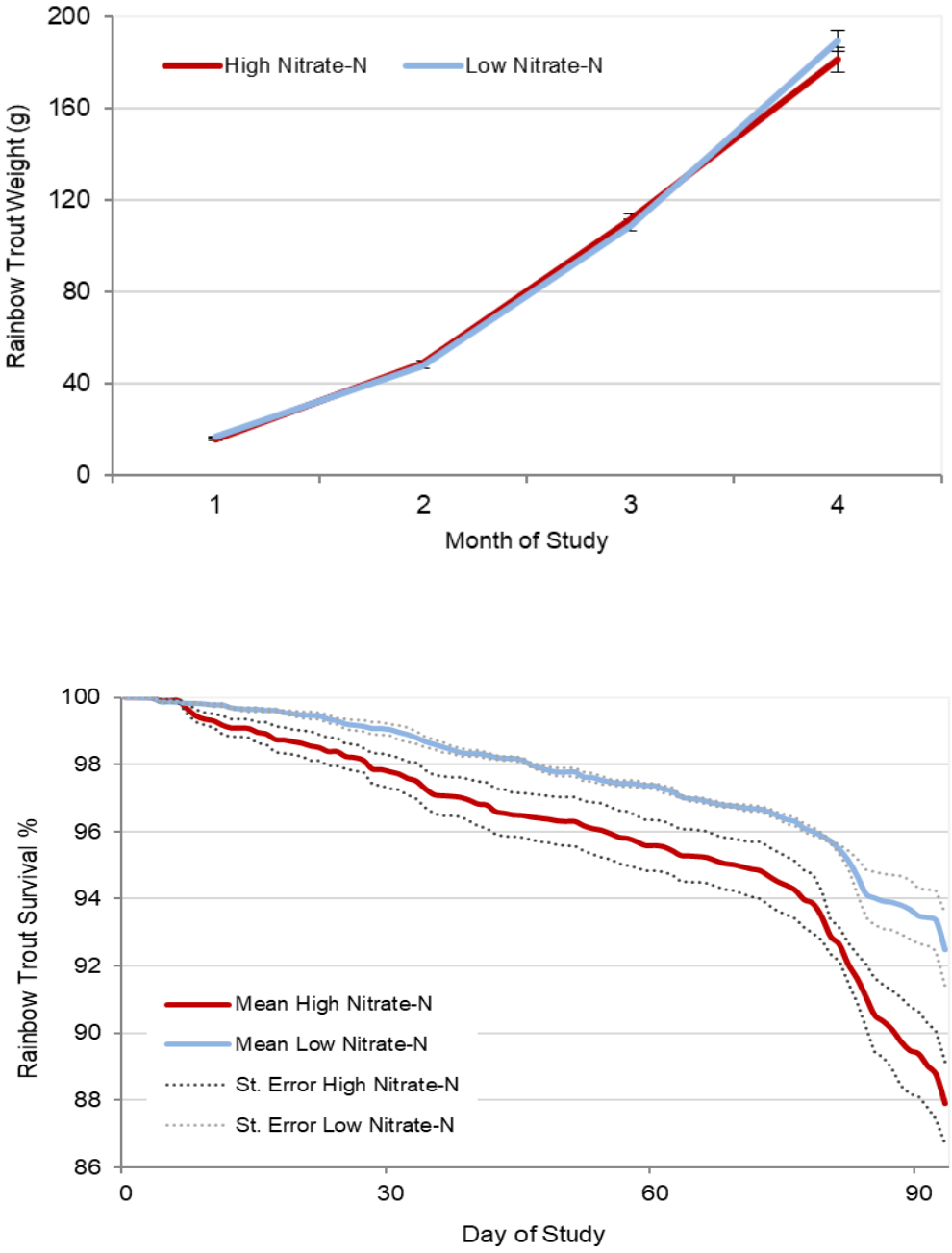

Fig. 10. Rainbow trout growth rates (top) and survival curves (bottom) resulting from exposure to high and low nitrate-nitrogen levels in replicated RAS. Davidson et al. (2014 – **Study 3**)

3.4. Nitrate threshold for post-smolt Atlantic salmon

Rapid development of a land-based RAS industry for the production of larger post-smolts and market-size Atlantic salmon spurred a similar research trial evaluating NO_3-N limits for salmon reared in RAS. Using the findings from Davidson et al. (2014 – **Study 3**) as a guide, an 8-month study was conducted comparing the effects of "high" (99 ± 1 mg/L NO_3-N) versus "low" nitrate-nitrogen (10.0 ± 0.3 mg/L NO_3-N) on the health and performance of post-smolt Atlantic salmon cultured in freshwater RAS (Davidson et al., 2017 – **Study 4**). Atlantic salmon used for the trial were 102 ±1 g to begin. Like the former study, sodium nitrate was continuously dosed to create high nitrate conditions. However, Atlantic salmon performance (e.g. weight, length, condition factor, TGC, and FCR) was not negatively affected in RAS with 100 mg/L NO_3-N. At the end of the study, salmon from the high and low NO_3-N treatments weighed 1148 ± 22 and 1174 ± 8 g, respectively ($P > 0.05$), and cumulative survival was > 99% for both treatments. Further, plasma chemistry, tissue histopathology, fin quality, and general observations of fish behavior were statistically similar between treatments and within a normal range for acceptable fish health and welfare. Overall, this research provided initial evidence that NO_3-N levels $\leq$ 100 mg/L are safe for post-smolt Atlantic salmon production in RAS operated under similar conditions, e.g., freshwater RAS, salmon strain, post-smolt life stage, fish size, etc. In general, these findings also indicated that post-smolt Atlantic salmon are relatively tolerant to high NO_3-N concentrations. Additional onsite research is planned to evaluate higher NO_3-N levels up to 200 mg/L to determine the approximate concentration at which Atlantic salmon begin to demonstrate chronic toxicity symptoms.

3.5. Effects of ozone on RAS water quality and salmonid performance

Previous discussion focused on the effects of the fish culture environment on salmonid performance. In the following sections, water treatment approaches and their influence on water quality and fish production are described, starting with a look back to Davidson et al. (2011b – **Study 2**) which evaluated the use of low-dose ozone. During this research, the use of low-dose ozone to maintain an ORP of 250-290 mV resulted in significant reductions in TSS, TC (which is dictated by dissolved organic matter), $cBOD^5$, and dissolved Cu and Fe levels. Dissolved Zn and THBC were also consistently lower, albeit not significantly, in ozonated RAS, and UVT, the percentage of light passing through water at a certain wavelength, was significantly higher. In addition, during one of the trials described in Davidson et al. (2011b – **Study 2**), ozone created ambient water quality in low exchange RAS that was comparable to RAS operated with a 10-fold greater dilution rate. Despite this large difference in water flushing, rainbow trout performance was statistically similar between treatments. However, the most important outcome of these trials was demonstration of faster rainbow trout growth rates in RAS operated with ozone compared to non-ozonated RAS. For example, during the first study in this series, mean rainbow trout weight was significantly greater in ozonated RAS approximately one month after the trial began, and growth curves continued to separate over the remainder of the study (Fig. 11). At the end of the 4-month trial, rainbow trout cultured in RAS with and without ozone

weighed 1161 ± 6 and 993 ± 12 g, respectively (P<0.05). Interestingly, a recent study evaluating the effects of ozone on post-smolt Atlantic salmon performance, maturation, and water quality (Davidson et al., 2021 – **Study 7**) revealed a similar growth pattern (Fig. 11). Evidence of improved growth of Atlantic salmon post-smolts in ozonated RAS was noted during the earliest sampling event and continued over the duration of the 8-month trial. Atlantic salmon from RAS with and without ozone weighed 2,156 ± 101 and 1,810 ± 15 g, respectively by the end of the experiment (Davidson et al., 2021 – **Study 7**). Ozone did not inhibit early Atlantic salmon maturation during this study, but waterborne hormone concentrations (particularly estradiol) were reduced in ozonated RAS, and a range of water quality improvements were observed similar to those documented by Davidson et al. (2011a: 2011b – **Study 1 & 2**). For instance, TC, THBC, and dissolved Cu, Fe, and Zn levels were significantly lower in ozonated RAS.

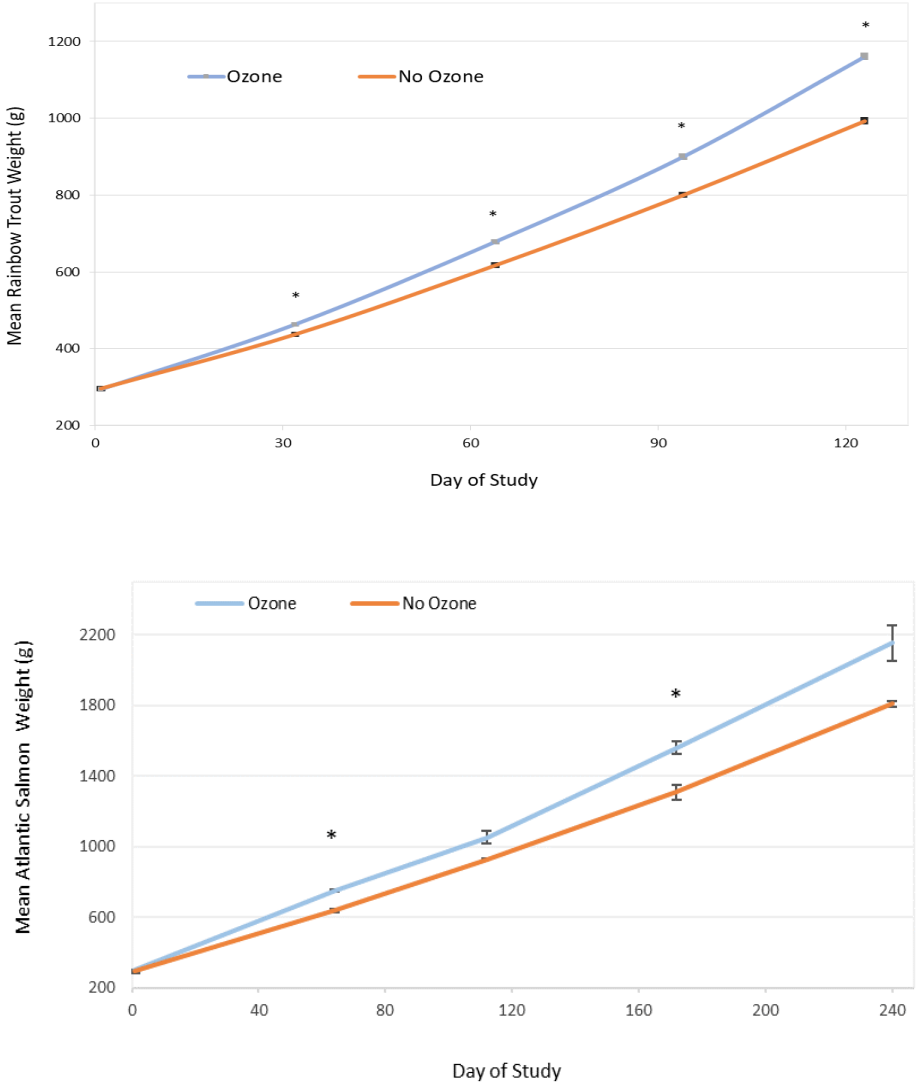

Fig. 11. Rainbow trout (top) and post-smolt Atlantic salmon growth (bottom) in RAS operated with and without low-dose ozone (Davidson et al. 20011b; 2021 – **Study 2 & 7**).

The exact mechanism of improved rainbow trout and Atlantic salmon growth related to ozone is unclear; however, Davidson et al. (2021 - **Study 7**) provided several plausible explanations. First, cumulative improvement to the culture environment instigated by ozone exists as a strong possibility. This assertion was supported by Powell and Scolding (2018) who suggested that improved fish growth in ozonated RAS could be explained by reduced energetic costs of fish exposed to an optimized environment. As previously described, ozone instigated a wide range of water quality improvements in RAS; therefore, it is difficult to pinpoint which enhancement or combination thereof was most supportive of increased fish growth. However, ozone effectively reduced dissolved Cu, which was flagged as a potentially harmful contaminant (Davidson et al., 2011a – **Study 1**), by approximately 3-fold. Reduced Cu and other dissolved heavy metals concentrations (Fe and Zn) coincided with improved rainbow trout and Atlantic salmon growth (Fig. 11) (Davidson et al., 2011b; 2021 – **Study 2 & 7**). Other water quality improvements included dramatic increases to water clarity as measured by TC and UVT. True color was 13 times lower in ozonated RAS during the Davidson et al. (2011a – **Study 1**) trial and 22 times lower in RAS operated with ozone for Davidson et al. (2021 – **Study 7**). Ultraviolet transmittance increased by approximately 27% as a result of ozonation during both of these studies. Clear water with reduced turbidity reportedly enhances the ability of salmonids to see and capture feed and can lead to increased growth (Sigler et al., 1984). In relatively shallow experimental tanks, nominal inhibition of fish sight could impact the effectiveness of feed capture due to short duration of feed availability in the water column. Evaluation of feed sinking rates after Davidson et al. **(**2021 **– Study 7)** indicated that feed was suspended in the water column of the 1.2-m deep tanks for < 10 sec and flushed from the tank in approximately 30 sec. As such, feed bypassing fish in turbid water of non-ozonated RAS would have resulted in reduced daily ration to control FCR and related limitations to fish growth. Overall, the use of ozone appears to improve the feasibility of rearing salmonid species in freshwater RAS operated with relatively low water exchange rates. Due in large part to this research, ozone is now used regularly in RAS at *FI* for salmonid research and at several commercial freshwater RAS facilities that are producing rainbow trout and Atlantic salmon in the United States.

3.6. An alternate to ozone? – Peracetic acid

Ozone addition resulted in a range of water quality benefits in RAS and a growth advantage for rainbow trout (Davidson et al., 2011b - **Study 2**) and Atlantic salmon (Davidson et al., 2021 – **Study 7**); however, as previously mentioned, the use of ozone also has certain disadvantages such as: i) negative consequences to fish and human health at relatively low water and air concentrations (Gearhart and Summerfelt, 2007), ii) operational complexity including a requirement for onsite generation, continuous monitoring, and alarming, and iii) less than trivial capital and operating costs (Summerfelt and Hochheimer, 1997; Gonclaves and Gagnon, 2011). In addition, ozone is not used extensively in saline aquaculture due to its propensity to react with naturally occurring bromides to form toxic byproducts such as bromine and bromate (Tango and Gagnon, 2003). With these drawbacks in mind, it was important to evaluate another water treatment approach in RAS as a possible alternative to ozone. Peracetic acid, is a relatively new

compound for use in aquaculture that has been described as a powerful oxidant capable of producing water quality improvements similar to ozone (Pedersen et al., 2015b). However, the water oxidizing capacity of PAA in RAS and its effects on fish performance required further investigation. To this end, a trial was carried out to comprehensively evaluate the effects of PAA on water quality and rainbow trout performance (Davidson et al., 2019a – **Study 5**). Peracetic acid was added semi-continuously to replicate RAS (N=3) using peristaltic pumps to achieve target concentrations of 0.05, 0.10, and 0.30 mg/L over one-month intervals. Three control RAS were operated with similar water flushing and feed loading rates, but without PAA addition.

Semi-continuous dosing of PAA was compatible with rainbow trout performance and RAS operation, i.e., rainbow trout growth was statistically similar between treatments and biofilter performance was not inhibited, but PAA did not create water quality improvements like those expected when applying low-dose ozone. In fact, the tested PAA doses generally did not improve RAS water quality, with the exception of minor reductions to TC. Nevertheless, several important outcomes resulted that could facilitate the use of PAA for other applications, such as a water disinfectant for pathogen control and fish health management (e.g., Meinelt et al., 2009; 2015). For example, this study was the first to report that ORP increases according to rising PAA concentration indicating potential for ORP monitoring and control of PAA residuals. In addition, a safe and effective PAA dosing approach was developed that included spill containment, air monitoring, and alarming that could be replicated elsewhere.

It is important to note that the semi-continuous PAA application approach used during Davidson et al. (2019b – **Study 5**) may not have been ideal. In fact, the authors hypothesized that semi-continuous PAA dosing may have facilitated periodic bacterial spikes of a nonpathogenic microorganism called *Flectobacillus roseus* that had not been observed onsite until this study. *Flectobacillus roseus* did not cause significant effects to fish health, but in some instances the bacteria clouded RAS water and reduced water clarity. Similarly, Liu et al. (2017) reported that continuous application of 0.2 mg/L PAA resulted in significant biofilm formation in flow through systems with rainbow trout, but pulsed addition of 1 mg/L PAA resulted in stable pH, higher O_2 levels, and inhibited biofilm formation. Further, Good et al. (2020) found that pulsed application (once daily) of 0.2, 0.5, and 1.0 mg/L PAA resulted in significantly reduced external saprolegniasis and higher survival rates in juvenile Atlantic salmon following vaccination compared to RAS controls without PAA. Although PAA does not appear to be a viable replacement for ozone for water conditioning, this oxidant may offer other advantages when applied to RAS differently. It should be noted that PAA is approved for use in aquaculture systems with fish in Europe, but has only been approved for surface disinfection of tanks and aquaculture equipment in the U.S. (European Union, 2014: Straus et al., 2018; Straus and Meinelt, 2019).

3.7. Integrating denitrification – Membrane biological reactors

The previously described trials utilized a RAS design that required dilution to maintain suitable water quality concentrations, namely nitrate, for rainbow trout and Atlantic salmon production. Decisions to design and adopt RAS that rely on dilution for nitrate management hinge on water availability and biomass production goals. Quite simply, at a certain scale of fish production (mT) the nitrate dilution approach becomes limiting. However, adding denitrification within RAS reduces water use and eliminates the necessity to dilute nitrate via flushing (van Rijn, 2006). Considering the massive scale of prospective commercial salmonid RAS facilities (EY Global, 2019) and the relatively large water footprint required to support planned production goals, denitrification technology will likely be required within commercial RAS designs. Therefore, a study was designed to evaluate the feasibility for integrating a specialized denitrification technology within RAS – membrane biological reactors (MBRs) (Davidson et al., 2019b – **Study 6**).

Triplicate RAS with and without MBRs (controls) were evaluated with particular focus on effects to rainbow trout performance, water quality, and water use. Single vessel reactors received solids laden backwash from the drum filter and radial flow settler, which otherwise would have been pumped to separate waste treatment systems and replaced with new water. Microbial populations within the accumulating activated sludge of the MBR facilitated denitrification, while a semi-purified filtrate was created from a concentrated solution that was forced through a series of fine pore membranes and returned to the RAS pump sump (Fig. 12). Ultimately, integration of MBR's within RAS created a nearly 100% recycle loop, where minimal water loss occurred due to system overflow, splashing, and evaporation.

Due to reduced water exchange, concentrations of certain water quality parameters accumulated and were significantly greater in RAS with MBRs including chloride, CO_2, THBC, pH, NO_3-N, TAN, total P, and TC, as well as dissolved concentrations of Ca, Cu, Mg, and S, while Alk and UVT levels were significantly lower. These culture environment differences did not negatively affect rainbow trout growth, feed conversion, or survival ($P > 0.05$); however, the lack of negative impact was surprising given that NO_3-N levels exceeded those that were deemed chronically toxic during Davidson et al. (2014 – **Study 3**). For example, mean NO_3-N measured in systems with and without MBRs was 201 ± 11 and 117 ± 3 mg/L, respectively, but Davidson et al. (2014 – **Study 3**) suggested that rainbow trout begin to exhibit chronic exposure symptoms ("side swimming" and rapid swimming velocity) when NO_3-N accumulates to approximately 80-100 mg/L. Further discussion related to this discrepancy is provided in Section 3.8.1.

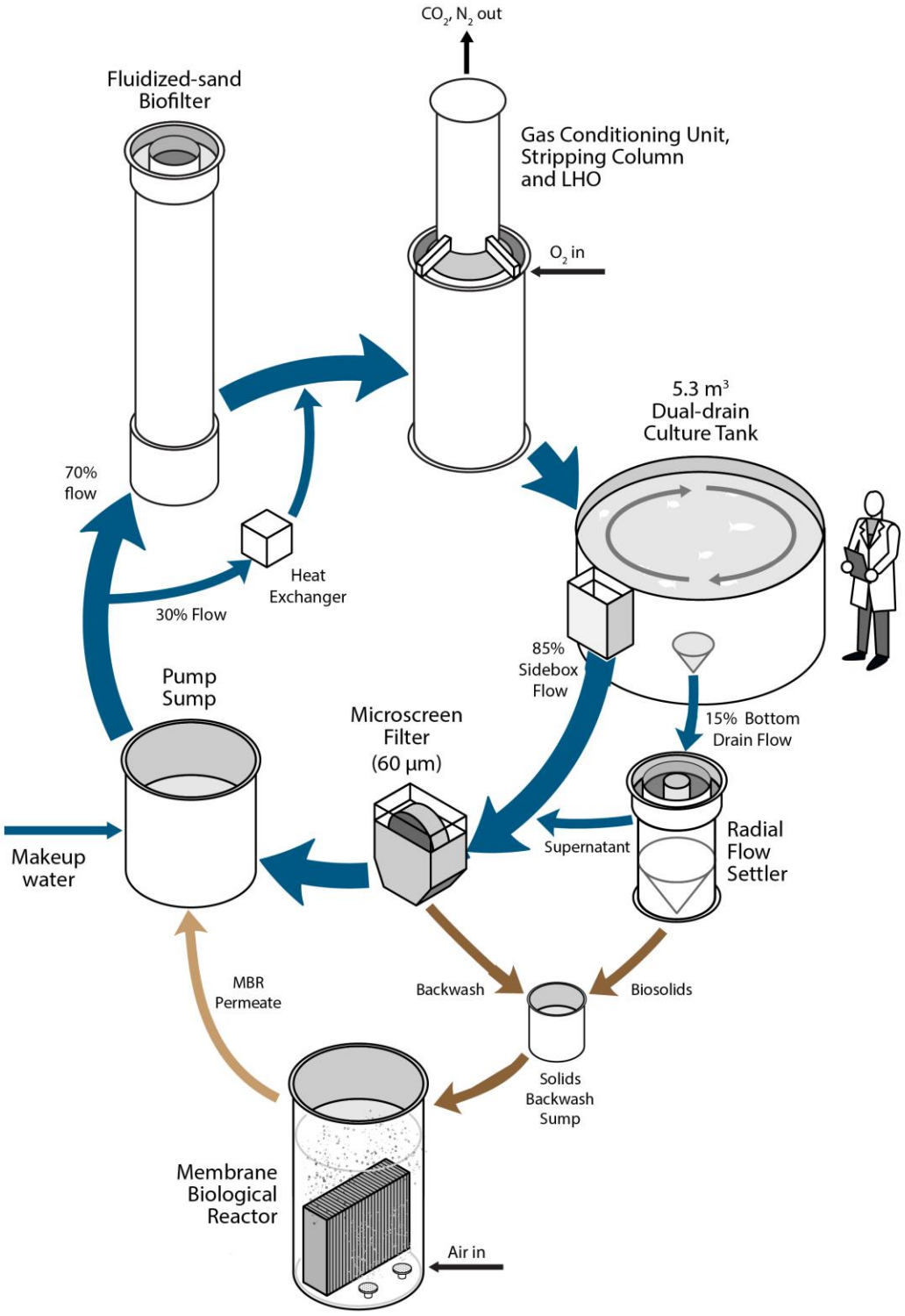

Fig. 12. Water flow and process design for an individual RAS with an integrated MBR used during Davidson et al. (2019b – **Study 6**).

Another important finding of Davidson et al. (2019b – **Study 6**) was the significant water savings facilitated by MBR integration. RAS with MBRs used approximately 6.5 times less water compared to RAS without MBRs, resulting in mean system HRTs for RAS with and without MBRs of 104 ± 31 and 13 ± 1 days, respectively, and average daily water replacement of 1.2 ± 0.4 and 7.8 ± 0.5% ($P < 0.05$). In light of this difference, it is worth noting that NO_3-N levels in the control RAS were approaching those of RAS with MBRs by the end of the trial (Fig. 13). Nitrate-nitrogen levels in RAS with MBRs stabilized following addition of sugar as an external carbon source to boost denitrification at approximately Day 70, but NO_3-N continued to climb in the control RAS. If the study had continued for another month, NO_3-N levels in control RAS likely would have surpassed those in RAS with MBRs, despite the 6.5-fold difference in water flushing. In addition, substantial water reduction and acceptable trout performance were achieved even while identifying deficiencies in MBR operation including slower than expected permeate production rates due to membrane clogging and incomplete denitrification. Additional onsite research is planned to re-evaluate MBR integration within RAS after correcting the recognized deficiencies.

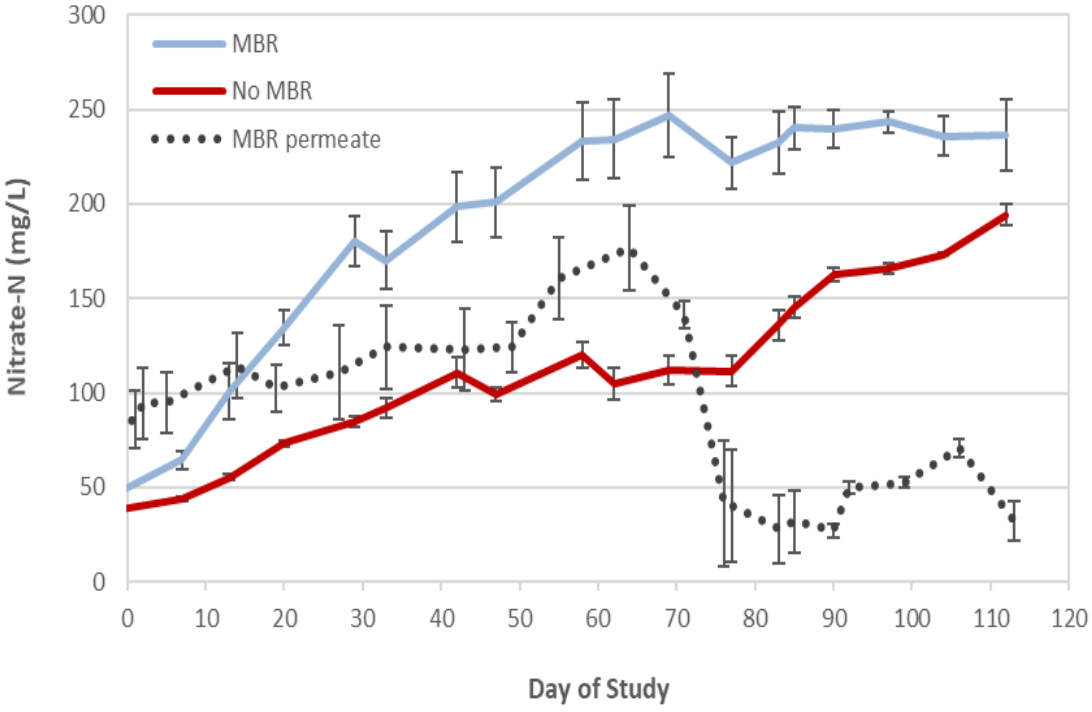

Fig. 13. Nitrate-nitrogen concentrations (mean ± standard error; N = 3) measured weekly in RAS operated with and without MBRs and within the MBR permeate.

Table 2. Water flushing summary from highlighted trials with corresponding species and fish health, performance, and welfare observations.

Thesis Study	Davidson et al. Citation	Treatment	Species	HRT (days)	FLR (kg feed/ m^3 water/day)	L/ kg feed/day	Fish Health, Welfare Issues?	Fish Growth, Health, Welfare Observations
-	2009	High Flush	RBT	0.7	0.5	2577	No	-
-	2009	Low Flush	RBT	6.7	5.3	290	No	-
1, 2	2011a, b [1]	Ozone	RBT	6.9	4.0	250	No	improved growth
1, 2	2011a, b [1]	No Ozone	RBT	7.2	3.7	268	No	-
1, 2	2011a, b [2]	Low Flush, Ozone	RBT	6.7	4.1 *	295 *	Yes	side-swimming; rapid swim speed
1, 2	2011a, b [2]	High Flush, No Ozone	RBT	0.7	0.4	2772	No	-
1, 2	2011a, b [3]	Near-Zero Flush - Ozone	RBT	95-196	71-147	7-14	Yes	Increased mortality, unusual swimming; deformities
1, 2	2011a, b [3]	Very Low Flush - No Ozone	RBT	31-66	23-44	21-44	NA	-
3	2014	High NO_3-N	RBT	1.4	0.8	1329	Yes	increased mortality; side-swimming; rapid swim speed
3	2014	Low NO_3-N	RBT	1.3	0.7	1444	No	-
4	2017	High NO_3-N	AS	1.7	0.2	4306	No	-
4	2017	Low NO_3-N	AS	1.7	0.2	4294	No	-
5	2019a	PAA	RBT	2.7	1.5	667	No	-
5	2019a	No PAA	RBT	2.7	1.6	640	No	-
6	2019b	MBR	RBT	104	39.3 †	33 †	No	-
6	2019b	No MBR	RBT	13	5.2	207	No	-
7	2021	Ozone	AS	13.5	3.6	281	No	improved growth
7	2021	No Ozone	AS	16.3	3.6	276	No	-

* Anomaly to safe rainbow trout production where low-level welfare impacts were observed

† Extreme anomaly where negative rainbow trout health and welfare effects were not observed despite extremely low water flushing conditions

3.8. Value-added analysis - Required water metrics

Three areas of value-added analysis related to RAS water exchange were recognized while reviewing the highlighted articles: i) summary of flushing rates that support optimal rainbow trout and Atlantic salmon performance in freshwater RAS, ii) establishment of trendlines that facilitate prediction of water flushing rates and expected nitrate concentrations, and iii) a decision-making matrix for integrating various unit processes within RAS, particularly denitrification based on nitrate accumulation and its effect on fish performance.

3.8.1. Optimal flushing rates for rainbow trout in freshwater RAS

Water flushing rates used for each study were tabulated alongside respective observations of rainbow trout and Atlantic salmon growth, health, and welfare responses, and the suitability of these conditions for salmonid production was ascertained (Table 2). This exercise separated water use metrics that supported optimal fish performance, and, conversely, shed light on flushing criteria that resulted in negative health and welfare effects to rainbow trout under the described conditions. In general, optimal rainbow trout health, welfare, and performance was maintained when RAS were operated with feed loading rates $\leq$ 5.3 kg feed/m^3 makeup water/day and $\geq$ 250 L makeup water/ kg daily feed. Specifically, feed loading rates of 0.4-5.3 kg feed/m^3 makeup water/day and flushing rates equating to 250-2,772 L makeup water/ kg daily feed were generally safe for rainbow trout production in freshwater RAS (Table 2), corresponding with a system HRT of approximately one to seven days. Overall, these metrics provide a general guide for maintaining optimal water quality in similarly designed freshwater RAS while rearing rainbow trout.

Nevertheless, several discrepancies were identified (Table 2) that require further discussion. For example, rainbow trout reared in low exchange RAS with ozone (4.1 kg feed/m^3 makeup water/day; 295 L makeup water/ kg daily feed) exhibited mild chronic toxicity symptoms including increased prevalence of side-swimming and rapid swimming speeds (Davidson et al., 2011a – **Study 1**). These water flushing values fall within the limits that were deemed acceptable for rainbow trout production in RAS; however, it is notable that growth and survival of these trout populations was unaffected, and side-swimming fish appeared to be feeding and in good health. Subsequent onsite research carried out by Good et al. (2014) found mild, but significantly reduced performance attributes in side-swimming trout compared to normal conspecifics including slightly lower mean weight and length and reduced fillet yield. These effects were likely caused by increased physical exertion and muscle activity required by this unusual side-swimming condition (Good et al., 2014). Whole-blood chemistry results (e.g. reduced pH, increased $P_{CO2,}$ and increased blood glucose) supported this assertion. In addition, swim bladder malformations were generally present in side-swimming fish, suggesting that loss of neutral buoyancy may have originated from this abnormality. Ultimately, Good et al. (2014) concluded that efforts should be made to eliminate this condition in rainbow trout populations.

Additionally, anecdotal observations indicate that there may be a genetic link to side-swimming in rainbow trout. First, side-swimming trout were observed during Davidson et al. (2011a – **Study 1**) regardless of flushing treatment and culture environment differences (Fig. 7),

suggesting that this group of trout was predisposed to this abnormality. Further, different rainbow trout cohorts provided by the same supplier, as well as genetic lines of trout procured elsewhere, have not always exhibited the side-swimming behavior even when fish are cultured under similar conditions. For instance, rainbow trout provided as fingerlings by the National Center for Cool and Cold Water Aquaculture (USDA-ARS; Leetown, WV, USA) and cultured in the same RAS with mean NO_3-N > 200 mg/L and hydraulic retention times extending beyond 100 days did not exhibit the side-swimming behavior or other ill effects during a recent trial (Davidson et al., 2019b – **Study 6**). The reason for this discrepancy is unknown; however, nitrate toxicity in fish reportedly varies based on exposure time, life stage, and interacting water quality parameters (Camargo et al., 2005). With this in mind, it may be important to note that the mean water temperature recorded during Davidson et al. (2014 - **Study 3**) and Davidson et al. (2019b - **Study 6**) was 15.5 vs. 13.8-13.9 ^{o}C, respectively. Although, Davidson et al. (2014 – **Study 3**) did not discuss a potential interaction of nitrate and temperature, subsequent review indicates that a steeper mortality trend occurred at the end of the study (Fig. 10) coinciding with maximum RAS temperatures of 17-18 ^{o}C. Sprague (1985) described water temperature as one of many abiotic factors that can modify the toxicity of compounds to aquatic organisms; however, research evaluating temperature interactions with nitrate toxicity to RAS-produced fish is lacking. A study evaluating different genetic lots of rainbow trout tagged for identification and exposed to high nitrate concentrations at different water temperatures would provide insight into the varying health and welfare responses observed among studies. Identification of rainbow trout strains with high nitrate tolerance could have tremendous implications for water use and the necessity to incorporate denitrification technologies in RAS.

3.8.2. Optimal flushing rates for Atlantic salmon in freshwater RAS

Davidson et al., (2021 – **Study 7**) determined that post-smolt Atlantic salmon performance was improved in ozonated RAS operated with relatively low water exchange rates. For example, no significant health or welfare concerns were noted when RAS were operated with a feed loading rate of 3.6 kg feed/m^3 makeup water/day or 276-281 L makeup water/kg feed/day (Table 2), indicating that these water use metrics are suitable for post-smolt Atlantic salmon production in similar freshwater RAS. In light of the varying chronic toxicity responses described for different populations of rainbow trout, it is imperative that the suggested flushing rates be understood with context to specific study conditions, i.e., Atlantic salmon strain, freshwater, interacting water quality parameters, and RAS design criteria. Nevertheless, these flushing rates represent a reduction in necessary water use compared to the 803 L/ kg feed requirement estimated by Liu et al. (2016) for a 3,300 mT head-on-gutted Atlantic salmon RAS facility. It is important to note, however, that the water footprint suggested by Liu (2016) also considered water required for depuration of off-flavor and flushing to maintain an upper NO_3-N limit of 75 mg/L based on the findings of Davidson et al. (2014 – **Study 3**). Further, Davidson et al. (2017 – **Study 4**) found that post-smolt Atlantic salmon were tolerant of mean NO_3-N concentrations up to 100 mg/L and maximum levels up to 150 mg/L. These findings imply that post-smolt Atlantic salmon can be reared in freshwater RAS with even greater feed loading rates

and less water use compared to the values reported in Table 2 and those reported by Liu et al. (2016). Additional onsite research is planned to assess post-smolt Atlantic salmon tolerance to higher NO_3-N levels up to 200 mg/L. Establishment of a finite NO_3-N threshold for post-smolt Atlantic salmon production in RAS will provide additional guidance regarding suitable flushing rates and will direct the necessity for inclusion or exclusion of denitrification technologies within commercial RAS.

3.8.3. Differences in rainbow trout and Atlantic salmon production

The observed differences between rainbow trout and Atlantic salmon relative to NO_3-N toxicity thresholds and proposed RAS operational metrics may be surprising given the close phylogenetic relationship of these two salmonid species. After all, both genera *Oncorhynchus* and *Salmo* are categorized within the same family Salmonidae and the same subfamily Salmoninae (Sanford, 1990; Stearley and Smith, 1993). Rainbow trout and Atlantic salmon are, in fact, so closely related that at one time rainbow trout was assigned to the genus *Salmo*, i.e. *Salmo gairdneri* (Stearley and Smith, 1993). Basic separation of the genera *Salmo* and *Oncorhynchus* has only recently been settled as describing salmonids of Pacific and Atlantic origin, respectively (Stearley and Smith, 1993), and only fine differences in morphology truly separate these species. All members of the Salmoninae subfamily including rainbow trout and Atlantic salmon spawn in freshwater, and full freshwater and anadromous life history paths are represented for both species (Sanford, 1990). However, Stearley and Smith (1993) noted that "the terms trout and salmon refer roughly to life history modes, and not phylogenetic relationships." Accordingly, rainbow trout are more commonly landlocked, spending their entire life from egg to spawning adult in freshwater, while Atlantic salmon strains are typically anadromous, but exhibit a highly plastic and diverse range of life history forms and reproductive tactics that is unmatched by most vertebrates (Good and Davidson, 2016). Further, the history of domestication for each of these species is vastly different. For example, rainbow trout have been artificially propagated in fish hatcheries since the late 1800's (Schley, 1971), with genetic selection beginning in the early 1930's (Gjedrem, 1992). In contrast, selective breeding of Atlantic salmon for aquaculture did not begin until the 1960's in Norway (Houston and Macqueen, 2019). Further, all-female rainbow trout stocks have been commercially available for decades and are now commonly used for rainbow trout farming (Johnstone et al., 1979; Bye and Lincoln, 1986), while commercial development of all-female Atlantic salmon eggs began more recently (Johnstone and MacLachlan, 1994; Lee, 2004) and has gained increased attention due to challenges with early maturation of mixed sex Atlantic salmon populations in RAS (Good and Davidson, 2016). Further, recently published research indicates that the use of triploid all-female salmon may be preferential in freshwater RAS due to the sterility of these fish, as well as demonstration of relatively comparable growth performance (Crouse et al., 2021). Differences in fish physiology and culture environment requirements between rainbow trout and Atlantic salmon have likely developed within this complex matrix of differences in life history, selective breeding, gender selection, and ploidy options. These differences appear to dictate adoption of species-specific fish husbandry practices and rearing protocols. For example, Atlantic salmon

utilize photoperiod as an important environmental cue to initiate smoltification, the physiological process that prepares fish for transition from freshwater to seawater. As such, photomanipulation using an artificial winter photoperiod is generally used to trigger smoltification during Atlantic salmon production, while perpetual 24-h overhead lighting is often used throughout the rainbow trout production cycle. In addition, onsite fish production experience has also demonstrated different feeding behaviors, density tolerance limits, and growth rates between rainbow trout and Atlantic salmon; therefore, specific fish husbandry protocols have been adopted for each species. Overall, in context to this thesis, it is important for RAS investors, operators, and stakeholders to understand that although rainbow trout and Atlantic salmon are closely related, these two species have inherent differences that dictate establishment of species-specific culture requirements in RAS. More research is still required, but varying tolerance to water quality thresholds, such as nitrate, could also influence system design, such as inclusion of denitrification technologies.

3.8.4. Flushing rates for dilution vs. denitrification

Integration of denitrification technologies in RAS appears to be part of the next frontier of RAS research and development, as supported by trends discussed in this manuscript. In fact, the future may be here, as several commercial land-based salmonid facilities are already utilizing denitrification within RAS. With this in mind, the primary objective of this section was to theoretically define the point at which RAS design and operation shifts from utilization of flushing for nitrate dilution to the necessary integration of denitrification technologies, such as MBRs. The initial driver for this decision, is, of course, the nitrate tolerance threshold of salmonid species, as studied during several of the highlighted trials (Davidson et al., 2014; 2017 – **Study 3, 4**). However, NO_3-N threshold values don't provide information about water flushing requirements to achieve necessary nitrate dilution. Therefore, for predictive purposes, average feed loading rates (FLR) were plotted along with associated NO_3-N concentrations, utilizing data from a multitude of onsite studies carried out in the same RAS (Davidson et al., 2011a; 2011b; 2014; 2017; 2019a; 2019b; 2021 – **Studies 1-7**; Davidson et al., 2009; 2013; 2016).

When plotting FLR, which accounts for daily water flushing and feeding, alongside corresponding NO_3-N levels, several interesting trends were revealed. First, a bimodal pattern became evident, where FLR values from 0-10 kg feed/m^3 makeup water/day followed a relatively steep slope represented by the light blue data points with red dotted trendline (Fig. 14). The R^2 value for this data grouping was relatively high at 0.7647, indicating a positive relationship between FLR (0-10 kg feed/m^3 makeup water/day) and corresponding NO_3-N levels (0-150 mg/L). Beyond this range, however, a different trendline is apparent, represented by the orange points and black trendline. The FLR x NO_3-N relationship of the second trendline is also relatively strong (R_2 = 0.7826). The delineation between these two plots suggests that passive denitrification (i.e., indirect, or unintended nitrate conversion) was taking place at feed loading rates > 10 kg/m^3 makeup water/day and NO_3-N concentrations >150 mg/L. To further demonstrate the likelihood of passive denitrification taking place within these RAS, a theoretical trendline (based on mass balance calculations described in Timmons et al. (2018)) was added in Fig. 15 to show the expected NO_3-N concentrations in RAS water in the absence of

denitrification. The difference in slope and trajectory of the theoretical plot versus the real data is profound (Fig. 14, 15).

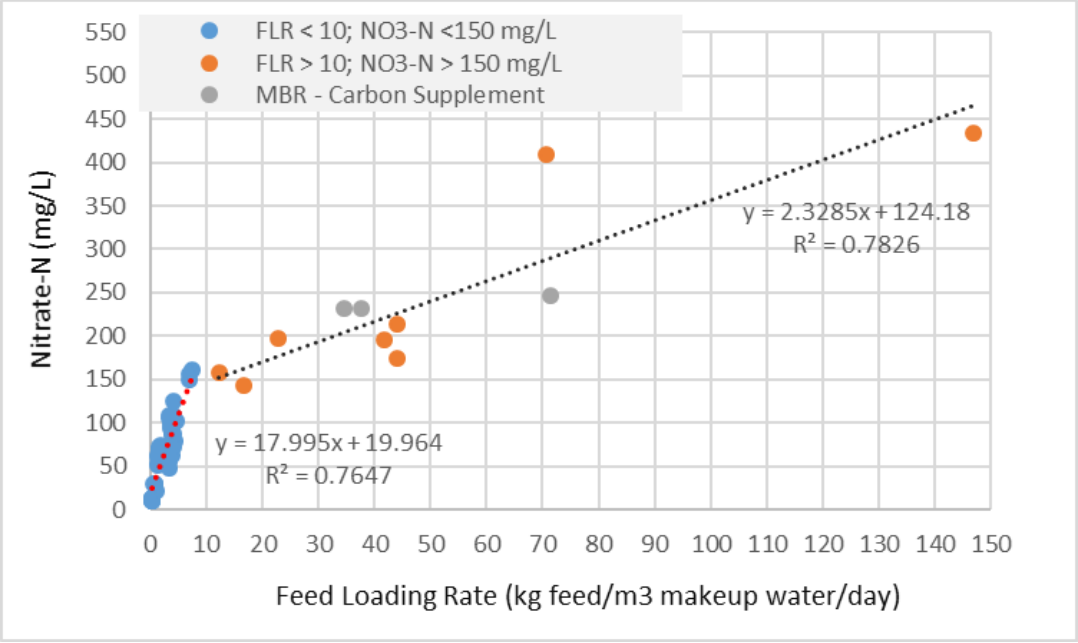

Fig. 14. Feed loading rates and corresponding nitrate-nitrogen levels in freshwater RAS used to rear rainbow trout and Atlantic salmon during various onsite trials (Davidson et al., 2011a; 2011b; 2014; 2017; 2019a; 2019b; 2021 – **Studies 1-7**; Davidson et al., 2009; 2013; 2016).

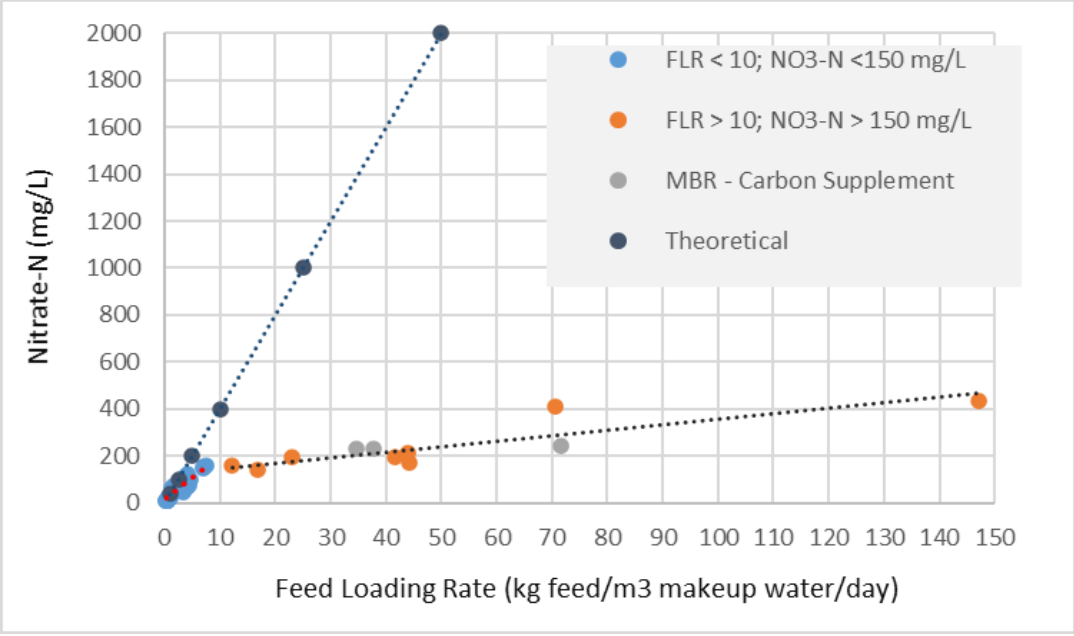

Fig. 15. Feed loading rates and corresponding nitrate-nitrogen levels in freshwater RAS used to rear rainbow trout and Atlantic salmon. (Davidson et al., 2011a; 2011b; 2014; 2017; 2019a; 2019b; 2021 – **Studies 1-7**; Davidson et al., 2009; 2013; 2016). Theoretical nitrate nitrogen levels depicted by the dark blue line were calculated using mass balance formulas described in Timmons et al. (2018).

First mention of passive denitrification occurring in the same replicate RAS was made by Davidson et al. (2011b - **Study 2**), who suggested that NO_3-N reduction via passive denitrification occurred with increasing feed loading rates. Data presented in Figures 14 and 15 expands on that analysis through data inclusion from many onsite trials. Further literature review suggests that a likely location for passive denitrification to occur in these RAS is the fluidized sand biofilter (FSB). Tsukuda et al. (2015) reported that similarly designed FSBs used to treat aquaculture wastewater provided low-level denitrification without addition of an external carbon source. Fluidized sand biofilters provide tremendous surface area for microbial attachment and biofilm growth which can result in diminished O_2 levels in the passing water due to microbial respiration. For example, Summerfelt (2006) reported O_2 levels as low as 2.5 mg/L in the effluent of an FSB. In addition, Dalsgaard and Revsbech (1992) observed denitrification within layers of biofilm in a trickling filter, typically 0.2-0.3 mm beneath the biofilm surface. Davidson et al. (2011b) suggested a similar mechanism, stating that passive denitrification likely occurred after NO_3-N diffused deeply into biofilms where denitrifying bacteria were sheltered from aerobic conditions. Further, van Rijn (2006) reported that biofilms within anoxic microsites of RAS could support passive denitrification. Van Rijn (2006) also suggested that ANNAMOX (anaerobic ammonium oxidizing bacteria) could be partly responsible. In this microbial process, nitrite and ammonium ions are converted directly to nitrogen gas and water instead of nitrate. Additional research is required to understand why nitrate is being reduced far below expected levels in the replicated freshwater RAS utilized at the *FI*, including microbial DNA sequencing of biofilms within the fluidized sand biofilter.

Lastly, it is important to note that the gray coordinates in Figs. 13 and 14 represent data collected from Davidson et al. (2019b – **Study 6**) for the three RAS with integrated, denitrifying MBRs. Interestingly, these data points fall close to the secondary FLR x NO_3-N trendline, affirming that complete denitrification was not achieved by integrated MBRs. Davidson et al. (2019b – **Study 6**) recognized this deficiency, concluding that fish solids alone did not provide enough carbon for effective denitrification, while noting that increased water exchange between RAS and MBR is required to effectively reduce system nitrate. Intensive water sampling and analysis of NO_3-N levels across various unit processes along with subsequent mass balances would facilitate an improved understanding of nitrogen removal in these RAS.

3.8.5. Decision-making matrix for RAS design

As a final exercise, I established a decision-making matrix that can be referenced when planning and designing freshwater RAS facilities for Atlantic salmon and rainbow trout (Table 3). I must emphasize that these are estimated values based on specificity to conditions of freshwater production and RAS design at the Freshwater Institute. Additionally, unexplained differences in NO_3-N threshold for various rainbow trout cohorts and incomplete information regarding finite NO_3-N thresholds should be considered. Overall, Table 3 indicates that greater water flushing (as influenced by water availability) dictates reduced complexity of the RAS design. For example, availability of large volumes of flushing water support selection of a simple partial reuse design with gas conditioning and solids removal processes where nitrogen levels are

diluted by rapid water exchange. As water availability diminishes, the necessity to include additional water treatment processes increases. For instance, biofilter inclusion becomes necessary at approximately 1,000 L of new water/ kg feed/day, and addition of unit processes for fine particle control, such as ozone, become necessary at ~250-1,000 L new water/ kg feed/day when effective dilution of fine solids and accumulating compounds is no longer possible. Water chilling may also become necessary within this range or beyond, depending on climate, because RAS water warms as the amount of cool makeup water mixing with system water is reduced. Lastly, the decision to incorporate denitrification technologies is based on the intersection of species-specific NO_3-N threshold, water availability, and overall fish production goals (mT). Additional research evaluating higher NO_3-N limits for Atlantic salmon and possibly different strains of rainbow trout is necessary before the exact threshold for including or excluding denitrification can be definitively selected.

3.8.6. Practical value

The practical value of the analyses presented in Section 3.8. is two-fold. First, if passive denitrification is taking place within freshwater RAS, the necessity for incorporating denitrification technologies may be diminished, thereby reducing RAS complexity, associated capital costs of added water treatment, and limiting the amount of water required to safely culture salmonids. Second, a potential increase in the safe NO_3-N threshold for rainbow trout or Atlantic to 200 mg/L based on strain or species-specific tolerance would confer similar benefits. Further, a NO_3-N tolerance shift overlapping with the increased propensity for passive denitrification at higher feed loading rates could result in combined benefits. It should be duly noted that passive denitrification may be specific to this system design, which incorporates a fluidized sand biofilter; therefore, additional research is required to understand the microbial dynamics and specificity of this phenomenon to RAS design or biofilter type. Additionally, while trends and scenarios have been presented that may facilitate reduced water use in RAS, a conservative approach to water use should be applied when planning and designing RAS facilities to ensure that ample water is available to maintain optimal water quality for salmonid production.

Table 3. Approximated water flushing x system design matrix for rainbow trout and Atlantic salmon production in freshwater RAS based on data collected from highlighted studies and data analysis provided in Figs. 14 and 15.

System Type/ Water Flushing Regimen	Partial Water Reuse	High Exchange RAS	Medium to Low Exchange RAS	Nearly Closed Loop RAS	Closed Loop RAS
Nitrate-Nitrogen Threshold	NA	NA	< 75-100 mg/L	>100 mg/L	200 mg/L
FLR (kg feed/ m^3 makeup water/day	< 0.05 - 0.10	0.10 - 1.0	1-4	> 4-5	15-30
Liters / kg feed /day	10,000 - 20,000	1,000 - 10,000	250 - 1,000	< 250	33 - 67
General Unit Process Configuration	Gas Conditioning Solids Removal	Gas Conditioning Solids Removal Biofiltration	Gas Conditioning Solids Removal Biofiltration Particle Control Water Chilling *	Gas Conditioning Solids Removal Biofiltration Particle Control Denitrification Water Chilling	Gas Conditioning Solids Removal Biofiltration Particle Control Denitrification † Water Chilling

* Necessity for water chilling is estimated in this instance but will depend on local climate. Warmest summer conditions should also be considered.

† Adoption of dentification will be dictated under these conditions may be dictated by additional research to definitively establish nitrate thresholds for Atlantic salmon and rainbow trout.

4. Summary and conclusions

A decade of research related to characterization and optimization of the RAS culture environment for the freshwater production of rainbow trout and Atlantic salmon was summarized in this manuscript. Important findings from highlighted research, in part, helped to shape the development, design, and operation of freshwater RAS for salmonids and filled critical knowledge gaps. For instance, accumulating water quality concentrations were ascertained in freshwater RAS operated with various water exchange rates, and dissolved Cu, K, and NO_3-N were identified as parameters of concern for rainbow trout production. Literature review and subsequent research revealed that nitrate was the primary cause of chronic toxicity effects to rainbow trout at concentrations as low as 80-100 mg/L, justifying adoption of 75 mg/L NO_3-N as the upper threshold for safe rainbow trout culture. However, subsequent observations indicate that rainbow trout do not always display chronic toxicity responses at or above this prescribed threshold. These findings indicate that NO_3-N toxicity can be modified by abiotic variables (e.g., alkalinity, hardness, salinity, and temperature), varying unit process designs (e.g. biofilter type; denitrification), and/or biotic factors (fish life stage, genetics, or species). Although post-smolt Atlantic salmon were unaffected by mean NO_3-N levels of 100 mg/L during one of the described

trials, the potential for variation in biological responses based on the aforementioned criteria should be considered. Ultimately, the results from these trials should not be generalized to all circumstances and conditions, and additional research may be required when substantial variations to environment, technology, or fish biology are applied.

This body of research also revealed that low-dose ozone (250-320 mV ORP) imparts relatively profound growth benefits when rearing rainbow trout and post-smolt Atlantic salmon in freshwater RAS. These positive growth effects appear to be related to a range of water quality improvements including reduced TSS, fine particles, water clarity (TC and UVT), THBC, and dissolved metals concentrations. The dramatic improvements in salmonid growth performance observed when operating RAS with ozone could significantly reduce the duration of the fish production cycle, thereby resulting in cost benefits for RAS farmers. Further, the compilation of research presented in this thesis exemplifies how water quality, fish health and performance, technology, and RAS operation interact and ultimately affect important decisions surrounding water use and water treatment process selection for RAS. As knowledge was gleaned about the RAS environment, particularly related to accumulating nitrate as a limiting factor, the research focus progressed toward evaluating denitrification within RAS. The first attempt at integrating denitrifying MBRs within the water recycle loop of RAS resulted in a 6.5-fold reduction in water use compared to traditionally operated RAS, while maintaining suitable rainbow trout health and performance. These findings occurred even while identifying operational deficiencies to MBRs such as incomplete denitrification and slower than expected membrane permeate rates that resulted in reduced water exchange between the MBR and RAS. Follow-up research is planned at the Freshwater Institute to re-evaluate MBR integration with RAS in conjunction with several improvements and optimizations.

Lastly, the holistic nature of this project resulted in newly gleaned information including: i) updated perspective related to potassium and nitrate toxicity for rainbow trout and Atlantic salmon, ii) a comprehensive summary of water flushing rates that support optimal performance of these species in freshwater RAS, iii) added knowledge regarding the potential for passive denitrification in similarly designed and operated RAS, and iv) a general guide and decision-making matrix for selection of salmonid RAS unit processes and flushing rates. Overall, this research significantly adds to the body of knowledge regarding freshwater production of rainbow trout and post-smolt Atlantic salmon in RAS and guides future research that will support the advancement of this growing industry sector.

5. Future research

Knowledge gleaned from each study described in this thesis emphasized the need for additional research and scientific validation. For instance, based on this body of research, additional study is required to understand the anomaly in nitrate toxicity responses between different rainbow trout populations. A critical upper limit of 75 mg/L NO_3-N was suggested; however, a recently studied rainbow trout cohort did not exhibit chronic toxicity symptoms when NO_3-N levels far exceeded this threshold. Additional research evaluating the effect of rainbow trout genetic strain and/or interacting environmental conditions, such as water temperature would

provide important follow-up information. Research is currently underway at *FI* to evaluate the performance of rainbow trout cohorts from different commercial suppliers in a semi-commercial scale RAS; however, a trial specifically focused on evaluating tagged rainbow trout of various genetic backgrounds exposed to different NO_3-N levels (e.g., 75 vs. 150 mg/L) would provide greater insight. Follow-up research is also needed to ascertain the critical upper NO_3-N limit for post-smolt Atlantic salmon in freshwater RAS. Research presented in this thesis suggested that post-smolt Atlantic salmon are tolerant of NO_3-N levels as high as 100 mg/L, but a critical upper limit has yet to be identified. Research evaluating the effects of 100 vs. 200 mg/L NO_3-N on post-smolt Atlantic salmon performance, health, and welfare is planned at *FI*. Nevertheless, because *FI* is focused on freshwater production of salmonids, similar research may be necessary in brackish or saltwater RAS. Ultimately, these follow-up studies could result in selection of genetic lots of salmonids that are best suited for RAS production under specific conditions (e.g. freshwater, saltwater), adjustment to originally suggested NO_3-N limits for rainbow trout and post-smolt Atlantic salmon, and further knowledge that directs decisions surrounding system design (denitrification) and operation (water use/flushing rates).

Further, research presented in this thesis indicated that days to months could be eliminated from market-size rainbow trout and Atlantic salmon production cycles when using low-dose ozone in freshwater RAS. Additional research should be carried out to assess the economic tradeoffs of ozone use and operation (energy, capital costs) compared to the benefits gleaned from improved fish growth and production efficiencies. While the growth benefits defined by this research are obvious, the decision to incorporate ozone in a RAS design should also be validated through economic analysis. In addition, it is unclear whether the growth differences observed between ozonated and non-ozonated RAS were partly influenced by tank size, where shallow tanks with slightly stained water in the absence of ozone may have impacted feed capture response. Similar research evaluating salmonid production with and without ozone should be carried out in larger, semi-commercial scale RAS. In the event that this type of research is not possible due to replication of very large RAS, time series production trials in large RAS could be considered if environmental and biological conditions can be effectively replicated.

In addition, follow-up research evaluating the integration of MBRs with RAS is planned with changes to system design and operation. Although incorporating MBRs within RAS was deemed as a feasible method that substantially reduced water use while maintaining suitable rainbow trout performance, a range of deficiencies were identified. Weaknesses included incomplete denitrification, inadequate MBR permeate production rates, limited water exchange across the MBRs, and complexity of balancing microbial processes in a single-vessel unit where anoxic and aerobic processes are required. The improved MBR design to be tested will utilize a multi-compartment system where anoxic and aerobic processes are separated and where membranes are not submerged in the viscous activated sludge. Regular membrane cleaning protocols will also be adhered to and permeate assist pumps will be incorporated in order to maintain permeate flow rates and associated water exchange across the MBRs. In addition, ozone will be applied in combination with the use of MBRs with expectation that ozone will

improve water clarity and reduce accumulating water quality constituents that result with exponential reductions to water exchange and dilution.

Lastly, further research is necessary to gain insight into the phenomenon of passive denitrification that was revealed when overlapping NO_3-N data versus flushing rate. Research designed to answer the following questions would provide additional insight: i) Is this phenomenon enhanced in fluidized sand biofilters or is it also prevalent in other filter types such as moving and fixed bed biofilters? ii) what are the specific microbial populations that are responsible for imparting the benefit of passive denitrification in RAS of different designs and environmental conditions (freshwater, saltwater), and iii) where is the brunt of passive denitrification taking place, e.g., the biofilter or elsewhere in RAS? A greater understanding of the passive denitrification process could enable replication within RAS and promotion of or development of a supplemental microbial consortium to facilitate the process. Ultimately, the ability to replicate passive denitrification could help to simplify RAS designs through elimination of intended denitrification unit processes.

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Study I

Abnormal swimming behavior and increased deformities in rainbow trout Oncorhynchus mykiss cultured in low exchange recirculating aquaculture systems

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Abnormal swimming behavior and increased deformities in rainbow trout *Oncorhynchus mykiss* cultured in low exchange water recirculating aquaculture systems

John Davidson*, Christopher Good, Carla Welsh, Steven T. Summerfelt

The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, United States

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ABSTRACT

Two studies were conducted to evaluate rainbow trout *Oncorhynchus mykiss* health and welfare within replicated water recirculating aquaculture systems (WRAS) that were operated at low and near-zero water exchange, with and without ozonation, and with relatively high feed loading rates. During the first study, rainbow trout cultured within WRAS operated with low water exchange (system hydraulic retention time (HRT) = 6.7 days; feed loading rate = 4.1 kg feed/m^3 daily makeup flow) exhibited increased swimming speeds as well as a greater incidence of "side swimming" behavior as compared to trout cultured in high exchange WRAS (HRT = 0.67 days; feed loading rate = 0.41 kg feed/m^3 daily makeup flow). During the second study, when the WRAS were operated at near-zero water exchange, an increased percentage of rainbow trout deformities, as well as increased mortality and a variety of unusual swimming behaviors were observed within WRAS with the highest feed loading rates and least water exchange (HRT $\geq$ 103 days; feed loading rate $\geq$ 71 kg feed/m^3 daily makeup flow). A wide range of water quality variables were measured. Although the causative agent could not be conclusively identified, several water quality parameters, including nitrate nitrogen and dissolved potassium, were identified as being potentially associated with the observed fish health problems.

1. Introduction

Water recirculating aquaculture systems (WRAS) offer many advantages (Summerfelt and Vinci, 2008); however, recent studies have indicated that accumulating water quality concentrations could be problematic when these systems are operated with minimal water exchange. Several studies have examined the effects of accumulating water quality parameters within low exchange WRAS on the performance of various species including: common carp (Martins et al., 2009a); hybrid striped bass *Morone chrysops* × *Morone saxatilis* and tilapia *Oreochromis* spp. (Brazil, 1996; Martins et al., 2009b); European sea bass *Dicentrarchus labrax* (Deviller et al., 2005), and rainbow trout *Oncorhynchus mykiss* (Davidson et al., 2009; Good et al., 2009). Martins et al. (2009a) concluded that ortho-phosphate-P, nitrate, and heavy metals (arsenic and copper) accumulated to levels that likely impaired the embryonic and larval development of common carp. Martins et al. (2009b) reported that larger tilapia showed a trend towards growth retardation in the lowest flushing WRAS, but small individuals seemed to grow faster in such systems. Deviller et al. (2005) attributed a 15% growth reduction in European sea bass cultured within WRAS to an unknown "growth-inhibiting substance" and suggested that metals accumulation could have contributed to reduced fish performance. Davidson et al. (2009) concluded that certain water quality constituents (e.g., dissolved copper, total suspended solids, and fine particulate matter) can accumulate to concentrations that are potentially harmful to salmonid performance and welfare when makeup water is reduced within WRAS and systems are operated with relatively high feed loading rates ($\geq$4 kg daily feed per m^3 daily makeup water).

Other studies have also indicated that certain water quality constituents measured within fish culture systems can cause skeletal deformities. Baeverfjord et al. (2009a) reported that anecdotal evidence from intensive Atlantic salmon *Salmo salar* smolt production trials indicated that some aspect of the water quality was associated with skeletal deformity, but could not pinpoint a specific parameter. Additionally, Baeverfjord et al. (2009b) attributed increasing levels of carbon dioxide (up to 30 mg/L) to a shortening of the body in cultured rainbow trout. Shimura et al. (2004) suggested that elevated nitrate nitrogen (100 mg/L) contributed to skeletal deformity observed in juvenile Medaka *Oryzias latipes* during a long-term toxicity challenge in aquaria. Many studies

* Corresponding author. Tel.: +1 304 876 2815x221; fax: +1 304 870 2208.
E-mail address: j.davidson@freshwaterinstitute.org (J. Davidson).

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have indicated that elevated concentrations of various water quality parameters in natural settings have caused increased skeletal deformities in fish including: heavy metals (Bengtsson et al., 1988; Lall and Lewis-McCrea, 2007); zinc (Bengtsson, 1974; Sun et al., 2009); cadmium (Pragatheeswaran et al., 1987), lead (Sun et al., 2009); selenium (Lemly, 2002); ammonium and low dissolved oxygen (Sun et al., 2009). Lall and Lewis-McCrea (2007) suggested that skeletal deformities in fish could also be caused by insecticides, pesticides, organochlorine, and other chemicals. Many of the aforementioned studies also discussed changes in fish behavior that were likely associated with elevated water quality concentrations.

A series of controlled studies have been conducted in six replicated WRAS to identify how fish growth, survival, health, and welfare metrics are impacted under various culture conditions (Davidson et al., 2009, 2011; Good et al., 2009, 2010). The primary objective of this paper is to describe fish health and welfare observations (unusual swimming behaviors, increased prevalence of deformities, and decreased survival) from several of these studies (Davidson et al., 2011), as well as the corresponding water quality conditions, when WRAS were operated at low and near-zero water exchange.

2. Methods

2.1. Experimental systems and treatments

Rainbow trout performance, health, and welfare metrics as well as system water quality were evaluated within six identical 9.5 m^3 WRAS during two studies. These systems are described in detail in Davidson et al. (2009, 2011). Treatment metrics for the present studies are outlined in Table 1. Study 1 – Three WRAS were operated with "low" water exchange and ozone vs. three WRAS operated with "high" water exchange without ozone. Mean system hydraulic retention times (HRT) for the low and high exchange WRAS were approximately 6.7 and 0.67 days, respectively; and mean feed loading rates were 4.1 and 0.41 kg feed per cubic meter of daily makeup water, respectively (Davidson et al., 2011). WRAS described as operating at low and high water flushing rates continuously exchanged 0.26% and 2.6% of the total recycled flow. Study 2 – The original study design was to evaluate three WRAS operated at near-zero water exchange (i.e., backwash replacement only) with ozone compared to three WRAS operated at near-zero water exchange without ozone. During this study, periodic drum filter failures occurred within four of six WRAS which resulted in increased and variable dilution amongst WRAS. Additionally, drum filter backwash spray was found to be added as additional makeup water to some WRAS and not others, which also contributed to differences in dilution. Due to the variability in flushing during Study 2, individual WRAS turnover rates varied from <10 days to as high as 180 days and feed loading rates ranged from 4 to 147 kg feed per cubic meter of daily makeup water. In order to evaluate the potential correlation of feed loading rate and accumulating water quality to the observed fish health and welfare issues during the present studies, WRAS were separated into two treatment groups based on feed loading rate and HRT: (1) very low exchange – WRAS with mean HRT's of $\leq$36 days and mean feed loading rates $\leq$44 kg feed/m^3 makeup water/day compared to (2) near-zero exchange – WRAS with HRT's $\geq$103 days and feed loading rates $\geq$71 kg feed/m^3 makeup water/day. For comparative purposes, data generated from WRAS 3 was excluded. WRAS 3 had the least flushing of any WRAS and also used ozone; therefore this system could not be categorized with other WRAS that did not use ozone and had significantly different flushing rates.

2.2. Rainbow trout

All female, diploid, rainbow trout (Kamloops strain) obtained as eyed eggs from Troutlodge Inc. (Sumner, WA, USA) were used. All experimental fish were hatched on-site within a recirculating incubation system and then cultured within flow through systems prior to use in the present studies. Equal numbers of fish were stocked in each WRAS to begin each study. Rainbow trout were 151 ± 3 g to begin Study 1 and 18 ± 1 g to begin Study 2. Initial stocking densities for Studies 1 and 2 were 30 and 12 kg/m^3, respectively. Maximum densities were maintained at $\leq$80 kg/m^3.

2.3. Photoperiod and feeding

A constant 24-h photoperiod was provided. Fish were fed a standard 42:16 trout diet (Zeigler Brothers, Inc., Gardners, PA, USA). Equal daily rations were delivered to each WRAS with feeding events occurring every other hour, around the clock, using automated feeders (T-drum 2000CE, Arvo-Tec, Finland). Additional detail relative to feeding methodology was described in Davidson et al. (2011).

2.4. Sampling protocols

Fish were sampled for lengths and weights on a monthly basis and mortalities were removed and recorded daily to assess cumulative survival. During the final fish sampling event of Study 2, notations were made for fish that had any form of curved spine, including kyphosis and lordosis (ventral and dorsal spinal deviations in the axial plane, respectively); scoliosis (spinal deviations in the sagittal plane); or any combination of these observable abnormalities. Skeletal deformities were then summed and divided by the total number of fish sampled per WRAS to determine a percentage of the population affected.

Water samples were collected weekly from the side drain of each tank and tested for a variety of parameters and a series of dissolved metals and elements were analysed when fish were at near-maximum densities and feed loading rates (Davidson et al., 2011). Specific methodologies and laboratory information for all water quality analyses were described in Davidson et al. (2011).

2.5. Rainbow trout swimming speed and behavior observations

Two distinct differences in rainbow trout swimming behavior were observed between treatments during these studies: (1) swimming speed and (2) prevalence of side swimming fish, i.e., fish swimming oriented on their side. Swimming speeds were quantified weekly by timing individual fish passing between marked locations distanced 3 ft apart and then adding the water rotational velocity within 30 cm of the tank wall. Swimming speeds of 15 fish were measured within each tank weekly, including five fish near the top, middle, and bottom of the tanks. Swimming speed measurements for Study 1 began after 7 weeks when it became evident that a distinct difference existed between the high exchange and low exchange treatments. During Study 2, measurements were taken only during the first 9 weeks of the study when water quality was still clear enough to observe fish in the non-ozonated WRAS.

Side swimming behavior was assessed during Study 1 by positioning a video camera directly above the center of each tank. Video footage was collected for the first time approximately 4 months into the study. Five minutes of video were collected for each WRAS. Black and white snap shots of the video were then clipped out at 1 min intervals and side swimming fish, which had a distinct white appearance in the picture, were manually counted. Mean numbers of side swimmers were then calculated and compared between treatments. Side swimming was not quantified during

Table 1
Experimental design overview of water exchange, feed loading rate, hydraulic retention time, and use of ozone for each treatment utilized during Studies 1 and 2.

Water exchange	Number of WRAS	Feed loading (kg feed/m^3 makeup flow/day)	Hydraulic retention time (days)	Ozone
Study 1				
High	3	0.41	0.67	0 of 3
Low	3	4.10	6.70	3 of 3
Study 2				
Very low	3	17–44	25–36	0 of 3
Near-zero	2	71–147	103–180	2 of 2

Note: WRAS 3 was excluded from most analyses, because of the low exchange systems for Study 2, it was the only system that used ozone and also had significantly greater flushing and significantly lower feed loading rates. Therefore, it was considered an outlier.

Study 2, because the water quickly became too turbid to observe this behavior in the non-ozonated WRAS.

2.6. Statistical analysis

Statistical comparisons of swimming speed and number of side swimming fish were made using a Student's t-test. Transformations were applied to abnormally distributed data. All parameters that were sampled during multiple events over time from the same location, such as water quality parameters were analysed using a Hierarchical Mixed Models approach called Restricted Maximum Likelihood (REML), which allows the assignment of "Tank" as a random factor, thus buffering the main treatment effect from potential variation arising from tank effects. A hierarchical approach was recommended by Ling and Cotter (2003), who suggested that the random variation between replicated tanks represents a "nuisance factor" in aquaculture experiments. A probability value (α) of 0.10 was used to determine significance for each statistical test as opposed to the traditional 0.05 due to a relatively low n-value (three WRAS per treatment). Statistical correlation analysis was used to evaluate the strength of relationships between various fish health and welfare metrics and specific water quality parameters. Statistical analyses were carried out using SYSTAT 11 software (2004).

3. Results

3.1. Rainbow trout health and welfare

3.1.1. Swimming speed

Rainbow trout swimming speed generally increased as the WRAS flushing rate decreased and when the system hydraulic retention time was longer. For example, during Study 1, mean swimming speeds in WRAS operated at high exchange were 35.9, 17.5, and 15.9 cm/s; while trout within WRAS operated at low exchange swam at mean speeds of 49.3, 48.1, and 42.6 cm/s ($P = 0.056$). Statistical comparison indicated that trout within the low exchange WRAS swam at a significantly greater mean speed (1.4 ± 0.1 body lengths/s (bl/s)) than fish cultured within WRAS operated at high exchange (0.7 ± 0.2 bl/s) ($P = 0.041$) (Fig. 1). Feed loading rate (kg feed/day per m^3 makeup water/day) appeared to be a more correlative metric with rainbow trout swimming speed rather than flushing rate alone. Daily feeding gradually decreased over the course of the study as fish grew larger, thus feed loading decreased and the concentrations of various water quality components were reduced. These changes occurred in unison with the reduction in rainbow trout swimming speed that was evident from the third to sixth month of Study 1 (Fig. 1). Daily observations indicated that trout tended to maintain the described swimming speeds for each condition continuously without rest. During Study 1, fish within the low exchange WRAS were always observed swimming faster than the water rotational velocity, while fish within the high exchange WRAS generally held position in the water column.

During Study 2, rainbow trout swimming speed was generally greater, but not significantly, in WRAS with higher HRT's and feed loading rates. Fish within the very low exchange WRAS had mean swimming speeds of 44.8, 30.6, and 44.1 cm/s, while swimming speeds in the near-zero exchange WRAS were 45.7 and 46.1 cm/s. Rainbow trout stocked during Study 2 were smaller (18 g) than those stocked during Study 2 (151 g), thus swimming speed relative to body length was greater and ranged from 2.1 to 3.4 bl/s.

3.1.2. Rainbow trout swimming behavior

In addition to the swimming speed differences measured between treatments during Study 1, other obvious differences in rainbow trout swimming behavior were observed. Specifically, a statistically greater portion of the population within the low exchange WRAS were observed swimming on their sides in comparison to the high exchange WRAS ($P = 0.001$) (Figs. 2 and 3). Count data from video snap shots taken 4 months into Study 1 indicated 42 ± 1 side swimming trout in the low exchange WRAS and 10 ± 2 side swimming trout in the high exchange WRAS. Figs. 2 and 3 illustrate the statistically greater number of side swimmers within the low exchange WRAS. Video recordings taken near the end of Study 1, i.e., after 6 months, indicated similar results. At that time, counts of side swimming trout from the low and high exchange WRAS were 26 ± 7 and 10 ± 1 side swimmers, respectively. During Study 2, trout within the near-zero exchange WRAS exhibited additional unusual behaviors including erratic swimming, swimming near the water surface (surface swimming), and periodically swimming at an oblique angle to the surface with their nose out of the water.

3.1.3. Rainbow trout deformities

During Study 1, a difference in the prevalence of rainbow trout deformities was not observed between treatments. However, during Study 2, a higher incidence of skeletal deformities (as pictured in Fig. 4) were observed, particularly in WRAS operated with the

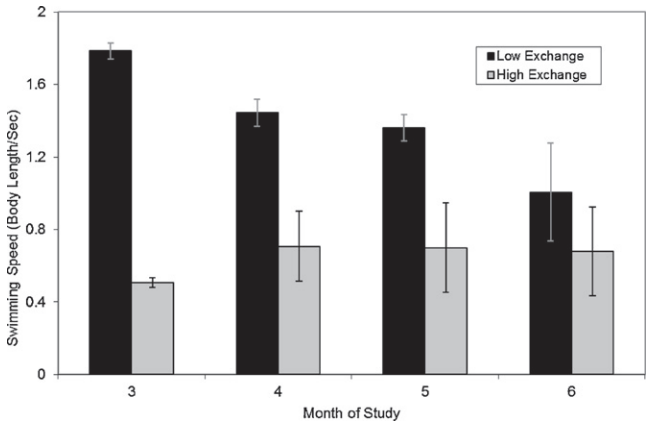

Fig. 1. Mean rainbow trout swimming speeds (±1 standard error) measured from the third to sixth month of Study 1 within WRAS operated with high water exchange vs. low water exchange ozone.

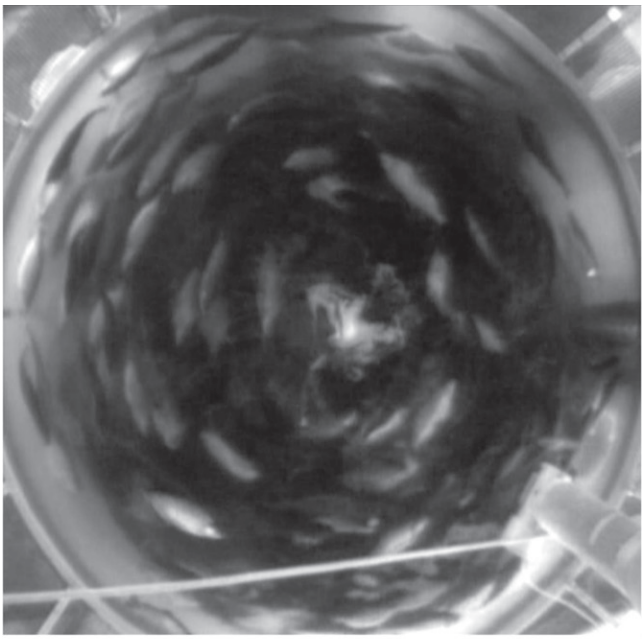

Fig. 2. Video frames of "side swimming" rainbow trout within WRAS operated at low water exchange with ozone and high water exchange without ozone (Study 1).

least flushing and greatest feed loading, i.e. near-zero exchange. For example, the WRAS with the least flushing (HRT = 180 days) had the greatest prevalence of skeletal deformities, 38%, while WRAS with the greatest flushing (HRT = 5 days), had no observable skeletal deformities, 0%. Fish within the near-zero exchange WRAS were also observed as having stiffened musculature during handling.

3.1.4. Decreased survival

During Study 1, rainbow trout survival was excellent for all WRAS and was similar between low exchange WRAS and high exchange WRAS, i.e. 93.3 ± 1.6% and 93.1 ± 0.5%, respectively. Therefore, the flushing and/or feed loading rates did not appear to impact survival during Study 1. During Study 2, WRAS operated at near-zero exchange had substantially greater mortality in

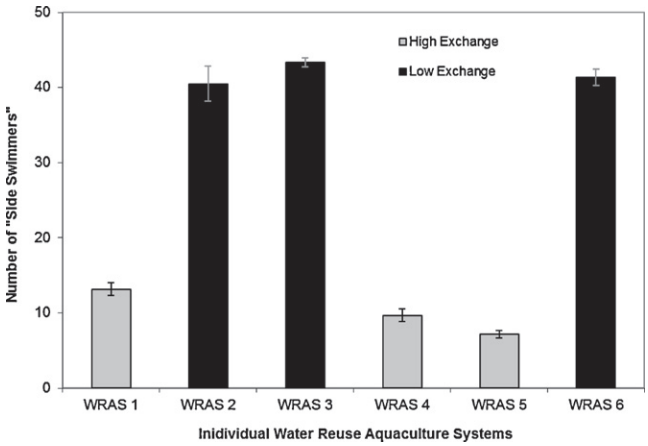

Fig. 3. Number of side swimming rainbow trout (±1 standard error) counted from video frames from individual WRAS during Study 1, comparing WRAS operated at low water exchange with ozone vs. high water exchange without ozone.

comparison to all other WRAS. Mean cumulative survival for the near-zero exchange WRAS (mean HRT ≥103 days and feed loading ≥71 kg feed/day per m^3 makeup water/day) was 85.7 ± 1.9%, while mean survival for the very low exchange WRAS (HRT's of ≤36 days and mean feed loading rates ≤44 kg feed/m^3 makeup water/day) was 94.6 ± 0.4%.

3.2. Water quality concentrations

An extensive suite of water quality parameters were analysed during both studies. Water quality concentrations measured over the duration of each study are presented in Table 2 and dissolved metals concentrations from samples taken during near-maximum feed loading periods are presented in Table 3.

Fig. 4. Examples of skeletal deformities observed in rainbow trout cultured within near-zero exchange WRAS during Study 2.

Table 2
Mean water quality values (±1 standard error) at the tank side drains over the duration of Studies 1 and 2 between systems operated at various water exchange rates. Means for most parameters during Studies 1 and 2 derived from 22 and 17 weekly sampling events, respectively.

	Study 1		Study 2	
Treatment	Low exchange	High exchange	Very low exchange	Near-zero exchange
TAN*1*2	0.31 ± 0.02	0.45 ± 0.01	0.92 ± 0.09	0.77 ± 0.05
NH_3	0.003 ± 0.000	0.003 ± 0.000	0.008 ± 0.001	0.005 ± 0.000
NO_2–N	0.11 ± 0.04	0.08 ± 0.00	0.13 ± 0.01	0.13 ± 0.09
NO_3–N*1*2	13 ± 0	99 ± 7	171 ± 16	422 ± 13
Alkalinity*1	224 ± 3	200 ± 1	216 ± 3	209 ± 3
pH*1*2	7.61 ± 0.01	7.47 ± 0.01	7.54 ± 0.03	7.44 ± 0.02
CO_2	10 ± 1	11 ± 0	14 ± 1	16 ± 0
$cBOD_5$ *1*2	2.5 ± 0.1	3.0 ± 0.2	11.8 ± 2.7	3.7 ± 0.2
TOC	11.2 ± 2.1	17.9 ± 2.8	–	–
DOC	9.0 ± 1.2	16.1 ± 1.6	–	–
True color*1*2	12 ± 0	5 ± 1	157 ± 25	5 ± 1
UV transm. (%)*1*2	89 ± 0	77 ± 2	30 ± 2	61 ± 0
Phosphorous*1*2	0.8 ± 0.0	3.9 ± 1.0	5.2 ± 0.0	9.3 ± 0.8
TSS*1*2	3.4 ± 0.1	4.6 ± 0.5	18.9 ± 1.1	3.5 ± 0.6
Heterotrophic bacteria	117 ± 23	114 ± 19	825 ± 407	61 ± 7
Temperature (°C)	12.9 ± 0.0	13.0 ± 0.1	15.7 ± 0.0	15.6 ± 0.1
Conductivity	–	–	2.7×10^3	4.7×10^3
DO*1*2	10.4 ± 0.0	10.6 ± 0.0	9.7 ± 0.0	11.1 ± 0.1
ORP*1*2	195 ± 8	238 ± 2	156 ± 12	265 ± 6

Note: Mean ORP levels include days when ozone was turned off and are therefore slightly below the ORP ranges described in Section 2.
* Indicates statistically significant between treatments ($P < 0.10$), 1, or 2 following * indicates study 1 or 2.

4. Discussion

4.1. Health and welfare

A variety of unusual swimming behaviors were noted during Studies 1 and 2 that correlated with WRAS water exchange and feed loading rates. The prevalence of each of these behaviors was always greater within WRAS that were operated with less water exchange or greater feed loading, which in turn contained the highest ionic and water quality concentrations.

The authors hypothesize that the increased swimming speeds were a physiological response (similar to a flight response) caused by chronically stressful water quality concentration(s). The observations of increased rainbow trout swimming speed with increasing HRT are important from a fish health and welfare perspective for several reasons: (1) the increased swimming speeds represented a deviation from typical swimming behavior. Given a sufficient rotational velocity, salmonids generally hold position in the water column, as was observed in the high exchange WRAS during Study 1. (2) Increased swimming speeds can result in dramatic increases in oxygen consumption in fish (Brett, 1973; Forsberg, 1994). (3) The mean swimming speeds measured during Study 2 (2.1–3.4 bl/s) and those measured during the third month of Study 1 (1.8 ± 0.0 bl/s) (Fig. 1), exceeded the recommendations of Davison (1997), who provided an overview of literature on the effects of exercise training in fish. 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. Additionally, Jain et al. (1997) determined that the "fatigue velocity" for rainbow trout was 2.1 ± 0.1 bl/s; therefore, it is possible that rainbow trout were swimming at exhaustive speeds during Study 2. (4) Lastly, excessive swimming activity can cause the accumulation of lactic acid in the blood (lactic acidosis), which can contribute to mortality when fish are severely exercised (Wedemeyer, 1996).

The authors have observed side swimming behavior in a small percentage of rainbow trout previously cultured on-site. The percentage of side swimming trout observed during Study 1 far

Table 3
Mean dissolved metal and nutrient concentrations (mg/L) (±1 standard error) at the tank side drain outlets when WRAS were operated near-maximum feed loading and fish density during Studies 1 and 2. Means for Study 1 derived from one sampling event at near-maximum feed loading. Means for Study 2 derived from two sampling events at near-maximum feed loading.

	Study 1		Study 2	
Treatment/parameter	High exchange	Low exchange	Very low exchange	Near-zero exchange
Barium*1	0.055 ± 0.001	0.043 ± 0.001	0.367 ± 0.066	0.228 ± 0.011
Boron	<MDL	<MDL	0.061 ± 0.011	0.079 ± 0.000
Calcium*1*2	108 ± 0	104 ± 1	99 ± 2	71 ± 1
Copper*1*2	0.014 ± 0.002	0.038 ± 0.004	0.119 ± 0.008	0.050 ± 0.010
Iron*2	<MDL	<MDL	0.041 ± 0.013	0.006 ± 0.001
Magnesium*1*2	12.1 ± 0.1	14.8 ± 0.4	19.8 ± 0.4	26.2 ± 0.1
Manganese	<MDL	<MDL	0.008 ± 0.004	<MDL
Phosphorous*1*2	0.5 ± 0.1	2.7 ± 0.2	7.0 ± 1.6	14.5 ± 0.0
Potassium*1*2	5 ± 0	25 ± 3	44 ± 7	112 ± 10
Silicon	48 ± 0	43 ± 2	44 ± 1	41 ± 2
Sodium*1*2	5 ± 0	164 ± 20	346 ± 86	753 ± 70
Strontium*1*2	0.90 ± 0.00	0.83 ± 0.01	0.89 ± 0.02	0.72 ± 0.00
Sulfur*1*2	9.5 ± 0.2	18.4 ± 1.1	26.7 ± 2.0	48.3 ± 1.7
Zinc	0.011 ± 0.003	0.007 ± 0.002	0.128 ± 0.023	0.082 ± 0.000

<MDL = less than minimum detection limit of the test. *Notes:* The following elements were below the minimum detection limit within the culture water for all treatments during both studies: aluminum, arsenic, beryllium, cadmium, chromium, cobalt, lead, mercury, molybdenum, nickel, and selenium.
* Indicates statistically significant between treatments ($P < 0.10$), 2, or 3 following * indicates study 2, or 3.

exceeded that of previously cultured cohorts and therefore was viewed as a potential concern for the health and welfare of the fish. Unfortunately, very little information is available in the literature regarding side swimming behavior in fish. The authors hypothesize that constant increased swimming speeds in the same continuous circular pattern without rest could have caused physiological or morphological changes, such as imbalance in musculature symmetry, skeletal deformities, or a deviation of swim bladder shape and positioning, which may have contributed to the increased side swimming behavior observed in the population. The anatomical and physiological changes associated with side swimmers, however, require further investigation to provide greater understanding of this phenomenon.

Other unusual behaviors were observed during Study 2 in rainbow trout cultured within WRAS with the least flushing and greatest feed loading rates. Specifically, rainbow trout within the near-zero exchange WRAS began to swim erratically several months into the study. Some fish swam near the surface with their bodies at an oblique angle as opposed to fish swimming normally, parallel to the water column. Many of the erratically swimming fish broke the surface of the water with their nose pointed up and exhibited a yawning or gulping action with their mouth. Observation of these unusual swimming behaviors increased as the study progressed and could be defined as severe near the end of the study. The authors hypothesize that the various abnormal swimming behaviors observed during Study 2 could have induced the increase in skeletal deformities. Divanach et al. (1997) concluded that intense posterior muscular activity in sea bass exposed to consistently strong currents induced lordosis. Therefore, it is feasible that rainbow trout swimming at increased speeds always in the same circular direction could have been prone to skeletal deformation during the present studies. Additionally, Kitajima et al. (1994) associated lordotic deformation of the skeleton in hatchery-bred physoclistous fish with an abnormal swimming behavior in which fish swam at an oblique angle to the water surface to compensate for deflated swim bladders. The behavior observed during Kitajima et al.'s study caused a V-shape curvature of the backbone in fish displaying this behavior. During Study 3, rainbow trout were noticed swimming at an oblique angle to the water surface in the near-zero exchange WRAS. Based on Kitajima et al.'s findings, this behavior could have been related to the increase in skeletal deformities observed within these systems, particularly for deformed trout with heads that appeared to curve upward, causing a V-shape of the spine (Fig. 4; fish at top). Skeletal deformities can be a serious economic problem in commercial aquaculture. Deformed fish are often culled from the population or have reduced market value.

Many water quality parameters have been cited as causes, as previously discussed. Although it is apparent that elevated concentrations of various water quality criteria can contribute to skeletal abnormalities, many other parameters have also been implicated. For example, skeletal deformities in cultured salmonids have been attributed to: incubation temperature (Lein et al., 2009), diet and nutrition (Madsen and Dalsgaard, 1999; Power, 2009), genetics (McKay and Gjerde, 1986), and methods used to induce triploidy (Madsen et al., 2000; Sadler et al., 2001; Fjelldal and Hansen, 2010). The fish that were used during the present studies were hatched under the same conditions at the same time, were from the same diploid cohort, and were fed the same diet throughout their life cycle. The skeletal deformities that were observed during Study 2 materialized during the study period, and were therefore at least partially, if not entirely, related to the environmental conditions created during this study.

In addition to the fish health and welfare issues observed, survival also appeared to be related to flushing and feed loading rate during Study 2. Therefore, some aspect(s) of the water quality within the near-zero exchange WRAS likely reached chronic to slightly acute concentrations in order to cause the low level mortality observed.

Each of the aforementioned fish health and welfare metrics appeared to be correlated to feed loading and system flushing rates which suggests that accumulating water quality constituents were related to the observed problems. Therefore, a brief review of the water quality concentrations measured during each of these studies is warranted and provides valuable information and direction for future studies designed to identify accumulating water quality variables that become problematic in low and near-zero exchange WRAS.

4.2. Water quality

Of the water quality parameters measured during Study 1 (Tables 2 and 3), some could systematically be excluded as potential causative agents of the aforementioned health and welfare problems due to: (1) lack of detection during laboratory analyses; (2) concentrations that were significantly lower within WRAS in which health and welfare issues were observed; and (3) concentrations that were not significantly different between WRAS in which health and welfare problems were observed. The remaining water quality parameters that were significantly greater within WRAS in which fish health and welfare issues occurred (i.e., low exchange (Study 1) and near-zero exchange (Study 2)) were further considered as potential causative agents of the observed problems. Water quality parameters are grouped within each of the aforementioned statistical categories in Tables 4 and 5.

The potential toxicity of each water quality concentration that was statistically greater within the low exchange WRAS (Study 1, Table 4) and near-zero exchange WRAS (Study 2, Table 5) were assessed based on toxicity information available in the literature. Davidson et al. (2009, 2011) reviewed recommended upper limits for a variety of metals and water quality parameters as reported in literature (Piper et al., 1982; Meade, 1989; Heinen, 1996; Wedemeyer, 1996; EPA, 1987, 1996, 2002, 2007; Colt, 2006; Boyd, 2009). Of these parameters, nitrate nitrogen, copper, and potassium were categorized as existing at potentially toxic concentrations during Study 1. Statistical correlation analysis indicated that copper, potassium, and nitrate nitrogen correlated well with the number of side swimming fish, as well as fish swimming speed during Study 1. Pearson's correlation coefficient (R) for copper, potassium, and nitrate nitrogen was 0.937, 0.960, and 0.977, respectively, relative to the number of side swimming fish; and 0.916, 0.935, 0.881, respectively, relative to rainbow trout swimming speed.

During Study 2, statistical analysis indicated that copper did not correlate well with swimming speed, deformity, or survival, but indicated a strong correlation of potassium and nitrate nitrogen to each of these metrics. Pearson's correlation coefficient for copper, potassium, and nitrate nitrogen was 0.052, 0.636, and 0.667, respectively, relative to swimming speed; 0.049, 0.609, and 0.762, respectively, relative to deformity; and 0.396, 0.880, and 0.971, respectively, relative to survival.

The authors are fully aware that all water quality parameters that could accumulate within low and near-zero exchange WRAS were not measured during the present studies. Concentrations of other unmeasured parameters could have been related to the fish health and welfare problems observed. For example, pheromones secreted by the fish could accumulate within WRAS and could cause an alarm reaction or other impacts to fish behavior (Solomon, 1977; Colt, 2006). In addition, endocrine disrupting chemicals including pesticides, natural and synthetic hormones, and PCB's could accumulate within WRAS if present within the makeup water supply and could cause adverse effects to fish (Damstra et al., 2002; Colt, 2006). Furthermore, plasticizers and/or trace contaminants from

Table 4
Systematic grouping of measured water quality parameters relative to statistical analysis, used to facilitate identification of water quality parameters that could have been related to the fish health and welfare problems observed during Study 1.

<Detection within low exchange	Significantly < within low exchange	No significant difference between high and low exchange	Significantly > within low exchange
Aluminum	Barium	Unionized ammonia	Copper
Arsenic	Calcium	Nitrite nitrogen	Magnesium
Beryllium	Strontium	Carbon dioxide	Phosphorous
Boron	True color	Total organic carbon	Potassium
Cadmium	UV transmittance	Dissolved organic carbon	Sodium
Chromium		Heterotrophic bacteria	Sulfur
Cobalt		Temperature	Total ammonia nitrogen
Iron			Nitrate nitrogen
Lead			Alkalinity
Manganese			pH
Mercury			Biochemical oxygen demand
Molybdenum			Total suspended solids
Nickel			Dissolved oxygen
Selenium			ORP

PVC or fiberglass could leach from system components, accumulate within WRAS, and potentially cause negative impacts to cultured species (Carmignai and Bennett, 1976; Zitko et al., 1985; Colt, 2006). In addition, interacting or combined effects of various water quality parameters (measured and/or unmeasured), as well as the overall conductivity or ionic concentration of the culture environment could have been responsible for the observed fish health and welfare problems.

The following discussion is meant to focus on the few measured parameters that were separated as being potentially related to the described fish health and welfare issues, which will be beneficial to future research regarding the toxicity of specific water quality parameters within low and near-zero exchange WRAS.

4.2.1. Ozone

Aside from water exchange rate, a distinct difference between treatments during Studies 1 and 2 was the use of ozone; therefore a brief toxicity review was warranted. During each study, ozone was generally used within WRAS that were operated at lower water exchange rates, i.e., WRAS in which the majority of abnormal swimming behaviors and other negative health and welfare effects were observed. Therefore, ozone toxicity was stringently evaluated. Bullock et al. (1997) suggested that an ORP level of 300 mV was safe for rainbow trout, and Summerfelt et al. (2009) reported that mean dissolved ozone concentrations were 0 ppb at a mean ORP ≤340 mV. To ensure that ozone did not remain in the culture water at toxic concentrations during the present studies, ozone residual was monitored and controlled using ORP. Mean ORP levels recorded over the duration of Studies 1 and 2 within WRAS operated with ozone were 238 ± 2 and 265 ± 6 mg/L, respectively (Table 2), thus ORP was maintained well below the threshold at which ozone residual becomes problematic for fish (Bullock et al., 1997; Summerfelt et al., 2009). Study 2 results further vindicated ozone residual as a cause for the negative impacts on fish health and welfare, because WRAS 3, which was operated with ozone, did not exhibit the previously described abnormal rainbow trout swimming behaviors, skeletal deformities, or decreased survival. In addition, previous on-site studies have been conducted using a similar ozone dose within a commercial scale WRAS culturing salmonids (Summerfelt et al., 2009), without any of the consequences to fish that are described in this paper. Thus, ozone residual was not suspected as a possible cause of the observed fish health and welfare problems.

4.2.2. Copper

Davidson et al. (2009) provided an overview of literature regarding the toxicity of copper to salmonids. In summary, the chronic-acute limits for dissolved copper are 0.022–0.037 mg/L at a corresponding water hardness of 300 mg/L as $CaCO_3$ (Alabaster and Lloyd, 1982; EPA, 2002). Water hardness measured during the present studies ranged from 290 to 312 mg/L as $CaCO_3$. In addition to hardness, alkalinity, pH, temperature, dissolved organic carbon (DOC), and TSS (Spear and Pierce, 1979; Alabaster and Lloyd, 1982; Sprague, 1985; U.S. EPA, 2002, 2007), can interact to alter copper toxicity. Updated U.S. EPA (2007) guidelines for copper toxicity which account for hardness, as well as DOC indicate that the chronic-acute copper toxicity limits could have been at least four times greater (≥0.088–0.148 mg/L) than earlier EPA models predicted (0.022–0.037 mg/L) at the same alkalinity (200 mg/L). Based on this toxicity review, rainbow trout in the low exchange WRAS

Table 5
Systematic grouping of water quality parameters based on statistical analysis, used to facilitate separation of water quality parameters that could have been related to the fish health and welfare problems observed during Study 2.

<Detection within near-zero exchange	Significantly < within near-zero exchange	No significant difference between very low and near-zero exchange	Parameters significantly > within near-zero exchange WRAS
Aluminum	Calcium	Barium	Magnesium
Arsenic	Copper	Boron	Phosphorous
Beryllium	Iron	Silicon	Potassium
Cadmium	Strontium	Zinc	Sodium
Chromium	Total ammonia nitrogen	Nitrite nitrogen	Sulfur
Cobalt	Unionized ammonia	Alkalinity	Nitrate nitrogen
Lead	pH	Carbon dioxide	UV transmittance
Mercury	Biochemical oxygen demand	Heterotrophic bacteria	Conductivity
Manganese	True color	Temperature	Dissolved oxygen
Molybdenum	Total suspended solids		ORP
Nickel			
Selenium			

during Study 1 and in all WRAS during Study 2 would have been negatively impacted by the measured dissolved copper concentrations (0.038–0.119 mg/L) (Table 3) in the absence of other buffering water quality parameters.

4.2.3. Potassium

Potassium accumulated with increasing feed loading rate and HRT (Davidson et al., 2011); thus, mean dissolved potassium levels during Study 1 were approximately five times greater (25 ± 3 mg/L) within the low exchange WRAS (Table 3) in which the abnormal swimming behaviors were observed as compared to the high exchange WRAS (5 ± 0 mg/L). During Study 2, potassium concentrations also accumulated relative to increasing feed loading rate and HRT (Table 3). Dissolved potassium concentrations in the very low exchange and near-zero exchange WRAS were 44 ± 7 and 112 ± 10 mg/L, respectively (Table 3).

Scientific literature typically discusses potassium toxicity relative to compounds such as potassium permanganate or potassium cyanide; therefore, little information is available regarding the toxicity of dissolved potassium alone. One study which evaluated the acute toxicity of potassium permanganate in African catfish *Clarius gariepinus* fingerlings, noted symptoms such as erratic swimming and gulping for air, which seem similar to observations during the present studies (Kori-Siakpere, 2008). Buhse (1974) reported that potassium >200 mg/L was toxic to fish in freshwater environments. Bell (1990) reported that 50 mg/L potassium could be toxic to fish, especially in soft water. Additionally, Heinen (1996) referenced literature that suggested that ≥10 mg/L potassium is acceptable for culture water with hardness >100 mg/L. Additionally, potassium levels of 100–130 mg/L were suspected as the cause for gill problems in rainbow trout (>400 g) in an aquaponics facility that supplemented potassium (personal communication, Marc Laberge, Cultures Aquaponiques Inc., Quebec, CA). With such a wide range of recommendations, it is unclear whether the potassium concentrations measured during the present studies were harmful to rainbow trout, thus further evaluation is needed.

4.2.4. Nitrate nitrogen

Several important publications have stated that NO_3–N is generally nontoxic to fish at concentrations that would be expected under typical culture conditions (Wedemeyer, 1996; Colt and Tomasso, 2001; Timmons and Ebeling, 2007; Colt, 2006). However, few specific studies have been conducted to evaluate the toxicity of NO_3–N to salmonids. Camargo et al. (2005) provided an overview of nitrate toxicity studies conducted with freshwater fish including salmonids. Several of these studies indicated that NO_3–N can be chronically toxic to salmonid eggs and larvae at concentrations <200 mg/L with sublethal effects occurring at <25 mg/L (Kincheloe et al., 1979; McGurk et al., 2006). However, establishment of acute, chronic, and sublethal NO_3–N levels would certainly depend upon life stage (Camargo et al., 2005). Only Westin (1974) evaluated the effects of NO_3–N to fingerling-sized rainbow trout (Camargo et al., 2005). 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 salmonids. During Westin's study, rainbow trout were reported to swim near the surface of the tank exhibiting a yawning or gulping action, and some broke the surface with their nose as if trying to escape. Interestingly, many of the rainbow trout swimming behaviors reported by Westin (1974) due to toxic nitrate nitrogen were similar to those reported during the present studies. In addition, unusual swimming behavior similar to that observed during the present studies including side swimming behavior, as well as stiffened or contracted musculature, have also been observed in seabream cultured in a zero-discharge WRAS when NO_3–N concentrations were 200–300 mg/L (personal communication, Jaap Van Rijn, Hebrew University of Jerusalem, Israel). Several other studies have also concluded that NO_3–N could be a parameter of concern for various species cultured in WRAS that are operated with low water exchange rates, including Martins et al. (2009a) – common carp; Hamlin (2006) – Siberian sturgeon *Acipenser baeri*; and Hrubec (1996) – hybrid striped bass *M. saxatilis* × *M. chrysops*. Therefore, more research is certainly needed to evaluate the chronic NO_3–N toxicity threshold for salmonids that are cultured in WRAS. Such research would enable the establishment of more concrete design limits for NO_3–N within low exchange WRAS used for salmonid culture.

5. Conclusion

The results of the present studies provide strong evidence that some aspect of the water quality environment within the low (HRT = 6.7 days; feed loading rate = 4.1 kg feed/m^3 daily makeup flow) and near-zero exchange (feed loading rate ≥71 kg feed/m^3 makeup flow/day; >103 days HRT's) WRAS caused negative impacts to rainbow trout health and welfare. Some of these impacts were subtle and are best described as chronic, such as increased swimming speeds and side swimming behavior. However, in WRAS with near-zero exchange rates, increased deformities and decreased survival occurred. Of the measured parameters, accumulating dissolved potassium and nitrate nitrogen were separated as possible causes of the observed fish health and welfare problems and should be further evaluated.

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Study II

The effects of ozone and water exchange rates on water quality and rainbow trout Oncorhynchus mykiss performance in replicated water recirculating systems

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The effects of ozone and water exchange rates on water quality and rainbow trout *Oncorhynchus mykiss* performance in replicated water recirculating systems

John Davidson, Christopher Good, Carla Welsh, Steven Summerfelt*

The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, United States

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ABSTRACT

Rainbow trout *Oncorhynchus mykiss* performance and water quality were evaluated and compared within six replicated 9.5 m^3 water recirculating aquaculture systems (WRAS) operated with and without ozone at various water exchange rates. Three separate studies were conducted: (1) low water exchange (0.26% of the total recycle flow) with and without ozone; (2) low water exchange with ozone versus high water exchange (2.6% of the total recycle flow) without ozone; and (3) near-zero water exchange (only backwash replacement) with and without ozone. Mean feed loading rates for WRAS operated at high, low, and near-zero exchange were 0.40, 3.98, and 55.9 kg feed/m^3 makeup water, respectively. Ozone significantly reduced total suspended solids, color, and biochemical oxygen demand and resulted in a significant increase in ultraviolet transmittance (%) ($P < 0.10$). Ozone also created ambient water quality within low exchange WRAS that was comparable to that of WRAS operated at high water exchange ($P > 0.10$). Additionally, dissolved copper and iron were significantly lower within WRAS operated with ozone ($P < 0.10$). Dissolved zinc was also consistently lower in WRAS operated with ozone, but not significantly ($P > 0.10$). In Studies 1 and 3, total ammonia nitrogen and nitrite nitrogen were slightly lower within the ozonated systems, but were not always significantly lower. In all studies, ozone did not prevent nitrate nitrogen accumulation. At the conclusion of Study 1, rainbow trout growth was significantly greater within low exchange WRAS operated with ozone ($P = 0.001$). At the conclusion of Study 2, rainbow trout growth was similar between treatments ($P = 0.581$), indicating that fish grew equally as well within ozonated WRAS operated at 1/10th the flushing rate as the non-ozonated and high flushing control systems. Overall, ozone created an improved water quality environment within low and near-zero exchange WRAS that generally resulted in enhanced rainbow trout growth rates, survival, feed conversion, and condition factor.

1. Introduction

A series of studies are being conducted to identify water quality parameters that could limit rainbow trout *Oncorhynchus mykiss* or Atlantic salmon *Salmo salar* performance (i.e. growth, health, welfare, and survival) within water reuse aquaculture systems (WRAS) that are operated at low water exchange with high feed loading rates (Davidson et al., 2009; Good et al., 2009) or with high carbon dioxide concentrations (Good et al., 2010). Davidson et al. (2009) pinpointed specific parameters that accumulated to potentially harmful levels when WRAS were operated at low exchange, i.e. 0.26% of the total recycle flow. Negative impacts on fish were not apparent; however, literature indicated that dissolved copper, fine and suspended solids, nitrate nitrogen concentrations, as well heterotrophic bacteria counts were a concern (Davidson et al., 2009; Colt, 2006). Several other studies have examined accumulating water quality parameters within low exchange WRAS and their effect on the performance of various species. Martins et al. (2009a) concluded that ortho-phosphate-P, nitrate, and heavy metals (arsenic and copper) accumulated to levels that likely impaired the embryonic and larval development of common carp *Cyprinus carpio*. Deviller et al. (2005) attributed a 15% growth reduction in European sea bass *Dicentrarchus labrax* cultured within WRAS to an unknown "growth-inhibiting substance" and implied that metals accumulation could have been the cause.

The accumulation of potentially harmful water quality concentrations in low exchange WRAS could represent a substantial barrier to the expanded utilization of this sustainable technology, particularly for species that require clean water such as salmonids. Therefore, methods that reduce and/or control accumulating water quality parameters within low exchange WRAS need to be evaluated.

Previous research from the water treatment and aquaculture industries has shown that ozone can reduce and control a variety

* Corresponding author. Tel.: +1 304 876 2815x211; fax: +1 304 870 2208.
E-mail address: s.summerfelt@freshwaterinstitute.org (S. Summerfelt).

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of water quality parameters, depending on where it is used and the dose of ozone applied. In varying applications, ozonation of water has been found to effectively reduce biochemical oxygen demand, chemical oxygen demand, dissolved organic carbon, color, nitrite, turbidity, total organic carbon, and/or total suspended solids (Rosenthal and Kruner, 1985; Hozalski et al., 1995; Brazil, 1996; Summerfelt and Hochheimer, 1997; Summerfelt et al., 1997; Tango and Gagnon, 2003; Summerfelt et al., 2009a,b). Ozone has also been used to control algae (Rice et al., 1981; Plummer and Edzwald, 2002), improve micro-flocculation of fine particulates (Rice et al., 1981; Rueter and Johnson, 1995), increase unit process efficiency (Rosenthal and Otte, 1980; Paller and Lewis, 1988; Summerfelt et al., 1997), reduce heavy metals such as iron and manganese (Rice et al., 1981; Langlais et al., 1991), and reduce bacterial populations depending on ozone dose, contact time, and operation with or without ultraviolet irradiation (Summerfelt, 2003; Sharrer and Summerfelt, 2007; Summerfelt et al., 2008, 2009a,b). Ozone has also been found to reduce off-flavor producing compounds such as MIB and geosmin in drinking water (Terishima, 1988; Nerenberg et al., 2000; Park et al., 2007) using dosages that are orders of magnitude greater than those typically utilized in WRAS. However, the use of ozone at dosages that achieve water quality improvements within WRAS, but are insufficient to maintain an ozone residual or disinfect the water, has not proven effective at reducing off-flavor compounds (Schrader et al., 2010). Ozone also reacts rapidly within water and produces few harmful byproducts in freshwater, where it forms dissolved oxygen as a reaction end product (Summerfelt and Hochheimer, 1997; Summerfelt, 2003).

A few studies have also indicated that the water quality improvements initiated by ozonation created a more optimal environment for growth and survival of various species cultured within WRAS. Suantika et al. (2001) found that ozonation ultimately improved rotifer production within a closed WRAS. In another study, ozonation of a seawater system culturing larval southern rock lobster *Jasus edwardsii* resulted in a significant decrease in bacterial populations and thus increased survival of lobster larvae (Ritar et al., 2006). Brazil (1996) reported that hybrid striped bass *Morone saxatilis x chrysops* growth was improved in ozonated systems. The potential advantages of ozonation have also been demonstrated within recirculating systems used for salmonid culture. Bullock et al. (1997) reported that ozonation applied within a recirculating system reduced concentrations of suspended solids, dissolved organic carbon, color, and nitrite, and eliminated the need for chemotherapeutic treatment to control bacterial gill disease (BGD) in rainbow trout. The ozone dose applied during Bullock's study (0.025–0.039 kg ozone/kg feed) was not sufficient to kill *Flavobacterium branchiophilum*, the causative agent of BGD, providing <1 log reduction of the bacteria in the system water and on the gill tissue, but improved the culture environment to a point that rainbow trout were not impacted by the disease (Bullock et al., 1997). Another study demonstrated the benefits of ozone for Atlantic salmon cultured within recirculating systems, including increased growth rates (Sutterlin et al., 1984).

The use of ozone in WRAS does not come without drawbacks. Excess ozone residual remaining in the culture water could cause significant harm or even catastrophic mortality to cultured species if not properly controlled (Summerfelt et al., 2004a). Ozone can also be hazardous to human health if air concentrations are not properly monitored and controlled (Summerfelt et al., 2009a,b). In-air monitors, alarms, and adequate ventilation systems are important to ensure worker safety when operating ozone. Lastly, ozone generation increases capital and operating costs.

As long as the potential hazards related to ozone are controlled, the benefits of ozone relative to water quality and fish performance appear to outweigh the potential drawbacks. Ozone application within WRAS could be the key to optimal water quality control and fish performance within low and near-zero exchange WRAS operated with high feed loading. Thus, three studies evaluating the use of ozone within low and near-zero exchange WRAS are discussed in this paper. The primary objectives were: (1) to determine if ozone creates a more favorable water quality environment for salmonids as measured by rainbow trout growth and survival, and (2) to determine which water quality parameters are improved as a result of ozone addition. These results will provide important information regarding the feasibility of operating WRAS at low and near-zero water exchange, or as completely closed systems for the commercial production of rainbow trout and other salmonids.

2. Methods

2.1. Experimental treatments

Rainbow trout performance and water quality criteria were evaluated and compared during three studies within six replicated water reuse aquaculture systems (WRAS), including WRAS operated with: (1) low water exchange rates with and without ozone; (2) low exchange rates with ozone versus high exchange without ozone; and (3) near-zero exchange with and without ozone. WRAS described as operating at "low" and "high" water exchange continuously replaced 0.26 and 2.6% of the total recirculating flow, respectively, while WRAS operated at "near-zero" water exchange replaced only the water that was lost as backwash or flushed from the radial flow settler. Makeup water was continuously added to the pump sump during Studies 1 and 2, but was introduced only as needed during Study 3 via a float valve located in the pump sump. Water replaced via the float valve during Studies 1 and 2 was not quantified because it represented <6% of the total daily makeup water addition within the "low" water exchange treatment and <1% of the total daily makeup water addition within the "high" water exchange treatment. Makeup flows were measured and calibrated several times per week during Studies 1 and 2. During Study 3, digital flow meters (Model C700, AMCO Water Metering Systems, Inc., Ocala, FL, USA) were installed on the makeup water lines of each WRAS to totalize the flow added via the float valve. During Study 3, periodic drum filter failures occurred within four of six WRAS, which resulted in increased and variable dilution amongst WRAS. Additionally, drum filter backwash spray was found to be added as additional makeup water to some WRAS and not others, also contributing to differences in dilution. Mean system hydraulic retention times relative to previously described flushing rates for the high, low, and near-zero exchange WRAS were approximately 0.67, 6.7 and 76 days, respectively. However, due to the variability in flushing during Study 3, mean hydraulic retention times for the individual WRAS varied from <10 days to as many as 196 days.

2.2. System description

Six identical 9.5 m^3 WRAS (Fig. 1), three per treatment, were used during each study and are described in detail in Davidson et al. (2009). To summarize, each system 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). The recirculating flow exchanged the culture tank water volume once every 15 min.

2.3. Ozone

Three WRAS were equipped with ozone generators (Model G22, Pacific Ozone Technology, Benicia, CA, USA). Approximately 1–6%

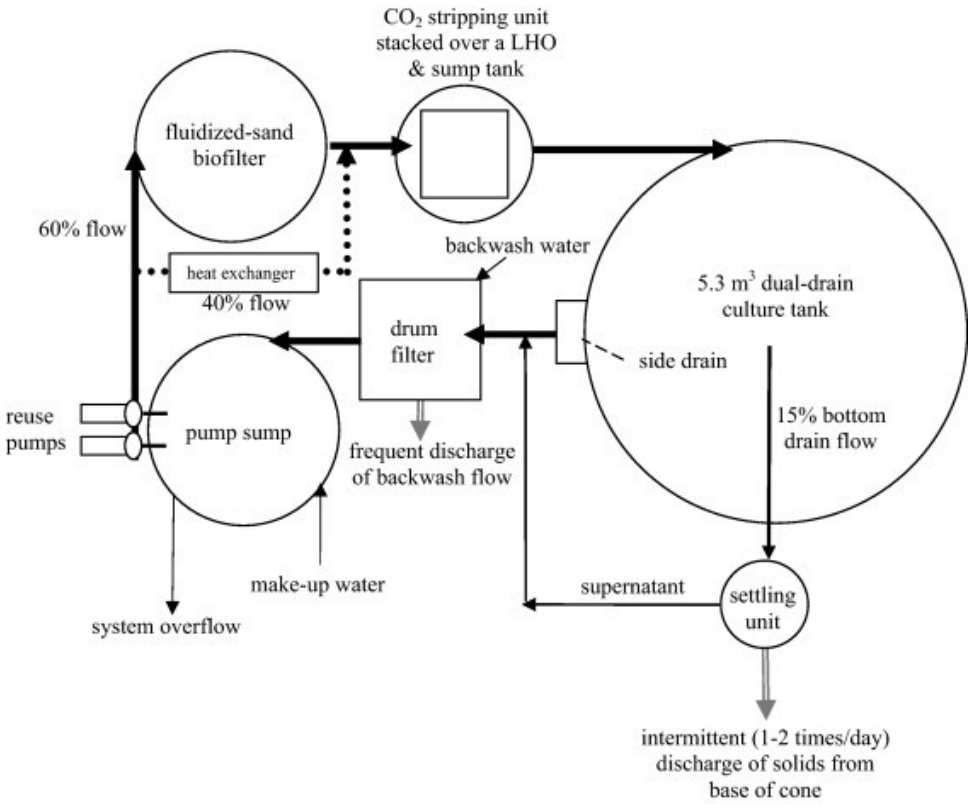

Fig. 1. Process flow drawing of individual 9.5 m^3 water recirculating aquaculture system used for the present studies.

of the >99% pure oxygen feed gas passing through the Corona discharge cell of each generator was converted to ozone and injected within the 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). During Studies 1 and 2, the SC100 was used to provide proportional-integral-derivative (PID) control of the ozone generator output in order to maintain an ORP set-point of 250 mV within the culture tank. During Study 3, the SC100 was used to provide on–off control of ozone generation to maintain an ORP set-point of 270–290 mV. ORP data was logged minute by minute over the duration of each study but was only available as raw data for Studies 2 and 3. The ORP set-points used during these studies were selected to prevent toxic ozone residuals from accumulating in the culture water in the absence of effective deozonation, such as the use of ultraviolet (UV) irradiation (Summerfelt et al., 2004a). The resulting ozone dose, which ranged from 20 to 25 g ozone/kg feed, was not intended to disinfect the water (i.e. reduce bacterial loads) at the ORP levels that were maintained, but was expected to improve general water quality.

2.4. Rainbow trout

Study 1—Rainbow trout (1000/tank), 74 ± 2 g, were stocked within each WRAS at a density of approximately 15 kg/m^3 and allowed eight weeks for acclimation and biofilter startup prior to ozone startup. The study began when ozone was turned on and was operating continuously 24 h per day. At the start of the study rainbow trout were 294 ± 3 g in systems operated with ozone and 296 ± 3 g in systems without ozone. *Study 2*—Rainbow trout (1000/tank), 151 ± 3 g, were stocked at a density of approximately 30 kg/m^3. Study 3—Rainbow trout (approximately 3600/tank), 18 ± 0 g, were stocked at a density of approximately 12 kg/m^3. For Studies 2 and 3, a one-week acclimation period was used following stocking. Optimal nitrification had already been established across the biofilters when these studies began.

2.5. Photoperiod and feeding

A constant 24-h photoperiod was provided for each 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). Previous research (Davidson et al., 2009) conducted within the same six WRAS indicated that the 24-h photoperiod and uniformly dispersed feeding events around the clock produced a relatively constant biological respiration rate as indicated by a nearly constant mean 24-h oxygen demand. Relatively constant daily water quality was required, because all water samples from the six systems could not be collected simultaneously. Feeding was estimated based on standardized feeding charts, as well as observations of feeding activity and wasted feed. If significant amounts of wasted feed and/or an obvious reduction in feeding response were observed, then feeding was adjusted for the respective WRAS. Thereafter, fish in each WRAS were fed to satiation but feeding was not necessarily equal between WRAS or between treatments. Feeding rates ranged from 1.0 to 3.0% of the fish biomass, depending on mean fish size. A standard slow-sinking trout diet (Zeigler Brothers, Inc., Gardners, PA, USA) with a protein: fat ratio of 42:16 was used throughout each study, with the exception of Study 3, during which a smaller pelleted 50:15 diet was fed during the first two weeks before switching to a 42:16 diet.

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

Parameter	Method of analysis	Frequency of testing
Dissolved oxygen	Hach SC100 Universal Controller & LDO® Probe	Recorded daily
Temperature	Hach SC100 Universal Controller & Differential ORP Sensor	Recorded daily
Oxidation reduction potential	Hach SC100 Universal Controller & Differential ORP Sensor	Recorded daily
pH	Hach Model HQ40D with digital pH sensor	Once weekly
Total ammonia nitrogen	Hach Method 8038—Nessler	Once weekly
Nitrite nitrogen	Hach Method 8507—Diazotization	Once weekly
Nitrate nitrogen	Hach Method 8171—Cadmium reduction	Once weekly
Total suspended solids	Standard methods 2540D—Dried at 103–105 °C	Once weekly
$CBOD_5$	Standard methods 5210B–5 day test	Once weekly
Total alkalinity	Standard methods 2320—Sulfuric acid titration	1–3 times weekly
Dissolved carbon dioxide	Hach Method 8223–Burret titration	Once weekly
Total heterotrophic bacteria	Standard methods 9215D—Membrane filtration and agar plate counts	Once weekly
Total coliform bacteria	Standard methods 9222B—Membrane filter and agar plate counts	1–2 times weekly Studies 1 and 2
Ultraviolet transmittance	Standard methods 5910B—ultraviolet absorption	Once weekly
True color	Hach Method 8025—platinum–cobalt	Once weekly
Particle size distribution	Hach 2200 PCX Particle counter modified set up with peristaltic pump, flow dampener, and stir plate	Once weekly Study 1
Total organic carbon	Standard methods 5310C—persulfate ultraviolet or heated persulfate oxidation	Once weekly Studies 1 and 2
Dissolved organic carbon	Standard methods 5310C—persulfate ultraviolet or heated persulfate oxidation	Once weekly Studies 1 and 2
Phosphorous	Hach Method 8190—acid persulfate digestion	Once weekly
Dissolved metals	Inductively coupled plasma atomic emission spectrometry technique (Cornell Nutrient Analysis Lab, Ithaca, NY, USA)	Consecutive days 1–2 weeks during near max feed loading
Dissolved ozone	HACH Method 8311—low range ozone AccuVac Reagent Ampuls Indigo Method	Two sampling events—Study 2
Bromine	HACH Method 8016	Five sampling events—Study 2
Bromide	EPA 300.0/SM4110B (Test America, Nashville, TN, USA) and EPA method 300.1 (Broward Testing Laboratory, Ft. Lauderdale, FL, USA)	Two sampling events—Study 2 (one at each lab); One sampling event—Study 3
Bromate	EPA method 317.0 (Test America, Nashville, TN, USA; Broward Testing Laboratory, Ft. Lauderdale, FL, USA)	One sampling event—Studies 2 and 3
Bromoform	EPA Method 8260B; Sample extraction methods—EPA Method 5035A; and EPA Method 5030B (Columbia Analytical Services, Inc., Kelso, WA, USA)	One sampling event of fish fillets Studies 2 and 3

2.6. Water quality control and sampling protocols

Alkalinity was maintained near 200 mg/L as $CaCO_3$ within low and near-zero exchange systems by adding sodium bicarbonate ($NaHCO_3$) in proportion to the daily feeding rate, i.e., 0.15 and 0.19 kg $NaHCO_3$ were added daily for every 1 kg of feed fed daily, respectively. Temperature was equalized amongst WRAS by adjusting water flow through the geothermal heat exchangers. The rapid culture tank hydraulic exchange rate (i.e., one exchange every 15 min) and the forced-ventilation stripping column were designed to maintain low carbon dioxide concentrations (typically <10 mg/L) that were equal amongst WRAS, even if feed loading levels differed slightly between culture tanks. The flow of oxygen feed gas to each LHO was manually adjusted when necessary to maintain dissolved oxygen concentrations in each culture tank at near 100% saturation for all WRAS.

Water samples were manually collected from the side drain of each tank and tested for a variety of parameters (Table 1). The majority of tests were carried out in-house by water chemistry staff. All water quality parameters were measured according to methods described in APHA (2005) and HACH (2003). During a two-week period of "intensive sampling" when fish had reached near-maximum feed levels and densities (80 kg/m^3), water samples were collected on consecutive days to compare culture tank water quality between treatments; including samples for the analysis of 27 dissolved metals, which were conducted by the Cornell Nutrient Analysis Laboratory (Ithaca, NY, USA) (Table 2). Water quality was also monitored on a weekly basis over the duration of each study.

In addition, water samples were collected to evaluate bromide and the oxidized toxic compounds that can be formed during ozonation. During Studies 2 and 3 the following parameters were evaluated: bromide, bromine, bromate, and bromoform (within fish tissues). For the bromoform analysis, three fish were filleted from each WRAS and samples were homogenized per WRAS. Analyses were conducted by the following laboratories: bromine (Freshwater Institute), bromide and bromate (Test America, Nashville, TN, USA; and Broward Testing Laboratory, Ltd., Fort Lauderdale, FL, USA), and bromoform (Columbia Analytical Services, Inc., Kelso, WA, USA).

The mean feed-specific NO_3-N production constant, $a_{nitrate}$ (kg NO_3-N/kg feed) was calculated for each of the WRAS by taking the difference in the NO_3-N concentration entering and exiting each WRAS divided by its corresponding feed loading rate (and converting units). The mean feed-specific NO_3-N production constant was calculated for each WRAS during each of the three studies when the WRAS were operated at near-maximum feeding rates. This calculation assumed that no denitrification or other NO_3-N removal process other than dilution was involved.

2.7. Fish sampling protocols

Fish were sampled for lengths and weights on a monthly basis. Sample size ranged from 50 to 120 fish and was calculated as follows: $n = (Z \times (\text{stdev. grams/accepted error grams}))^2$, where $Z = 1.65$ (relative to a 90% confidence interval) and accepted error was 5 g. Mortalities were removed and recorded daily to assess cumulative survival. During Study 3, mortalities related to a spike in ozone residual were excluded from the cumulative survival assessment, because the problem was associated with human error and not the specific treatment conditions. Fish were reared to a maximum density of 80 kg/m^3 and periodically thinned to approximately 50 kg/m^3. Thermal growth coefficients (TGC), condition factor (CF), and feed conversion ratios (FCR) were calculated during each study and compared between treatments. Calculations are as follows:

Table 2
Minimum detection limits for each metal/element analysis and upper recommended concentrations for each metal/element for salmonid culture as reported in the literature.

Parameters	Minimum detection limits (mg/L)	Recommended limits (mg/L)
Aluminum	0.130	0.01–1.00
Arsenic	0.019	0.05–0.40
Barium	0.002	5
Beryllium	0.002	0.01–1.10
Boron	0.200	5
Cadmium	0.004	0.0003–0.0700
Calcium	0.495	4–160+
Chromium	0.008	0.03–0.10
Cobalt	0.009	0.010–0.05
Copper	0.005	0.006–0.070
Iron $_{(Total)}$	0.200	0.1–1.1
Lead	0.200	0.01–4.0
Magnesium	0.031	15–28+
Manganese	0.002	0.05–1.00
Mercury	0.350	0.0001–0.0020
Molybdenum	0.011	8+
Nickel	0.016	0.01–0.40
Phosphorous $_{(Total)}$	0.019	3+
Potassium	0.332	5–10+
Selenium	0.075	0.005–0.020
Silicon	0.286	NA
Sodium	0.097	600–1500+
Strontium	0.002	NA
Sulfur	0.130	NA
Titanium	0.013	NA
Vanadium	0.018	0.1
Zinc	0.016	0.005–0.269

Recommended limits represent levels at which chronic or low-level mortality could occur. The toxicity of many parameters is dependent upon alkalinity, hardness, and other variables. Lower limits within each range are typically related to soft water and upper limits to hard water. *Limits are cited from*: Piper et al. (1982), Meade (1989), Heinen (1996), Wedemeyer (1996), EPA (1986, 1987, 1996, 2002, 2007), Boyd (2009).

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

$$\text{CF} = 100{,}000 \times \text{Weight}/(\text{Length})^3$$

$$\text{FCR} = \text{Cumulative Feed Delivered to Tank/Fish Biomass Gain.}$$

where weight is in grams, length is in millimeters, and temperature is in °C.

2.8. Statistical analyses

All parameters that were sampled during multiple events over time from the same location, such as water quality parameters measured during intensive sampling, as well as growth rates, were analyzed using multivariate repeated measures analysis of variance (MANOVA). Mean water quality data for the duration of each study, as well as metals data was compared between treatments using a Student's *t*-test. Normality was assumed for each test due to the relatively small sample size ($n = 3$). For Study 3, water quality variables were analyzed for differences between treatments using analysis of covariance (ANCOVA), with feed loading per unit makeup water as the covariate, due to the unexpected differences in flushing rates measured amongst WRAS. However, the ANCOVA model violated assumptions for the analysis of most water quality parameters, with the exception of total ammonia nitrogen and nitrate nitrogen. Water quality parameters that did not meet the assumptions of ANCOVA were analyzed using a *t*-test during Study 3. Survival percentages were converted for statistical analysis using an arcsine square-root transformation as recommended by Sokal and Rohlf (1981). A probability value (α) of 0.10 was used to determine significance for each statistical test as opposed to the traditional 0.05 due to sparsity of data, i.e. a relatively low $\underline{n}$-value (three WRAS per treatment). Replication of WRAS was considered a unique advantage during these studies; however, it was not feasible to construct more than six WRAS to further increase statistical power. A higher α value is warranted under these circumstances, because the presence of just one outlier would increase variation and thus impact the significance of results, possibly resulting in Type II error. Statistical analyses were carried out using SYSTAT 11 software (Chicago, IL, USA) (2004).

3. Results and discussion

3.1. Metals/trace elements

Concentrations of 11–14 dissolved metals/elements were detected during Studies 1–3 (Table 3). The only metals/elements that were significantly different between treatments during Study 1 were copper and sulfur. Copper was significantly greater within WRAS without ozone ($P = 0.005$), and sulfur was significantly greater in WRAS with ozone ($P = 0.051$). During Study 2, significant differences were expected between high exchange WRAS without ozone and low exchange WRAS with ozone due to the 10-fold difference in flushing rate and thus greater dilution of the high exchange systems. The following metals/elements were significantly greater within the low exchange WRAS with ozone: copper, magnesium, phosphorous, potassium, sodium, strontium, and sulfur ($P < 0.10$) (Table 3). Barium and calcium were significantly greater within the high exchange WRAS ($P < 0.10$). During Study 3, copper ($P = 0.005$) and iron ($P = 0.044$) were statistically greater within the near-zero exchange WRAS without ozone.

Effects of ozone (copper)—The use of ozone caused a 3–4-fold reduction of dissolved copper within low and near-zero exchange WRAS during Studies 1 and 3. Dissolved copper measured within WRAS with and without ozone during Study 1 was 0.021 ± 0.008 and 0.064 ± 0.001 mg/L, respectively (Table 3). During Study 3, dissolved copper within near-zero exchange WRAS operated with and without ozone was 0.041 ± 0.001 and 0.119 ± 0.008 mg/L, respectively (Table 3), providing repeated evidence that ozone reduced dissolved copper. During Study 2, copper was greater within the low exchange WRAS with ozone (0.038 ± 0.004 mg/L) as compared to high exchange WRAS without ozone (0.014 ± 0.002 mg/L); however, the dissolved copper concentrations within the low exchange WRAS were most likely reduced by ozone, because these systems received a continuous makeup flow that was 10 times less than the high exchange WRAS. Thus, ozone likely controlled dissolved copper during Study 2 as well, but not as effectively as a 10-fold increase in system flushing.

Effects of ozone (zinc)—Zinc was consistently lower within WRAS that used ozone, but not significantly. During Study 1 dissolved zinc was 0.005 ± 0.003 mg/L within low exchange WRAS without ozone and 0.001 ± 0.001 mg/L within low exchange WRAS with ozone (Table 3). During Study 2 dissolved zinc was 0.011 ± 0.003 mg/L within the high exchange WRAS without ozone and 0.007 ± 0.002 mg/L within the low exchange WRAS with ozone. For Study 3 dissolved zinc was 0.128 ± 0.023 mg/L within the near-zero exchange WRAS without ozone as compared to 0.078 ± 0.003 mg/L within the near-zero exchange WRAS with ozone. Like copper, the accumulation of zinc within low or near-zero exchange WRAS tended to be reduced by ozonation.

Effects of ozone (iron)—Dissolved iron was only detected in the culture water when WRAS were operated at near-zero exchange during Study 3. During Study 3, dissolved iron was 0.041 ± 0.013 mg/L within the near-zero exchange WRAS without ozone and 0.004 ± 0.002 mg/L within near-zero exchange WRAS operated with ozone. Therefore, ozone was also effective in reduction of dissolved iron, and therefore could be beneficial within

Table 3
Mean dissolved metal and nutrient concentrations (mg/L) at the tank side drain outlets when WRAS were operated near-maximum feed loading and fish density during Studies 1, 2, and 3.

Treatment/parameter	Study 1		Study 2		Study 3		All studies	
	Low exchange no ozone	Low exchange ozone	High exchange no ozone	Low exchange ozone	Near-zero exchange no ozone	Near-zero exchange ozone	Makeup water (PVC pipe)	Drum filter spray (copper pipe)
Barium*2	0.036 ± 0.005	0.038 ± 0.004	0.055 ± ± 0.001	0.043 ± 0.001	0.367 ± 0.066	0.233 ± 0.008	0.082 ± 0.028	0.241 ± 0.061
Boron*MW	<MDL	<MDL	<MDL	<MDL	0.061 ± 0.011	0.065 ± 0.014	0.003 ± 0.003	0.016 ± 0.005
Calcium*2	94 ± 5	100 ± 2	108 ± 0	104 ± 1	99 ± 2	84 ± 12	114 ± 4	121 ± 4
Copper*1, *2, *3, *MW	0.064 ± 0.001	0.021 ± 0.008	0.014 ± 0.002	0.038 ± 0.004	0.119 ± 0.008	0.041 ± 0.001	<MDL	0.023 ± 0.005
Iron*3	<MDL	<MDL	<MDL	<MDL	0.041 ± 0.013	<MDL	<MDL	0.018 ± 0.018
Magnesium*2, *MW	12.8 ± 0.7	13.7 ± 0.3	12.1 ± 0.1	14.8 ± 0.4	19.8 ± 0.4	23.7 ± 2.6	11.8 ± 0.3	13.3 ± 0.3
Manganese	<MDL	<MDL	<MDL	<MDL	0.008 ± 0.004	<MDL	<MDL	0.005 ± 0.004
Phosphorous*2	2.4 ± 0.1	2.2 ± 0.1	0.5 ± 0.1	2.7 ± 0.2	7.0 ± 1.6	11.8 ± 2.7	<MDL	<MDL
Potassium*2	17 ± 1	18 ± 0	5 ± 0	25 ± 3	44 ± 7	85 ± 27	2 ± 0	2 ± 0
Silicon	32 ± 2	36 ± 1	48 ± 0	43 ± 2	44 ± 1	43 ± 2	42 ± 2	46 ± 4
Sodium*2	125 ± 12	138 ± 2	5 ± 0	164 ± 20	346 ± 86	568 ± 189	5 ± 0	6 ± 0
Strontium*2	0.95 ± 0.06	0.90 ± 0.03	0.90 ± 0.00	0.83 ± 0.01	0.89 ± 0.02	0.80 ± 0.08	0.97 ± 0.02	1.02 ± 0.03
Sulfur*1, *2	15.7 ± 0.2	17.1 ± 0.4	9.5 ± 0.2	18.4 ± 1.1	26.7 ± 2.0	39.8 ± 8.6	8.2 ± 0.6	8.7 ± 0.2
Zinc*MW	0.005 ± 0.003	0.001 ± 0.001	0.011 ± 0.003	0.007 ± 0.002	0.128 ± 0.023	0.078 ± 0.003	0.016 ± 0.008	0.066 ± 0.010

*# Indicates statistically significant between treatments ($P < 0.10$), 1, 2, or 3 following *#; indicates Studies 1, 2, or 3, *# MW indicates significant difference between the two makeup water types for that parameter ($P < 0.05$). <MDL = less than minimum detection limit of the test. *Notes*: The following elements were below the minimum detection limit within the culture water, makeup water, and drum filter high pressure spray (backwash) for all treatments within all studies: aluminum, arsenic, beryllium, cadmium, chromium, cobalt, lead, mercury, molybdenum, nickel, and selenium. Reported values for boron, iron, and zinc, were within the instrument detection limits, but below the MDL.

aquaculture facilities that have relatively high levels of iron in their source water or within WRAS.

Ozone's impact on metals and other elements has not been documented within aquaculture systems; however, research from the water treatment industry indicates that ozone can enhance removal of dissolved iron and manganese (Rice et al., 1981; Langlais et al., 1991) and possibly other metals/elements due to its strong oxidizing capacity. The mechanism of removal of dissolved iron and manganese includes the oxidation and subsequent transformation of these metals from soluble to insoluble precipitates that can then be filtered (Langlais et al., 1991). Little information is available regarding removal of copper and other metals/elements by ozonation; however, it is possible that a precipitating reaction occurred in the present studies between ozone and the metals that were removed (copper, iron, and zinc), similar to the reaction that was reported between ozone with iron and manganese. Precipitated metals could have chelated with organic molecules, become incorporated into biofilms, or become entrapped with the biosolids that were eventually removed from the WRAS via the tank bottom drain and radial flow settler or the drum filter along with the concentrated biosolids.

Effects of ozone (potassium, magnesium, sulfur, phosphorus, and sodium)—Unlike copper, zinc, and iron, ozonation did not appear to decrease the accumulation of potassium, phosphorus, magnesium, sulfur, or sodium.

Origin of metals/elements in WRAS—Dissolved metals were analyzed within the makeup water entering the WRAS from two sources: (1) a PVC pipe that flows to the pump sump as the primary makeup water and (2) a copper pipe that supplies high pressure backwash for the drum filter. Copper was typically undetectable within the makeup water entering through the PVC pipe (0.001 ± 0.001 mg/L); while dissolved copper was consistently detected within the water entering as backwash spray from the copper pipe (0.013 ± 0.002 mg/L). However, the majority of the water entering via the copper pipe was removed from the system with the backwashed solids and therefore was not the main source of the accumulating copper. Previous mass balance calculations (Davidson et al., 2009) indicated that the majority of the accumulating dissolved copper was contributed by the feed. Other dissolved metals and trace elements were also likely introduced into the WRAS within the fish feed including: the majority of potassium, phosphorus, sulfur, and magnesium, which tended to accumulate in the water as system flushing was reduced. Sodium was introduced into the WRAS with daily addition of sodium bicarbonate (to replace alkalinity lost to nitrification) or occasional treatment of low-level bacterial gill disease with sodium chloride. Regardless of the origin of dissolved metals, it is apparent that metals, particularly copper, can accumulate to potentially harmful levels within low and near-zero exchange WRAS (without ozone) and should be monitored and controlled.

3.2. Nitrogenous waste

Effects of ozone – Study 1 – There were no significant differences between low exchange WRAS operated with and without ozone for total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N), or nitrate nitrogen (NO_3-N) during the week of intensive sampling (Table 4). TAN and NO_2-N were slightly lower within the ozonated systems, but not significantly. Mean TAN levels between WRAS with and without ozone were 0.59 ± 0.03 and 0.53 ± 0.02 mg/L, respectively ($P = 0.109$) (Table 4). NO_2-N levels in WRAS with and without ozone were 0.06 ± 0.01 and 0.05 ± 0.01 mg/L ($P = 0.782$). Similar trends were observed for TAN and NO_2-N over the study duration (Table 5). Nitrate nitrogen was similar between WRAS with and without ozone, i.e. 90 ± 1 and 91 ± 1 mg/L, respectively, during the week of intensive sampling ($P = 0.623$). However, NO_3-N was

Table 4
Mean water quality values (mg/L) at the tank side drain outlets for Studies 1–3 when WRAS were operated near-maximum feed loading and fish density.

Treatment	Study 1		Study 2		Study 3	
	Low exchange no ozone	Low exchange ozone	High exchange no ozone	Low exchange ozone	Near-zero exchange no ozone	Near-zero exchange ozone
TAN[*2]	0.59 ± 0.03	0.53 ± 0.02	0.55 ± 0.06	0.73 ± 0.04	1.23 ± 0.19	0.93 ± 0.01
NH_3	0.006 ± 0.000	0.005 ± 0.000	0.005 ± 0.000	0.005 ± 0.000	–	–
NO_2-N	0.06 ± 0.01	0.05 ± 0.01	0.17 ± 0.12	0.13 ± 0.04	0.21 ± 0.05	0.08 ± 0.03
NO_3-N[*2, *3]	91 ± 1	90 ± 1	17 ± 1	108 ± 18	191 ± 28	373 ± 66
Alkalinity[*1]	233 ± 4	208 ± 3	217 ± 4	187 ± 3	176 ± 24	182 ± 14
$cBOD_5$[*1, *3]	4.7 ± 0.9	1.8 ± 0.2	4.1 ± 0.7	4.7 ± 0.9	18.9 ± 6.2	4.3 ± 0.5
TSS[*1, *2, *3]	9.7 ± 1.4	4.7 ± 0.6	2.8 ± 0.2	5.1 ± 0.3	18.2 ± 5.9	3.5 ± 0.5
TOC	–	–	–	–	35.4 ± 7.1	28.8 ± 6.4
DOC	–	–	–	–	21.0 ± 2.6	18.6 ± 1.6
CO_2	12 ± 0	12 ± 0	15 ± 1	16 ± 0	21 ± 1	23 ± 1
O_2[*3]	9.7 ± 0.2	9.8 ± 0.2	10.6 ± 0.1	10.7 ± 0.1	8.4 ± 0.1	9.1 ± 0.0
Temperature (°C)	13.9 ± 0.1	14.0 ± 0.1	13.8 ± 0.0	14.0 ± 0.1	16.0 ± 0.2	16.3 ± 0.1

'*' Indicates statistically significant between treatments ($P < 0.10$), 1, 2, or 3 following '*' indicates Study 1, 2, or 3.

significantly greater within the ozonated WRAS for the study duration ($P = 0.038$), i.e., 84 ± 3 vs. 71 ± 1 mg/L in WRAS without ozone (Table 5). The greater NO_3-N concentrations within the ozonated WRAS for the study duration were attributed to slight differences in feeding between treatments. Slightly improved removal efficiency was observed across the biofilters in WRAS operated with ozone. Improved removal efficiency likely resulted due to cumulative water quality improvements initiated by ozone, especially the large reduction in carbonaceous biochemical oxygen demand (BOD) concentration entering the biofilter. Unit process removal efficiency measured during these studies will be described in another publication.

Study 2—Significant differences in water quality were expected because three WRAS were operated at high exchange and three at low exchange. Accordingly, TAN was significantly greater ($P = 0.073$) within the low exchange WRAS with ozone (0.73 ± 0.04 mg/L) as compared to the high exchange WRAS with no ozone (0.55 ± 0.06 mg/L) during the week of intensive sampling, as well as for the study duration ($P = 0.005$) (Tables 4 and 5). Despite the difference in dilution between treatments, NO_2-N was slightly greater, but not significantly, within the high exchange WRAS without ozone (0.17 ± 0.12 mg/L) as compared to the low exchange WRAS with ozone (0.13 ± 0.04 mg/L) during the week of intensive sampling ($P = 0.774$) (Table 4), as well as for the study duration ($P = 0.526$) (Table 5). The slightly lower NO_2-N levels within the ozonated systems were attributed to ozone's ability to oxidize nitrite. The comparable TAN and NO_2-N concentrations that were measured within the low exchange WRAS are indications of the effectiveness of the fluidized sand biological filter at converting TAN to NO_3-N in an ozonated system, despite the 10-fold difference in system flushing rate between treatments.

Nitrate nitrogen concentrations for WRAS operated at high exchange without ozone and WRAS operated at low exchange with ozone during the week of intensive sampling averaged 17 ± 1 and 108 ± 18 mg/L, respectively ($P = 0.008$) (Table 4). And, for the study duration, mean NO_3-N levels were 13 ± 0 and 99 ± 7 mg/L, respectively ($P = 0.005$). Because the makeup water supplied to these systems contained approximately 3 mg/L of NO_3-N, there was a nearly 10-fold difference in mean concentrations of NO_3-N that accumulated in the low and high flushing treatments over the duration of the study (Table 5), i.e., [99 − 3]/[13 − 3] = 9.6:1, which is representative of the 10:1 difference in dilution between these

Table 5
Mean water quality values at the culture tank side drains for Studies 1–3 over the duration of each study.

Treatment	Study 1		Study 2		Study 3	
	Low exchange no ozone	Low exchange ozone	High exchange no ozone	Low exchange ozone	Near-zero exchange no ozone	Near-zero exchange ozone
TAN[*2]	0.47 ± 0.01	0.45 ± 0.02	0.31 ± 0.02	0.45 ± 0.01	0.92 ± 0.09	0.72 ± 0.05
NH_3	0.006 ± 0.000	0.005 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.008 ± 0.001	0.005 ± 0.000
NO_2-N	0.05 ± 0.00	0.04 ± 0.01	0.11 ± 0.04	0.08 ± 0.00	0.13 ± 0.01	0.12 ± 0.05
NO_3-N[*1, *2]	71 ± 1	84 ± 3	13 ± 0	99 ± 7	171 ± 16	323 ± 87
Alkalinity[*1, *2]	205 ± 1	196 ± 1	224 ± 3	200 ± 1	216 ± 3	208 ± 3
pH	7.66 ± 0.01	7.60 ± 0.02	7.61 ± 0.01	7.47 ± 0.01	7.54 ± 0.02	7.46 ± 0.02
CO_2	10 ± 0	11 ± 1	10 ± 1	11 ± 0	14 ± 1	16 ± 0
$cBOD_5$[*1, *3]	3.6 ± 0.5	1.7 ± 0.1	2.5 ± 0.1	3.0 ± 0.2	11.8 ± 2.7	3.9 ± 0.2
TOC	15.9 ± 1.6	13.0 ± 1.3	11.2 ± 2.1	17.9 ± 2.8	–	–
DOC	15.3 ± 1.5	13.7 ± 1.4	9.0 ± 1.2	16.1 ± 1.6	–	–
True color[*1, *2, *3]	53 ± 2	4 ± 0	12 ± 0	5 ± 1	157 ± 25	5 ± 1
UV transm. (%)[*1, *2, *3]	60 ± 1	82 ± 0	89 ± 0	77 ± 2	30 ± 2	66 ± 4
Phosphorous[*2]	2.9 ± 0.0	3.0 ± 0.1	0.8± 0.0	3.9 ± 1.0	5.2 ± 0.1	7.4 ± 2.0
TSS[*1, *3]	8.7 ± 1.8	3.4 ± 0.4	3.4 ± 0.1	4.6 ± 0.5	18.9 ± 1.1	3.5 ± 0.6
Coliform bacteria	1.2×10^4	3.3×10^3	6.2×10^3	7.2×10^3	–	–
Heterotrophic bacteria	2.0×10^5	92	117 ± 23	114 ± 19	825 ± 407	77 ± 17
Temperature (°C)	15.1 ± 0.0	15.2 ± 0.0	12.9 ± 0.0	13.0 ± 0.1	15.6 ± 0.1	15.6 ± 0.0
DO[*2]	9.9 ± 0.0	9.8 ± 0.0	10.4 ± 0.0	10.6 ± 0.0	9.7 ± 0.0	11.0 ± 0.01
ORP[*1, *2, *3]	155 ± 1	248 ± 1	195 ± 8	238 ± 2	158 ± 12	269 ± 3

'*' Indicates statistically significant between treatments ($P < 0.10$), 1, 2, or 3 following * indicates Study 1, 2, or 3. *Note*: Mean ORP levels include days when ozone was turned off and are therefore slightly below the ORP ranges described in Section 2.

treatments. Thus, over the entire study, NO_3-N accumulation was directly proportional to the feeding rate and the mean hydraulic retention time of the WRAS, which did not provide a dedicated denitrification process. During the week of intensive sampling (Table 4), the mean concentrations of NO_3-N that accumulated in the low and high flushing treatments was only 7.5:1 (low:high); this discrepancy may have been created by passive denitrification, as discussed below (Study 3).

Study 3—TAN and NO_2-N concentrations were slightly lower within ozonated WRAS, replicating trends observed during Study 1. TAN concentrations within the near-zero exchange WRAS with and without ozone during intensive sampling were 0.93 ± 0.01 and 1.23 ± 0.19 mg/L, respectively, but this difference was not statistically significant ($P = 0.528$). TAN was also slightly lower, but not significantly, within the ozonated WRAS over the study duration, i.e. 0.72 ± 0.05 mg/L versus 0.92 ± 0.09 mg/L within the WRAS without ozone ($P = 0.123$). NO_2-N within near-zero exchange WRAS with and without ozone during intensive sampling was 0.08 ± 0.03 and 0.21 ± 0.05 mg/L, respectively ($P = 0.757$). Over the study duration NO_2-N was 0.12 ± 0.05 and 0.13 ± 0.01 mg/L, respectively ($P = 0.749$). Failure of the WRAS 2 ozone generator, at one point, caused an increase in mean NO_2-N for the ozonated WRAS. The increase in NO_2-N was attributed to either an increase in the carbonaceous BOD and TSS concentrations entering the biofilter (after ozone generator failure) or the biofilter had become accustomed to NO_2-N oxidation by ozone; therefore, when the ozone generator failed, sufficient nitrifying bacteria were not present to efficiently remove NO_2-N. Nitrite nitrogen peaked to 0.88 mg/L in WRAS 2 during this period. Despite this anomaly, mean NO2-N was still slightly lower within the ozonated WRAS.

Nitrate nitrogen accumulated to relatively high levels due to decreased water exchange during this study. Nitrate nitrogen within the near-zero exchange WRAS with and without ozone during intensive sampling was 373 ± 66 and 191 ± 28 mg/L, respectively (Table 4) and was significantly different between treatments ($P = 0.048$). Significant differences in NO_3-N were not attributed to the treatment (i.e. ozone or no ozone), as ozone has not been shown to reduce NO_3-N concentration in the literature. Instead significant differences were likely detected between treatments due to unexpected failures in drum filter flushing (explained in the Section 2) which created increased water exchange and dilution of NO_3-N, primarily within WRAS that were operated without ozone. The drum filter failures and subsequent increased flushing occurred during weeks prior to intensive sampling. Over the study duration NO_3-N within the near-zero exchange WRAS with and without ozone was 323 ± 87 and 171 ± 16 mg/L, respectively. Analysis of covariance, which was utilized to account for differences in flushing and feed loading rate between WRAS, indicated that there was not a significant difference between treatments for nitrate nitrogen over the study duration ($P = 0.644$) (Table 5).

Using data collected during the period of near-maximum feed loading during all three studies, the mean feed-specific NO_3-N production constant was found to decrease logarithmically as feed loading rate increased ($R^2 = 0.8591$), i.e., $a_{\text{nitrate}} = -0.0041 \times \text{LN}(\text{Feed Loading Rate}) + 0.0233$, from a high of 0.026 kg NO_3-N produced per kilogram feed fed at a feed loading of 0.61 kg/m^3 makeup flow to a low of 0.002 kg/kg at a feed loading rate greater than approximately 120 kg/m^3 makeup water (Fig. 2). At the same time, the NO_3-N concentration increased approximately logarithmically as feed loading rate increased (Fig. 2; $R^2 = 0.6318$). Extrapolating this relationship to a single-pass system with a feed loading rate of approximately 0.01 kg/m^3 makeup water, a mean feed-specific NO_3-N production constant of approximately 0.042 kg NO_3-N would be produced per kilogram feed fed, which is close to the total ammonia nitrogen production rate expected per unit of feed consumed. Thus, some portion of the NO_3-N that was produced was subsequently removed within the WRAS, and more NO_3-N was removed as feed loading rate increased. Thus, passive denitrification or other NO_3-N removal process occurred at higher feed loading rates (corresponding to longer system HRT) and with higher NO_3-N concentrations. We suspect that passive denitrification occurred after NO_3-N diffused deep enough into biofilms to effectively shelter the denitrifying bacteria from the aerobic conditions found elsewhere in the WRAS.

3.3. Alkalinity

Mean alkalinity was significantly different between treatments over the duration of Studies 1 and 2 ($P = 0.004$; 0.009), but was not different for Study 3 ($P = 0.169$) (Table 5). Alkalinity was generally lower within WRAS operated with ozone, but differences in alkalinity between treatments were attributed to slight variations in sodium bicarbonate addition. In low and near-zero exchange WRAS that use biofiltration, alkalinity is consumed during the nitrification process and must be supplemented to maintain homeostatic conditions (Loyless and Malone, 1997). In fact, nitrification efficiency drops when alkalinity falls below 40–80 mg/L as $CaCO_3$ (Paz, 1984; Biesterfeld et al., 2003). Chen et al. (2006) recommends maintaining an alkalinity of 200 mg/L $CaCO_3$ in WRAS operated with minimal water exchange for optimal biofilter performance. During Studies 1 and 2, approximately 0.15 kg of sodium bicarbonate/kg feed was added within WRAS operated at low exchange to maintain alkalinity near 200 mg/L. During Study 3, approximately 0.19 kg of sodium bicarbonate/kg feed was added within near-zero exchange WRAS to maintain alkalinity near 200 mg/L.

3.4. Total suspended solids

Effects of ozone – Study 1 – Total suspended solids (TSS) within WRAS operated with ozone were significantly lower during the week of intensive sampling (4.7 ± 0.6 mg/L) and for the study duration (3.4 ± 0.4 mg/L) as compared to WRAS operated without ozone during intensive sampling (9.7 ± 1.4 mg/L) and for the study duration (8.7 ± 1.8 mg/L) ($P = 0.032$; 0.001) (Tables 4 and 5). Thus, ozone effectively reduced TSS within low exchange WRAS.

Study 2—TSS was significantly greater within WRAS operated at low exchange with ozone (5.1 ± 0.3 mg/L) vs. WRAS operated at high exchange without ozone (2.8 ± 0.2 mg/L) during the week of intensive sampling ($P = 0.003$) (Table 4). Over the study duration, TSS within the low exchange WRAS with ozone averaged 4.6 ± 0.5 mg/L, while TSS within WRAS operated at high exchange without ozone averaged 3.4 ± 0.1 mg/L ($P = 0.107$) (Table 5). Differences in TSS during Study 2 must be kept in perspective because of the 10-fold difference in water flushing rate between treatments. Ultimately, ozone produced mean TSS concentrations within low exchange WRAS that were quite low and similar to WRAS operated with 10 times more water exchange.

Study 3—Mean TSS concentrations during the week of intensive sampling for near-zero exchange WRAS with and without ozone were 3.5 ± 0.5 and 18.2 ± 5.9 mg/L, respectively ($P = 0.069$) (Table 4). TSS levels over the study duration for WRAS operated with and without ozone were 3.5 ± 0.6 and 18.9 ± 1.1 mg/L, respectively ($P = 0.001$) (Table 5). Thus, TSS concentrations were significantly lower within ozonated WRAS during intensive sampling and over the study duration, indicating that ozone significantly reduced TSS concentrations within near-zero exchange WRAS. Maximum TSS concentrations measured within the near-zero exchange WRAS with and without ozone were 14.6 and 63.3 mg/L, respectively. The significantly lower TSS concentrations within the ozonated WRAS are even more impressive when viewed in light of the unexpected differences in flushing rate. By chance, two of three systems operated with ozone (WRAS 2

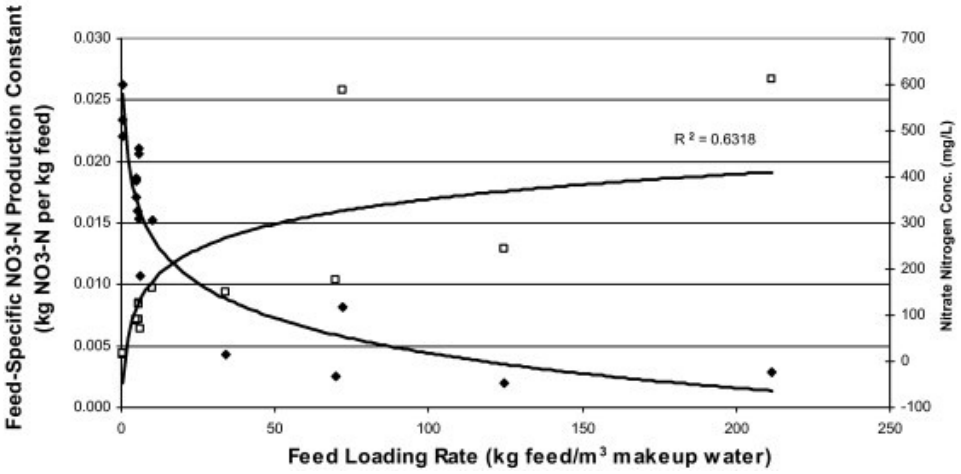

Fig. 2. The mean feed-specific NO_3-N production constant (solid diamonds), $a_{nitrate}$ (kg NO_3-N/kg feed), and corresponding nitrate nitrogen concentrations (non-filled squares) as plotted against feed loading rate from data collected from each WRAS when operated at near-maximum feed loading rates during Studies 1–3.

Table 6
Feed loading rate per unit of makeup flow (i.e., kg of feed delivered daily per cubic meters of makeup water supplied daily) calculated over the duration of each study as well as during near-maximum feeding for Studies 1–3.

	Duration	Duration	Duration	Near max feeding	Near max feeding	Near max feeding
	Study 1	Study 2	Study 3	Study 1	Study 2	Study 3
WRAS 1	3.73	0.41	44.1	5.44	0.59	69.9
WRAS 2	3.95	4.12	147	4.77	5.88	212
WRAS 3	4.34	4.04	4.1	4.90	6.37	10.2
WRAS 4	3.85	0.41	16.7	5.69	0.61	34.2
WRAS 5	3.73	0.40	22.9	4.81	0.60	124.4
WRAS 6	3.83	4.20	70.6	4.77	5.87	71.8

Note: The feed loading values for Study 1 and the low flushing WRAS (2, 3, and 6) of Study 2 could be reduced by an estimated 6% to account for radial flow clarifier flushing and replacement water added via the float valve. This flow was not measured during Studies 1 and 2, but was measured during Study 3.

and 6) were the least diluted systems and thus had much greater feed loading rates than other WRAS (Table 6). As a result, mean feed loading rates for WRAS operated with and without ozone were 74 kg feed/m^3 makeup water/day vs. 38 kg feed/m^3 makeup water/day, respectively over the study duration; and 98 kg feed/m^3 makeup water/day vs. 76 kg feed/m^3 makeup water/day, respectively, during intensive sampling. Thus, ozonation dramatically reduced TSS concentrations in comparison to WRAS operated without ozone, despite working against much greater feed loading rates.

TSS removal via ozonation has been previously demonstrated within WRAS. Summerfelt et al. (1997) observed a 35% reduction of TSS within a WRAS culturing rainbow trout in cross-flow raceways. In a much larger circular-tank-based WRAS, Summerfelt et al. (2009a,b) found that mean TSS concentrations within the culture tank could be reduced approximately 38–48%, from 4.0 mg/L (when the system water was not ozonated) to 2.1–2.5 mg/L, when ozone was applied. Tango and Gagnon (2003) found that ozone removed 40–50% of TSS within a marine recirculating system culturing Atlantic halibut *Hippoglossus hippoglossus*. Ozone causes microflocculation of fine solids (Maier, 1984), which leads to increased removal efficiency across solids removal devices. TSS removal efficiency during the present studies indicated that solids removal across the radial flow settler and drum filter was greater within WRAS operated with ozone, indicating that ozone likely caused flocculation of smaller particles and subsequent removal within these unit processes.

Effects of ozone—Fine particles—Analysis of particle counts and particle size distributions supports the theory of solids microflocculation and removal. Particle size distribution of the culture water during Study 1 (Fig. 3) was consistent with previous research, which reported that the majority of suspended solids present in WRAS are ≤20 μm (Patterson et al., 1999; Patterson and Watts, 2003; Chen et al., 1993). Mean total particle counts (2–60 μm) of samples collected for the duration of Study 1 were four times greater within WRAS without ozone (19,749 counts/mL) as compared to WRAS with ozone (4786 counts/mL). WRAS operated with ozone had substantially less fine particles for all size ranges (Fig. 3).

3.5. Carbonaceous biochemical oxygen demand

Effects of ozone – Study 1 – Carbonaceous BOD within low exchange WRAS with and without ozone averaged 1.8 ± 0.2 and 4.7 ± 0.9 mg/L, respectively, during the week of intensive sampling

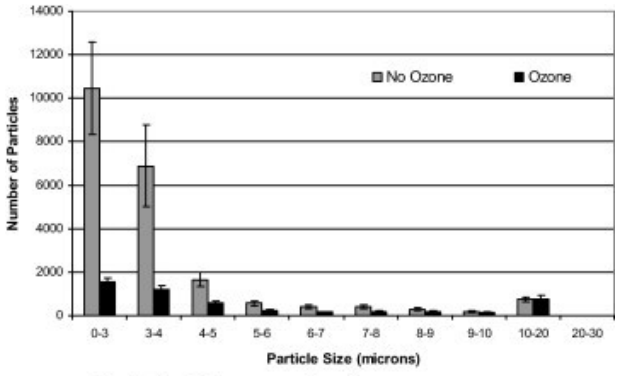

Fig. 3. Particle size distribution (mean ±standard error; $n = 3$) of the culture water from WRAS operated with and without ozone at low water exchange during Study 1.

and carbonaceous BOD was significantly lower within WRAS operated with ozone ($P = 0.034$). Carbonaceous BOD was also significantly lower within ozonated WRAS over the study duration ($P = 0.062$) (Table 5). Total organic carbon (TOC) and dissolved organic carbon (DOC) concentrations measured during Study 1 correlated with carbonaceous BOD results, as lower TOC and DOC concentrations were measured within WRAS operated with ozone (Tables 4 and 5).

Study 2—Carbonaceous BOD was similar between treatments during the week of intensive sampling and over the study duration ($P = 0.431$; 0.116) (Tables 4 and 5). During the week of intensive sampling, carbonaceous BOD within the high exchange WRAS without ozone was 4.1 ± 0.7 mg/L, and carbonaceous BOD within the low exchange WRAS with ozone was 4.7 ± 0.3 mg/L. Although significant differences between treatments were not detected, these results are quite noteworthy because carbonaceous BOD concentrations within the low exchange WRAS with ozone were similar despite operation with 10 times less water exchange than the high exchange WRAS.

Study 3—Results indicated that ozone effectively reduced the accumulation of carbonaceous BOD in the WRAS. Mean carbonaceous BOD concentrations were significantly greater within WRAS operated without ozone during the week of intensive sampling and over the study duration ($P = 0.079$; 0.096) (Tables 4 and 5). When WRAS were operated near-maximum feed loading, carbonaceous BOD concentrations in near-zero exchange WRAS operated with and without ozone averaged 4.3 ± 0.5 and 18.9 ± 6.2 mg/L, respectively.

The results from these studies indicated that ozone can substantially reduce carbonaceous BOD concentrations within WRAS, thus aiding in the optimization of the nitrification process. Carbonaceous BOD and TSS reduction via ozonation were likely related because a large portion of organic matter existed as measurable TSS.

3.6. Total heterotrophic bacteria plate count

The total heterotrophic bacteria plate counts that were quantified during these studies represent bacteria present within the sampled WRAS water that were able to colonize on the agar plates used for analysis. Thus, the total heterotrophic bacteria plate count data does not encompass all of the waterborne bacteria within the culture systems, but does provide a generalized comparison of total heterotrophic plate counts between treatments.

Effects of ozone – Study 1 – WRAS operated with low water exchange without ozone had approximately 2000-fold ($3 \log_{10}$) more heterotrophic bacteria counts as compared to low exchange WRAS operated with ozone, i.e. 2.0×10^5 vs. 92 counts/mL (Table 5). Despite this large disparity, a statistical difference was not detected due to widely variable heterotrophic counts between WRAS ($P = 0.240$). The mean bacteria count for the WRAS without ozone was elevated (5.6×10^5 counts/mL) due to a four-week period during which bacteria counts exploded in this treatment. This increase in bacteria did not occur within the low exchange WRAS operated with ozone (105 counts/mL). The authors hypothesize that the increase in bacteria within the low exchange WRAS without ozone could have resulted due to a turnover of bacteria populations within the biofilters. Aside from this four-week period, mean heterotrophic bacteria counts within the low exchange WRAS without ozone were much lower, i.e. 641 counts/mL, but were still approximately seven times greater than the bacteria levels observed in the WRAS with ozone. The ozone dose applied during these studies was not strong enough for disinfection, but other water quality improvements initiated by ozone, such as reduction of TSS and carbonaceous BOD, created an environment that was not as conducive to bacterial proliferation.

Study 2—Heterotrophic bacteria counts for the high exchange WRAS operated without ozone and the low exchange WRAS operated with ozone were 117 ± 23 and 114 ± 19 counts/mL (Table 5), respectively, and were not significantly different ($P = 0.933$). Therefore, ozone created ambient water quality at low exchange that was similar to a high exchange WRAS, with relatively low heterotrophic bacteria counts.

Study 3—Heterotrophic bacteria counts within the culture water of near-zero exchange WRAS operated with and without ozone were 77 ± 17 and 825 ± 407 counts/mL (Table 5). Despite the large disparity between treatments, a statistical difference was not detected due to variability in bacteria counts amongst WRAS operated without ozone ($P = 0.205$). However, the results were consistent with the other studies indicating that ozone indirectly minimized heterotrophic bacteria.

3.7. Visual observations, UV transmittance, and color

The reduction of TSS, carbonaceous BOD, bacteria, TOC, DOC, and refractory organic molecules via ozonation visually resulted in a culture environment with very clear water. In WRAS operated at low and near-zero exchange with ozone, the fish could easily be observed from above or through an observation window, while in WRAS operated at low and near-zero exchange without ozone, fish could barely be seen (Fig. 4). These differences were reflected in measurements of ultraviolet (UV) transmittance and true color over the duration of each study.

Effects of ozone—UV transmittance—UV transmittance is a measurement of the penetration of ultraviolet irradiation through a water sample. Mean UV transmittance for WRAS operated at low exchange with and without ozone was 82 ± 0 and $60 \pm 1\%$, respectively, for the duration of Study 1 ($P = 0.000$) (Table 5). During Study 2, UV transmittance was significantly lower within WRAS operated at low exchange with ozone ($77 \pm 1\%$) as compared to WRAS operated at high exchange without ozone ($89 \pm 0\%$) (Table 5), but this was partly due to the 10-fold difference in dilution between treatments ($P = 0.017$). UV transmittance during Study 3 measured within near-zero exchange WRAS operated with and without ozone was 66 ± 4 and $30 \pm 2\%$ (Table 5), respectively ($P = 0.002$). In summary, ozone significantly increased UV transmittance when systems were operated at comparable flushing rates.

Effects of ozone—Color—True color was significantly lower within the ozonated WRAS for Studies 1, 2, and 3 ($P = 0.000$; 0.022; 0.026). During Study 1, color measured in low exchange WRAS with and without ozone averaged 4 ± 0 and 53 ± 2 Pt-Co units, respectively (Table 5). During Study 2, color measured within the high exchange WRAS without ozone averaged 12 ± 1 Pt-Co units, while color within low exchange WRAS with ozone averaged 5 ± 1 Pt-Co units (Table 5). Ozone also dramatically reduced color during Study 3. Mean color measured within near-zero exchange WRAS with ozone was 5 ± 1 Pt-Co units, while mean color within near-zero exchange WRAS without ozone was 157 ± 25 Pt-Co units.

UV transmittance and true color measurements from all three studies demonstrated ozone's ability to provide clear water, which could be advantageous for both the fish and the fish farmer. A culture tank with clear water enhances the ability of the fish to see, feed optimally, and grow (Sigler et al., 1984) and also allows the farmer to better observe fish health, behavior, and feeding activity (Christensen et al., 2000). Increased UV transmittance in ozonated water allows for increased bacterial inactivation by UV and use of lower UV dosages to achieve the same level of disinfection.

3.8. Ozone residual, ORP, and ozone byproducts

Oxidation reduction potential (ORP) was measured during each study as an indirect measure of ozone residual. Bullock et al. (1997)

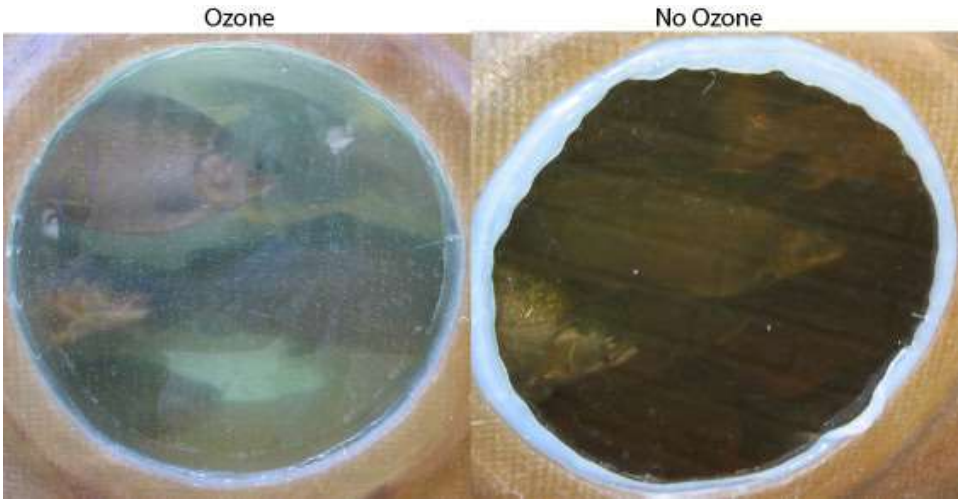

Fig. 4. Visual water quality differences observed through an observation portal for WRAS operated with and without ozone at low water exchange.

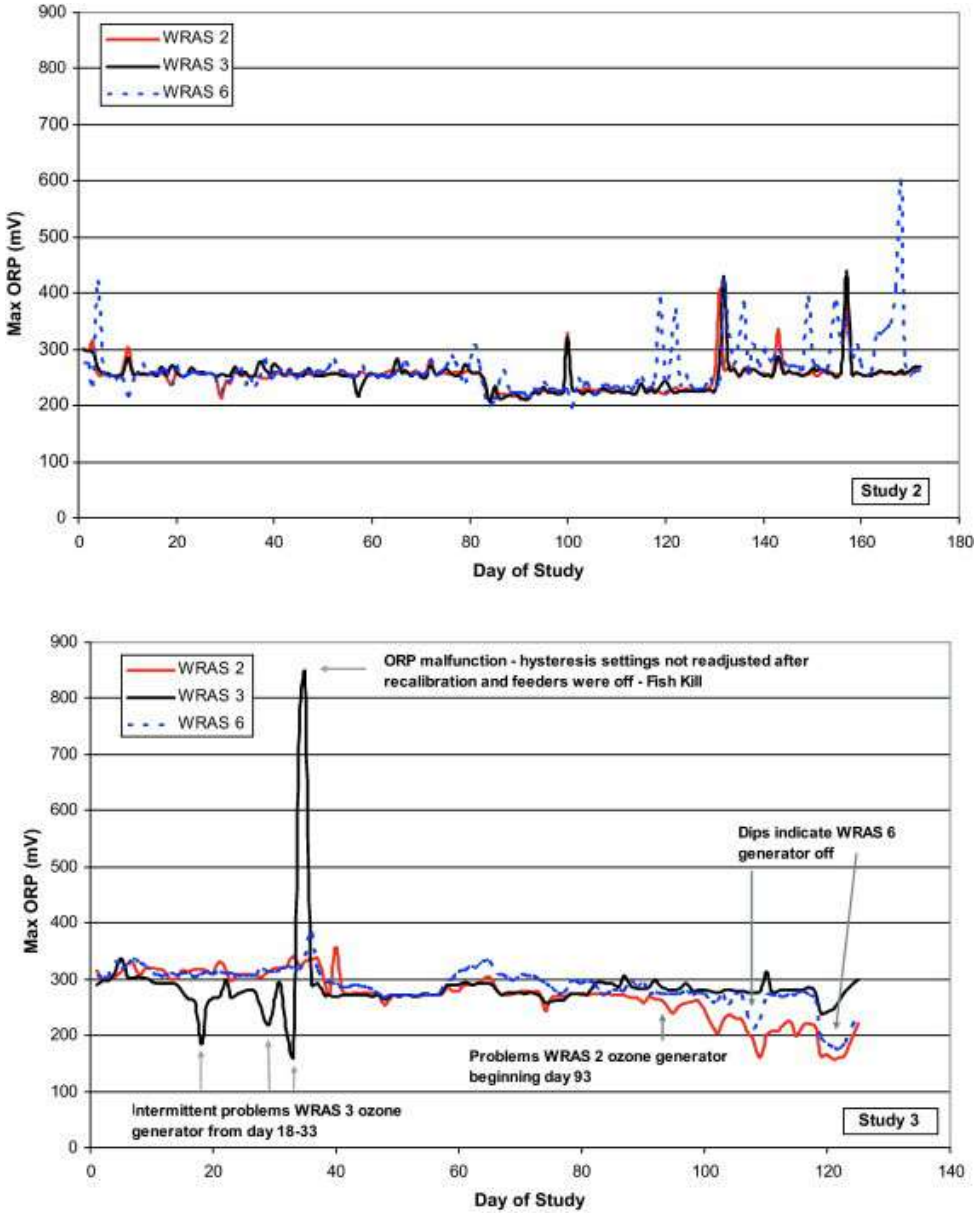

Fig. 5. Maximum oxidation reduction potentials (ORP) measured throughout Studies 1–3.

suggested that an ORP set-point of 300 mV was safe for rainbow trout, and Summerfelt et al. (2009a,b) reported that dissolved ozone concentrations were 0 ppb at an ORP up to 340 mV. ORP levels were maintained very close to the target levels (250 mV for Studies 1 and 2; 270–290 mV for Study 3) under most circumstances (Fig. 5). However, during Studies 1 and 2, multiple events occurred during which the SC100 Universal Controller's PID control loop severely overshot the set-point when auto-tuning after restart, allowing ORP to spike to 100–300 mV beyond the target set-point (Fig. 5, Study 2). Several ORP spikes (≥400 mV) indicated potentially dangerous levels of ozone residual in the culture water, including a few during Study 2 that resulted in low-level mortality. During Study 3, ORP was controlled via on/off set-points programmed within the SC100 Universal Controller to maintain an ORP of 270–290 mV. One major spike occurred during Study 3 within WRAS 3 (ORP = 850 mV; Fig. 5) that resulted in significant fish mortality, but this spike was attributed to human error, i.e., the control function was manually turned off and not turned back on until too late. Not withstanding human error, on/off ORP control via the SC100 unit proved to be a more stable ozone control method that was safer for fish.

Bromide ions (Br^-) are naturally occurring in seawater and many freshwater sources or can be present as impurities within salts such as sodium chloride (Grguric et al., 1994). Ozone reacts very little with chloride, but its reaction with bromide can form relatively toxic residuals, including bromine and bromate (BrO_3–) (Steslow, 1991; Grguric et al., 1994; Tango and Gagnon, 2003; Tanaka and Matsumura, 2002, 2003).

Bromide measured during Study 2 by Test America (Nashville, TN) using a MDL of 1 mg/L was undetectable for all six WRAS. However, results from the same sampling event that were sent to Broward Testing Laboratory (Ft. Lauderdale, FL, USA), which used an analytical technique with an MDL of 0.005 mg/L, indicated mean bromide levels of 0.020 ± 0.003 mg/L in the high exchange WRAS without ozone and 0.022 ± 0.007 mg/L (excluding WRAS 6) in the low exchange WRAS operated with ozone. Bromide within WRAS 6 was undetectable during this single sampling event, possibly indicating that bromide had been oxidized to bromine or bromate in this system. Natural salts containing bromide could have been present at low concentrations within the makeup water or bromide could have entered the WRAS as a contaminant within the sodium bicarbonate that was added daily to control alkalinity, or the sodium chloride that was added as a chemotherapeutic treatment as needed to counter low-level bacterial gill disease (BGD). Sodium chloride was added to the non-ozonated WRAS much more frequently than to the ozonated WRAS, due to higher incidence of BGD.

Bromate was non-detectable in all WRAS samples at a MDL of 0.001 mg/L, with the exception of the single sample from WRAS 6 (low exchange with ozone), which was 0.056 mg/L. This sample was taken shortly after the PID control loop for ORP had malfunctioned in WRAS 6, causing ORP in the culture tank to increase to at least 388 mV (Fig. 5). However, this measured bromate concentration was approximately 1000-times less than the concentration that Hutchinson et al. (1997) determined to be acutely toxic to rainbow trout. Bromine concentrations measured during Study 2 were less than or equal to the MDL's for the conducted assays: i.e. ≤0.03 mg/L. Supporting this MDL was the 0.02 ± 0.01 mg/L bromine concentration measured in the three WRAS where no ozone was added and bromine could not be present.

During Study 3, the mean concentrations of bromide and bromate within the ozonated WRAS were 0.050 ± 0.010 mg/L and 0.023 ± 0.040 mg/L, respectively. Thus, detectable bromate concentrations were measured within each of the three WRAS that were operated with ozone. Unfortunately, in hind sight, samples from the non-ozonated WRAS were not sent for analysis to test the analytical method against the non-ozonated control condition. As previously mentioned, these levels of bromate are far below those levels reported to be acutely toxic.

Bromoform was not detected (<0.050 mg/L MDL) within any fish tissue samples during Study 2 or 3.

3.9. Controlled water quality parameters

Dissolved oxygen, carbon dioxide, and temperature were controlled throughout Studies 1–3 and therefore were similar between treatments for each study (Tables 4 and 5). A significant difference for oxygen was detected between treatments during Study 2 for the study duration ($P = 0.006$) (Table 5), but the difference was only 0.2 mg/L, which was inconsequential to fish health or performance.

3.10. Growth, feed conversion, condition factor, and survival

3.10.1. Study 1

Rainbow trout growth was significantly greater within low exchange WRAS operated with ozone at the conclusion of Study 1 ($P = 0.001$). Mean final weights for low exchange WRAS operated with and without ozone were 1161 ± 6 and 993 ± 12 g, respectively, four months after initiation of treatments (Fig. 6). Mean thermal growth coefficients calculated for the study duration also reflected significantly faster growth rates for WRAS operated with ozone, i.e. 2.13 ± 0.01 vs. 1.83 ± 0.03 within WRAS without ozone ($P = 0.006$). Additionally, repeated measures analysis indicated a highly significant difference relative to a time × treatment interaction ($P = 0.000$) indicating that differences in mean fish weight occurred through time between the two treatments. Subsequent *t*-tests revealed that rainbow trout within the ozonated WRAS were significantly larger than trout in WRAS without ozone only one month after treatments were initiated. Fig. 6 illustrates the divergence of growth curves following the pre-study acclimation and set-up periods during which rainbow trout weights were equal.

Feed conversion ratios indicated that trout in WRAS with ozone generally consumed feed more efficiently than trout cultured in WRAS without ozone (Fig. 7). One month after initiation of treatments, substantial amounts of uneaten feed were observed within the radial flow settlers for WRAS operated without ozone. The increase in uneaten feed in WRAS without ozone coincided with the slower growth that occurred over the first month of the study. Feed conversion ratios for WRAS with and without ozone over the first month of ozone operation were 1.15 ± 0.06 and 1.38 ± 0.09, respectively ($P = 0.090$) reflecting the increase in uneaten feed in WRAS without ozone (Fig. 7). After the first month of the study, feeding was reduced to minimize uneaten feed, thus FCR's did not vary as much between treatments thereafter. However, overall FCR's were still slightly better (but not significantly) for fish cultured in WRAS with ozone. Cumulative FCR's for fish cultured in WRAS with and without ozone were 1.41 ± 0.03 and 1.51 ± 0.04, respectively ($P = 0.146$) (Fig. 7).

Condition factor at the conclusion of the study was significantly greater for WRAS operated with ozone, 2.03 ± 0.01, versus WRAS without ozone, 1.95 ± 0.02 ($P = 0.011$) (Fig. 8). The greater condition factors for the rainbow trout from the ozonated WRAS appear to be due to slightly greater weight for a given length. Rainbow trout length for WRAS with and without ozone was 384 ± 1 and 370 ± 1 mm, respectively ($P = 0.001$). A greater condition factor can be beneficial to a producer, because it typically indicates increased girth and potentially increased fillet yield.

Survival calculated over the study duration for WRAS with and without ozone was 99.3 ± 0.2 and 98.3 ± 0.5%, respectively ($P = 0.113$). Although a significant difference was not detected between treatments relative to survival, it is worth noting that ozonated WRAS had approximately half the mean cumulative

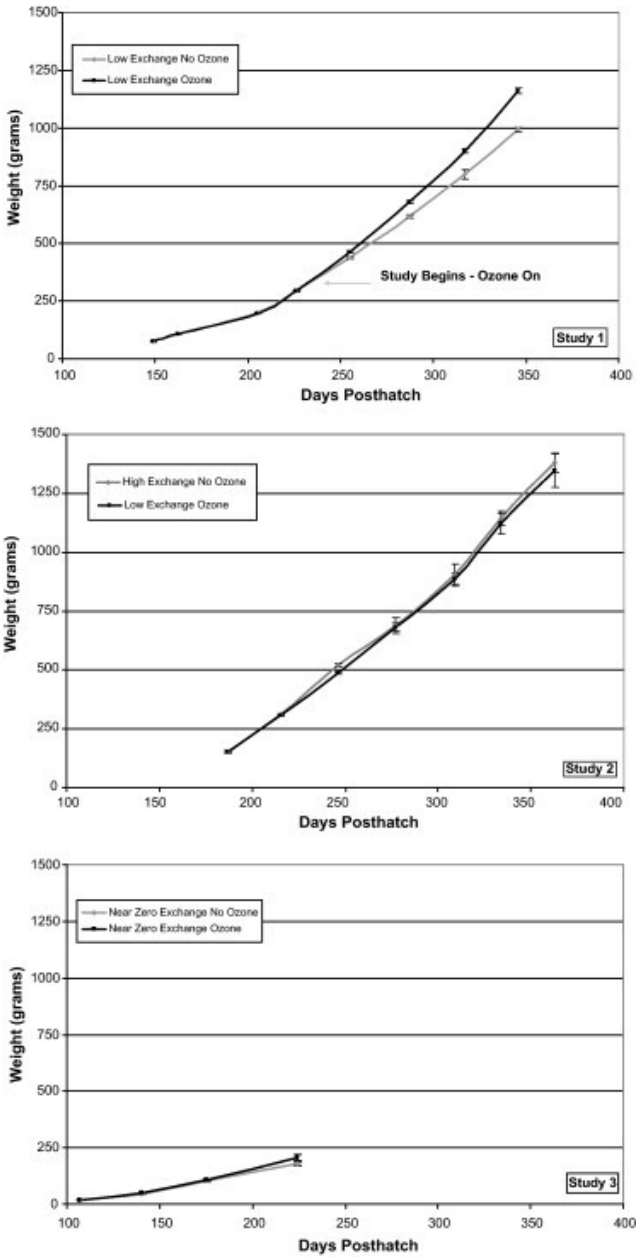

Fig. 6. Mean growth curves with one standard error for: Study 1—low water exchange ozone vs. no ozone; Study 2—low water exchange ozone vs. high water exchange no ozone; and Study 3—near-zero water exchange ozone vs. no ozone.

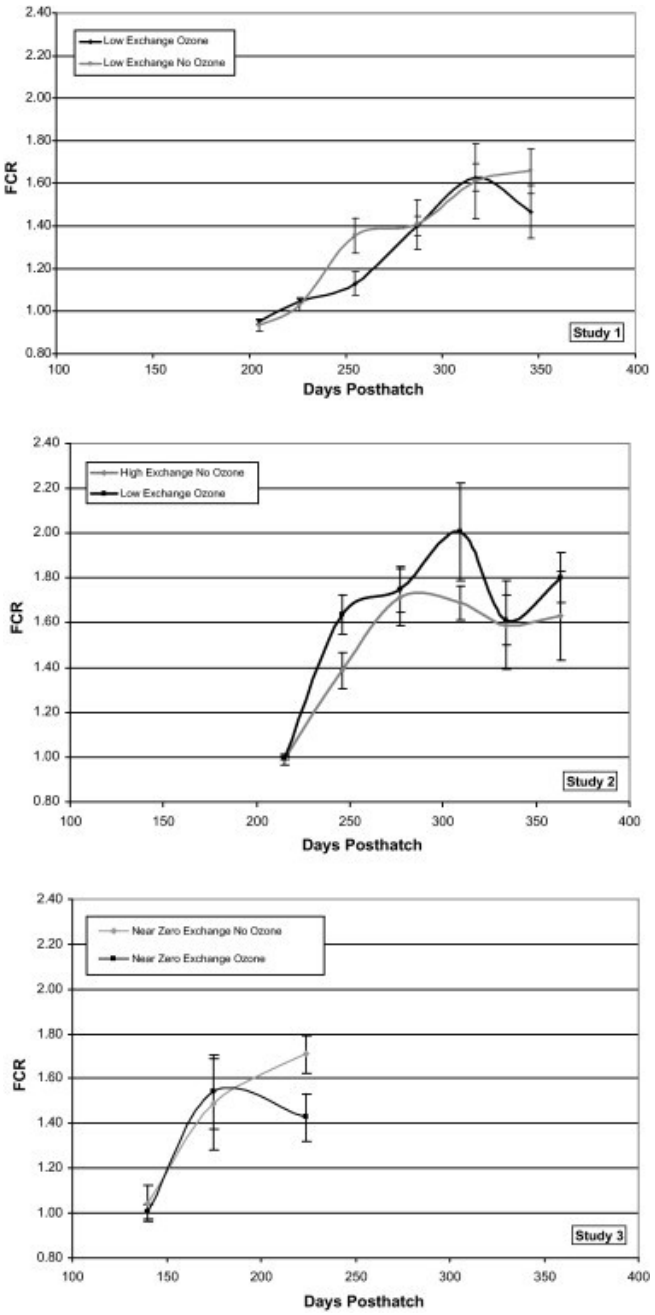

Fig. 7. Mean feed conversion ratios (FCR) with one standard error for: Study 1—low water exchange ozone vs. no ozone; Study 2—low water exchange ozone vs. high water exchange no ozone; and Study 3—near-zero water exchange ozone vs. no ozone.

mortalities as compared to WRAS without ozone, i.e. 7 ± 2 versus 17 ± 5 mortalities, respectively.

3.10.2. Study 2

Rainbow trout growth was similar between treatments throughout the duration of Study 2. At the conclusion, mean rainbow trout weights in WRAS operated at high exchange without ozone were 1379 ± 38 g versus 1348 ± 72 g in WRAS operated at low exchange with ozone (Fig. 6). Repeated measures analysis indicated that there was not a difference between treatments ($P = 0.581$) or a time × treatment interaction ($P = 0.991$), indicating that growth rates were similar throughout the study. Mean thermal growth coefficients for the high exchange WRAS without ozone and the low exchange WRAS with ozone were 2.58 ± 0.05 and 2.54 ± 0.10, respectively, reflecting equal growth rates ($P = 0.790$). Thus, rainbow trout performance within the low exchange WRAS with ozone was similar to that of a high exchange system without ozone, despite the 10-fold difference in flushing rate.

Feed conversion ratios were also similar between treatments (Fig. 7). The cumulative FCR for fish cultured in the low exchange WRAS with ozone averaged 1.52 ± 0.09 as compared to 1.43 ± 0.05 for fish cultured in the high exchange WRAS without ozone ($P = 0.254$) (Fig. 7).

Condition factor at the conclusion of Study 2 was significantly greater for WRAS operated at low exchange with ozone, 2.10 ± 0.04, versus WRAS operated at high exchange without ozone, 1.87 ± 0.01 ($P = 0.021$). Fig. 8 shows that the divergence in condition factor began very early in the study. The difference in condition factor was not related to differences in fish weight, because weights were

Table 7
Cumulative feed burden (mg feed per culture system per liter of makeup water) and feed loading rate (kg daily feed per cubic meters of daily makeup water) for various water exchange rates within various culture systems used for salmonids culture, including those used during the present studies.

Literature cited	Biofilter						
	Type of system	Cultured species	Nitr.	Denitr.	Makeup water % of recycle flow	Cumulative feed burden (mg/L)	Feed loading (kg feed/m^3 makeup water)
Roque d'Orbcastel et al. (2009a)	Single pass	Arctic char, trout			100	6–19	0.006–0.019
Roque d'Orbcastel et al. (2009b)	Single pass	Rainbow trout			100	17	0.017
Summerfelt et al. (2009a,b)	Partial reuse	A. salmon smolt			11–13	35	0.035
Summerfelt et al. (2004a,b)	Partial reuse	Arctic char, trout			17	67	0.067
Wolters et al. (2009)	Recirculating	A. Salmon brood	x		Appx. 2.5	49–108	0.049–0.018
Fischer et al. (2009)	Recirculating	Brook Trout	x		3.0	68	0.068
Roque d'Orbcastel et al. (2009b)	Recirculating	Rainbow trout	x		Appx. 15	111	0.111
Roque d'Orbcastel et al. (2009a)	Recirculating	Arctic char, trout	x		NA	125	0.125
Roque d'Orbcastel et al. (2009c)	Recirculating	Rainbow trout	x		Appx. 5	131	0.131
Wolters et al. (2009)	Recirculating	A. Salmon parr	x		Appx. 2.5	177	0.177
Wolters et al. (2009)	Recirculating	A. Salmon smolt	x		Appx. 2.5	136–213	0.136–0.213
Skybakmoen et al. (2009)	Recirculating	Arctic char	x		6.7–12.7	58–407	0.058–0.407
Couturier et al. (2009)	Recirculating	Salmon smolt	x		3.9–9.1	200–500	0.200–0.500
Davidson et al. (2009)	Recirculating	Rainbow trout	x		2.6	388–535	0.388–0.535
Present research—Study 2	Recirculating	Rainbow trout	x		2.6	360–362	0.360–0.362
Morey (2009)	Recirculating	Salmon	x		5.0	551	0.551
Martins et al. (2009a)	Recirculating	Common Carp	x		NA	660	0.660
Martins et al. (2009b)	Recirculating	Nile Tilapia	x		NA	920	0.920
Davidson et al. (2009)	Recirculating	Rainbow trout	x		0.26	3,950–5,256	3.95–5.26
Present research—Study 2	Recirculating	Rainbow trout	x		0.26	3,177–3,785	3.18–3.78
Present research—Study 1	Recirculating	Rainbow trout	x		0.26	3,441–4,065	3.44–4.07
Martins et al. (2009b)	Recirculating	Nile Tilapia	x	x	NA	14,312	14.3
Martins et al. (2009b)	Recirculating	Nile Tilapia	x	x	NA	33,374	33.4
Martins et al. (2009a)	Recirculating	Common Carp	x	x	NA	33,323	33.3
Present research—Study 3	Recirculating	Rainbow trout	x		0.001	1,702–146,834	1.70–147
Tal et al. (2009)*	Recirculating	Sea Bream	x	x	NA	29,762–130,952	29.8–131

Note: Feed burden ranges indicate lowest and highest mean of the six WRAS for the study duration. '*' For Tal et al. (2009) feed burden was calculated using the mean daily makeup flow for minimum and maximum daily feeds.

equal, but instead was attributed to a difference in fish length. Mean fish length at the conclusion of the study for WRAS operated at high exchange without ozone was 418 ± 3 mm, but only 399 ± 5 mm within low exchange WRAS with ozone ($P = 0.044$). We hypothesize that the difference in fish length and thus condition factor was related to a difference in fish swimming speed/exercise that was observed between treatments. Rainbow trout within the low exchange WRAS with ozone were consistently observed swimming forward at a much greater speed (i.e. 1.40 ± 0.06 body lengths/s) compared to fish cultured within WRAS operated at high exchange without ozone which generally held position in the water column, resulting in a swimming speed of 0.65 ± 0.18 body lengths/s ($P = 0.041$). Note that swimming speeds were measured by timing fish as they swam past marked tank locations and then by factoring in the rotational velocity of water circling the tank, as described in Davidson et al. (under review). A potential relationship was observed between rainbow trout swimming speed and certain constituents within water, such as nitrate nitrogen and dissolved potassium. Specifically, something in the water that was not related to ozone was causing sublethal effects that included abnormal swimming behavior and an increased incidence of spinal deformities. These finding are reported elsewhere (Davidson et al., under review).

Rainbow trout survival was similar between the two treatments, during Study 2. Survival within WRAS operated at low exchange with ozone was 93.3 ± 1.6% and 93.1 ± 0.5% in WRAS operated at high exchange without ozone ($P = 0.787$).

3.10.3. Study 3

Repeated measures analysis indicated that rainbow trout growth was similar between WRAS with and without ozone ($P = 0.267$) and a sample x treatment interaction indicated that differences in rainbow trout weights did not occur between treatment during any sampling point during the study ($P = 0.196$) At the conclusion of Study 3, rainbow trout within the near-zero exchange WRAS with and without ozone were 206 ± 14 and 180 ± 10 g, respectively (Fig. 6). Mean thermal growth coefficients for fish cultured within near-zero exchange WRAS with and without ozone were 1.79 ± 0.07 and 1.66 ± 0.07, respectively ($P = 0.257$).

Feed conversion ratios were slightly better for WRAS operated at near-zero exchange with ozone, but not significantly ($P = 0.339$). Mean FCR's over the study duration for the near-zero exchange WRAS with and without ozone were 1.30 ± 0.07 and 1.42 ± 0.08, respectively (Fig. 7).

Rainbow trout condition factor at the conclusion of the study was significantly greater within the near-zero exchange WRAS with ozone, i.e. 1.74 ± 0.07, as compared to the near-zero exchange WRAS without ozone, 1.40 ± 0.02 ($P = 0.034$) (Fig. 8). Although a statistical difference does exist between treatments, the authors do not believe that this difference is directly related to treatment. Instead, condition factor appears to correlate with differences in swimming behavior and the accumulation of certain constituents within the recirculated water that existed between treatments, as was also observed during Study 2 (Davidson et al., under review).

Rainbow trout survival was similar between the WRAS operated at near-zero exchange with and without ozone. Survival within WRAS operated at near-zero exchange with and without ozone was 88.8 ± 3.3% and 94.5 ± 0.5%, respectively ($P = 0.229$). It is important to note that the mortality event that occurred due to an ozone spike created by human error (described in Section 3.9) was excluded from the cumulative survival assessment. Although survival was lower for the ozonated WRAS, this difference was most likely not related to the ozone treatment, but instead to unexpected differences in flushing rates and the corresponding differences in water quality. For example, survival within one WRAS operated with ozone that had a HRT <10 days was 95.1%, while survival within WRAS operated with ozone with HRT's ≥94 days had cumulative survival of 83.8 and 87.6%. Thus, the overall decreased survival for

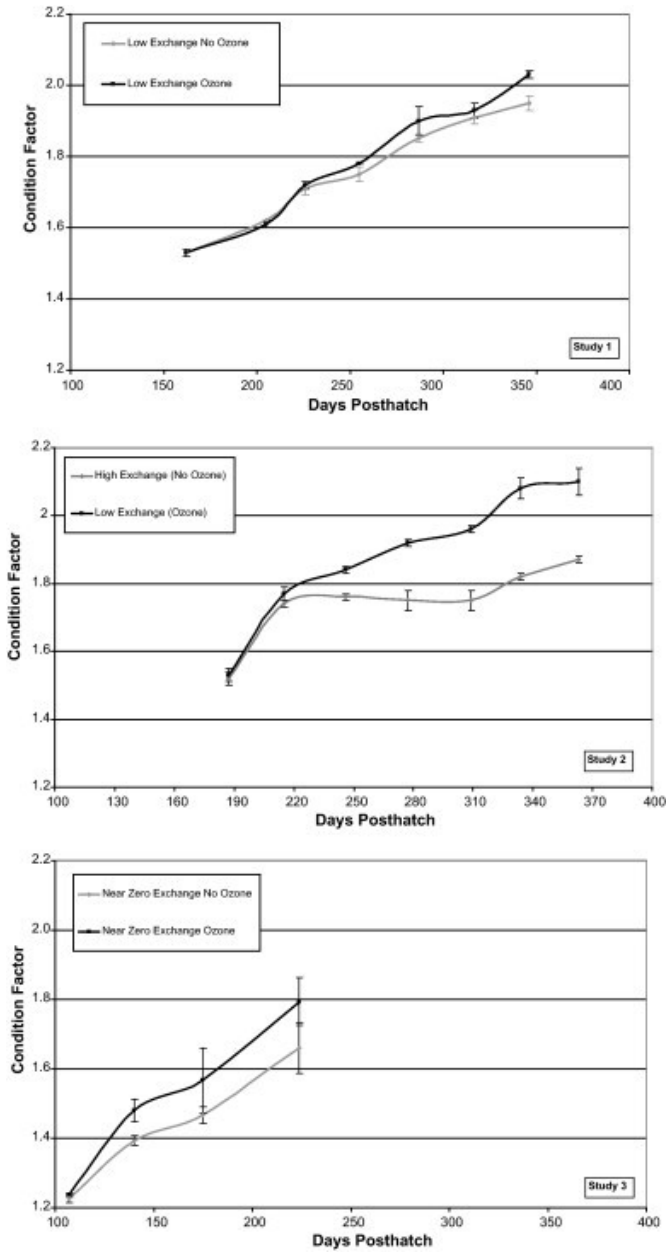

Fig. 8. Mean condition factor for the WRAS operated at high exchange without ozone and low exchange with ozone, with one standard error. Fulton's condition factor was used and was calculated as follows: ($10^5 \times$ Wt (g))/Length (mm).

WRAS operated with ozone was more likely related to some aspect of the water quality, because two of three ozonated WRAS had retention times ≥94 days and thus substantially greater concentrations of other parameters.

4. Summary and conclusions

These studies indicated that the use of ozone within low and near-zero exchange water recirculating systems significantly improved a variety of water quality conditions. Ozone effectively reduced TSS, carbonaceous BOD, and color, and resulted in a significant increase in ultraviolet transmittance. As a result of the improved water quality conditions, total heterotrophic bacteria counts were also lower within ozonated WRAS. Most notably ozone effectively reduced accumulating metals within low and near-zero exchange WRAS, particularly copper. Ozone also reduced other potentially toxic metals, including zinc and iron. During Study 2, ozone created water quality within low exchange WRAS that was similar to high exchange WRAS that were operated with 10 times greater water exchange; and, during Study 3, ozone created lower concentrations of most water quality parameters including copper, iron, TSS, and BOD, even within WRAS that had retention times ≥94 days as compared to WRAS operated without ozone with retention times of approximately 40 days. Overall, ozone created a more optimal water quality environment that generally led to increased growth, survival, feed conversion, and condition factor of rainbow trout.

These studies also indicated that heavy metals such as copper, zinc, and iron can accumulate within low and near-zero exchange WRAS without the use of ozone. In addition, ozone did not reduce dissolved potassium concentrations or nitrate nitrogen concentrations. The current research provided evidence that nitrate nitrogen could accumulate to very high levels (100–700 mg/L) within WRAS operated at low and near-zero exchange. Thus, WRAS operated at low and near-zero exchange could require denitrification unit processing (depending on exact feed loading and flushing rates) in order to avoid potentially toxic nitrate–nitrogen accumulation (Van Rijn et al., 2006). The potential toxicity of each water quality parameter measured during these studies will be presented in detail within a companion paper (Davidson et al., under review).

The flushing rates and subsequent feed loading rates that were used during the present studies represented the extreme or outer limit as compared to the recycle conditions that others have reported for fish culture, particularly for salmonids (Table 7). Table 7 provides a comparison of cumulative feed burden (mg of daily feed/L of makeup water used daily) and feed loading rate (kg feed/m^3 makeup water/day) for a variety of systems with various flushing rates that have been used for fish culture. Note that the authors could not identify other literature that reported cumulative feed burdens nearly as high as those that were used during the present studies for salmonid culture. During Study 3, feed burden was as high as 1.47×10^5 mg daily feed/L daily makeup water within WRAS that used the lowest water exchange rates (Table 7). The results from these studies suggest that the use of ozone enhances the culture tank water quality dramatically, thus increasing the feasibility of operating WRAS at extremely low water exchange rates that have not been previously attempted for the culture of a salmonid species such as a rainbow trout, which is relatively sensitive to deteriorating water quality conditions. Ultimately, these findings indicate an increased potential to locate fish culture facilities utilizing WRAS even in areas with very limited water resources, potentially near major cities with high demand for fresh seafood.

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Study III

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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John Davidson*, Christopher Good, Carla Welsh, Steven T. Summerfelt

The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, United States

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Nitrate toxicity

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

* Corresponding author. Tel.: +1 304 876 2815x221; fax: +1 304 870 2208.
E-mail address: j.davidson@freshwaterinstitute.org (J. Davidson).

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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.

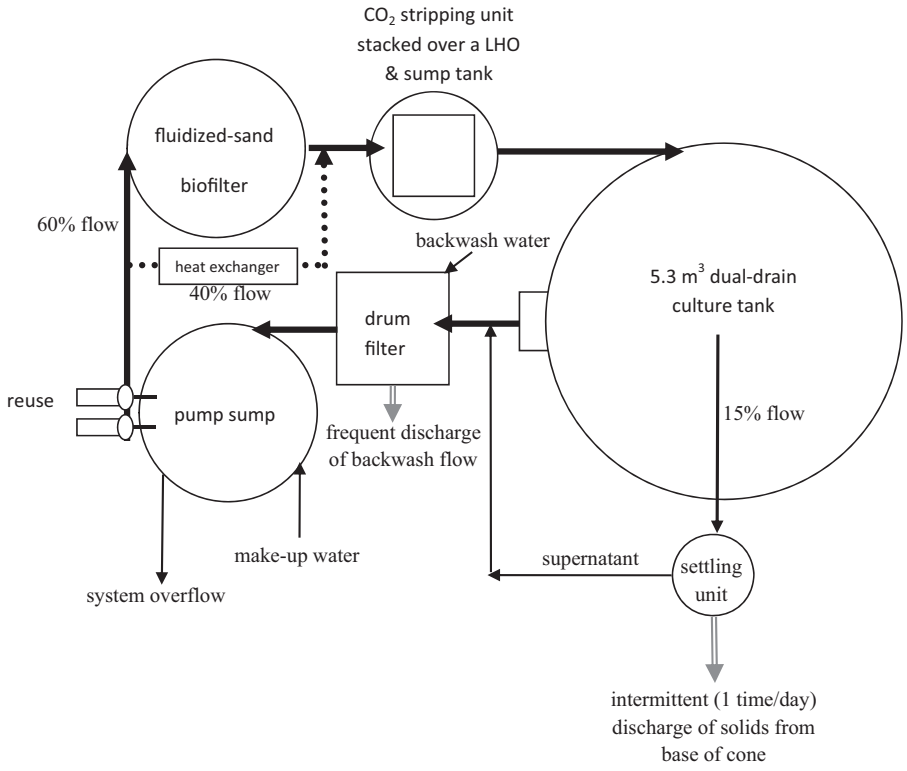

Fig. 1. Design schematic of an individual 9.5 m^3 experimental water recirculating aquaculture system. Arrows indicate direction of water flow through unit processes.

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,

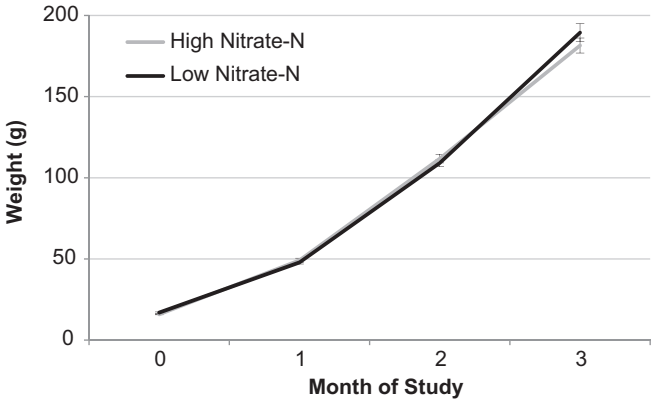

Fig. 2. Mean rainbow trout weight ± standard error (g) for the high and low NO_3-N treatments ($n = 3$).

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

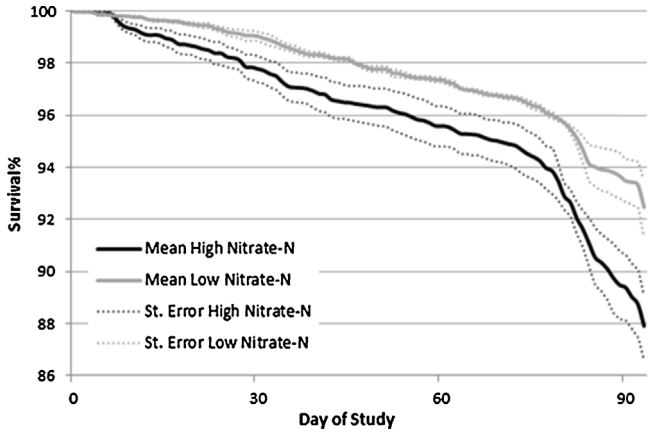

Fig. 3. Cumulative survival percentage (mean ± standard error) for rainbow trout from the high and low NO_3-N treatments ($n = 3$).

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

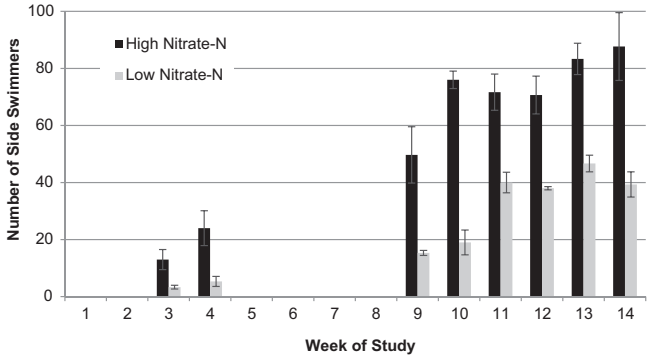

Fig. 4. Relative number of side swimming rainbow trout observed passing a given culture tank location for the high and low NO_3-N treatments ($n = 3$). Observations were not made during weeks 5–8 due to increased turbidity of the culture water.

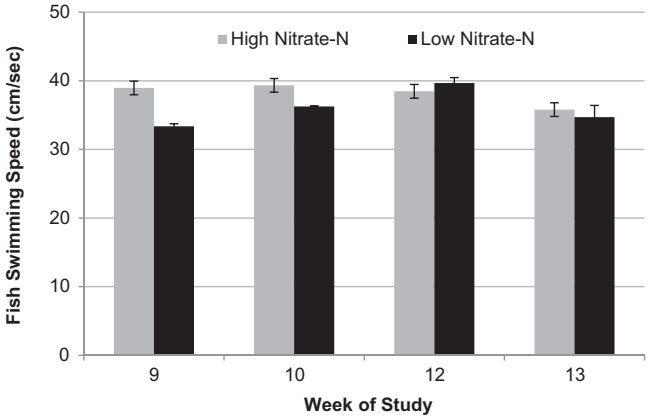

Fig. 5. Mean rainbow trout swimming speed ± standard error (cm/s) for the high and low NO_3-N treatments during weeks 9–13 of the study ($n = 3$). Note that week 11 data was excluded due to potential confounding caused by inconsistent hydraulics amongst systems, specifically related to position of the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

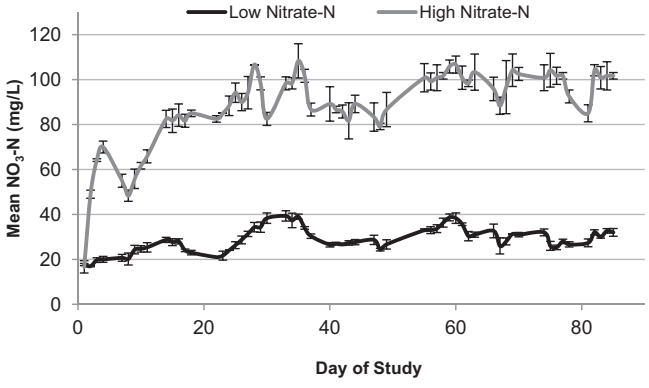

Fig. 6. Mean nitrate nitrogen concentrations ± standard error (mg/L) for the high and low NO_3-N treatments measured 4–5 times per week ($n = 3$).

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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Study IV

Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon Salmo salar in freshwater recirculation aquaculture systems

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Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon *Salmo salar* in freshwater recirculation aquaculture systems

John Davidson*, Christopher Good, Christina Williams, Steven T. Summerfelt

The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, United States

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ABSTRACT

Commercial production of Atlantic salmon smolts, post-smolts, and market-size fish using land-based recirculation aquaculture systems (RAS) is expanding. RAS generally provide a nutrient-rich environment in which nitrate accumulates as an end-product of nitrification. An 8-month study was conducted to compare the long-term effects of "high" (99 ± 1 mg/L NO_3-N) versus "low" nitrate-nitrogen (10.0 ± 0.3 mg/L NO_3-N) on the health and performance of post-smolt Atlantic salmon cultured in replicate freshwater RAS. Equal numbers of salmon with an initial mean weight of 102 ± 1 g were stocked into six 9.5 m^3 RAS. Three RAS were maintained with high NO_3-N via continuous dosing of sodium nitrate and three RAS were maintained with low NO_3-N resulting solely from nitrification. An average daily water exchange rate equivalent to 60% of the system volume limited the accumulation of water quality parameters other than nitrate. Atlantic salmon performance metrics (e.g. weight, length, condition factor, thermal growth coefficient, and feed conversion ratio) were not affected by 100 mg/L NO_3-N and cumulative survival was > 99% for both treatments. No important differences were noted between treatments for whole blood gas, plasma chemistry, tissue histopathology, or fin quality parameters suggesting that fish health was unaffected by nitrate concentration. Abnormal swimming behaviors indicative of stress or reduced welfare were not observed. This research suggests that nitrate-nitrogen concentrations ≤ 100 mg/L do not affect post-smolt Atlantic salmon health or performance under the described conditions.

1. Introduction

Atlantic salmon *Salmo salar* are being farmed more frequently using recirculation aquaculture systems (RAS) (Drengstig et al., 2011; Dalsgaard et al., 2013). Many Nordic salmon farms have transitioned a portion of their smolt production from traditional flow-through systems to RAS, or have plans to construct large RAS (Bergheim et al., 2009; Martins et al., 2010; Drengstig et al., 2011; Dalsgaard et al., 2013; Summerfelt et al., 2016), with some producers intending to raise smolts and post-smolts to larger sizes (250–1000 g) prior to transfer to sea cages (Dalsgaard et al., 2013). Moreover, there is growing interest in culturing Atlantic salmon to market-size in land-based systems that utilize RAS technology (Thorarensen and Farrell, 2011; Summerfelt and Christianson 2014; Davidson et al., 2016a; Liu et al., 2016).

Adoption of RAS by the Atlantic salmon industry has been driven by several factors, including the declining availability of freshwater resources (Kristensen et al., 2009), temperature control and related growth advantages, and the potential for larger post-smolts to be more robust at sea, less susceptible to sea lice, and to have a shorter grow-out period in net cages (Bergheim et al., 2009; Dalsgaard et al., 2013; Ytrestøyl et al., 2013). These trends have spurred research into the biological requirements and feasibility of raising post-smolt Atlantic salmon in RAS (Thorarensen and Farrell, 2011; Qiu et al., 2015; Davidson et al., 2016a; Liu et al., 2016).

Recirculation aquaculture systems provide a unique culture environment that generally contains greater concentrations of waterborne metabolites, nutrients, and dissolved metals (Davidson et al., 2009; Martins et al., 2009) compared to traditional production systems. Therefore, research focused on establishing water quality thresholds for RAS-produced Atlantic salmon is critical for this developing industry sector. In particular, nitrate is emerging as a water quality parameter of interest in RAS production. Recirculation aquaculture systems that are operated intensively with minimal water exchange and increased feed loads are prone to nitrate accumulation when denitrification is absent from the water treatment loop; however, the effects of nitrate have not been extensively studied for most RAS-produced species.

* Corresponding author.
E-mail address: jdavidson@conservationfund.org (J. Davidson).

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A review of physiological effects and known toxicity thresholds of nitrate for aquatic organisms is provided by Camargo et al. (2005). Physiological effects of nitrate to fish and other aquatic species include methemoglobinemia (Grabda et al., 1974; Cheng and Chen, 2002; Tilak et al., 2007), decreased immune response and osmoregulatory function (Hrubec et al., 1996, 1997), and endocrine system disruption and effects on reproductive maturation (Guillette and Edwards, 2005; Hamlin, 2007; Hamlin et al., 2008; Good and Davidson, 2016). In some cases, elevated nitrate-nitrogen (NO_3-N) levels have been found to cause fish mortality. Several lethal toxicity studies have been conducted with salmonids; namely, Westin (1974) reported 96-h LC_{50} concentrations of approximately 1355 and 1310 mg/L NO_3-N for rainbow trout *Oncorhynchus mykiss* and Chinook salmon *Oncorhynchus tshawytscha* fingerlings, respectively. Kincheloe et al. (1979) observed significant mortality in larval cutthroat trout *Oncorhynchus clarkii henshawi*, Chinook salmon, and rainbow trout at NO_3-N concentrations of only 2.3–7.6 mg/L, demonstrating a possible influence of salmonid life stage on nitrate toxicity. In contrast, Freitag et al. (2016) found no difference in survival of Atlantic salmon embryos exposed to mean NO_3-N levels of 4 or 93 mg/L. The response difference of juvenile salmonids to NO_3-N between these studies is surprising, but could be related to dissimilar water quality, such as hardness or sodium concentration, or the species under evaluation.

Recently, there is increasing evidence that elevated nitrate can chronically impact the general health and performance of fish cultured in RAS. Davidson et al. (2014) reported that rainbow trout exposed to 80–100 mg/L NO_3-N for three months demonstrated chronic health and welfare impacts including an increase in abnormal swimming behavior, increased swimming speeds, and mildly reduced survival. In addition, the growth of juvenile turbot *Psetta maxima* was negatively impacted at NO_3-N concentrations ≥ 125 mg/L, and health and feed efficiency was reduced at ≥250 mg/L (Van Bussel et al., 2012). Schram et al. (2014) also found that feed intake and growth rates of African catfish *Clarias gariepinus* were significantly reduced during 42-day exposure to 379 mg/L NO_3-N and therefore recommended an upper threshold of 140 mg/L NO_3-N for safe culture of this species.

Research assessing the effect of nitrate on Atlantic salmon is limited. Freitag et al. (2015) evaluated the endocrine disrupting potential of NO_3-N levels (5.3, 10.3, and 101.8 mg/L) in pre-smolt Atlantic salmon (102 g to begin). Plasma testosterone was significantly greater in salmon exposed to 10.3 mg/L NO_3-N compared to 5.3 and 101.8 mg/L NO_3-N; however, growth was unaffected. The authors concluded that Atlantic salmon may be a suitable species for RAS production based on the general lack of effect of NO_3-N on growth and most endocrine responses at concentrations up to 101.8 mg/L. The Freitag et al. (2015) study was a short-term trial lasting 27 days and only investigated the effects of nitrate on pre-smolt Atlantic salmon. Long-term research evaluating the effect of nitrate concentrations on Atlantic salmon is lacking, and a safe threshold for chronic exposure has not been fully established.

To this end, the present study was designed to evaluate the long-term effects of high (~100 mg/L) vs. low (~10 mg/L) NO_3-N concentrations on the health and performance of post-smolt Atlantic salmon cultured in RAS. This article expands on the findings of Good et al. (2016), which evaluated the influence of nitrate on post-smolt Atlantic salmon reproductive physiology during the same experiment.

2. Methods

2.1. Atlantic salmon

Fertilized Atlantic salmon eggs (mixed sex, diploid) were received from a commercial producer (Salmobreed, Bergen, Norway) and hatched on-site in a Heath-tray-style incubation system maintained at an average water temperature of 7.6°C. Hatched fry were acclimated to 13.0°C and transferred to a freshwater, flow-through system enclosed by an opaque tent where fish were reared under a light: dark (LD) 24:0 photoperiod until they reached approximately 40 g. A 6-wk LD 12:12 photoperiod was then instituted to simulate a winter photoperiod and thereby instigate smoltification; a LD 24:0 photoperiod was resumed thereafter. Nitrate-nitrogen (NO_3-N) levels in the pre-study culture systems were < 3 mg/L. When the salmon reached a mean weight of approximately 80 g, 336 fish were moved into each RAS used for the study. Salmon were acclimated to the new culture systems for one month, during which average NO_3-N levels were maintained at < 5 mg/L. When the study began, Atlantic salmon were 102 ± 1 g, and fish biomass density was 6–7 kg/m^3.

2.2. Culture systems

Six replicated RAS (9.5 m^3), first described in Davidson et al. (2009), were used as experimental units for the 8-month study. The culture systems recirculated a total freshwater flow of 375 ± 3 L/min (99 ± 1 gpm) through a 5.3 m^3 Cornell-style dual-drain culture tank, a radial flow settler, a microscreen drum filter with 60 μm screens, a fluidized sand biofilter containing 1 m of static sand, a geothermal heat exchanger, a media-packed degassing column, and a low head oxygenator (Fig. 1).

2.3. Experimental design

Three RAS were randomly assigned to each of two treatments, described herein as “high” and “low” NO_3-N with target concentrations of 100 and 10 mg/L NO_3-N, respectively. Sodium nitrate ($NaNO_3$; Tilley Chemical Company, Inc., Baltimore, MD, USA) stock solution was continuously dosed using Masterflex L/S peristaltic pumps (Model 07528-10, Cole Parmer, Vernon Hills, IL, USA) for the high NO_3-N treatment. The dosing rate was gradually increased over the first month of the study until 100 mg/L NO_3-N was achieved. Three RAS did not receive $NaNO_3$ and, as such, were operated with a mean NO_3-N concentration of 10 mg/L, resulting solely from nitrification. Sodium sulfate was selected as an innocuous chemical for conductivity control based on research by Elphick et al. (2011). Sodium sulfate (Tilley Chemical Company, Inc., Baltimore, MD, USA) stock solution was dosed via peristaltic pump to the low NO_3-N systems. Chemical stock solutions were continuously mixed with small submersible pumps and dosed into the pump sumps of respective RAS.

2.4. System operation

A continuous overflow (3.7 ± 0.03 L/min) from each RAS was calibrated daily to establish similar dilution rates among replicates. In addition, approximately 380 L of system water per RAS (a combination of drum filter backwash and solids-laden water manually flushed from the cone-bottom radial flow settlers) was discharged daily. Flushed water was sensed by a float valve which automatically replaced the lost volume with spring water. Cumulative spring (makeup) water addition was measured in each RAS by magnetic drive flow meters (Model C700, Elster AMCO Water Inc., Ocala, FL, USA). This water exchange strategy created an average system hydraulic retention time of approximately 1.7 days, or daily water exchange equivalent to 60% of the system volume. This relatively high flushing rate was necessary to limit the accumulation of water quality concentrations other than nitrate. Sodium bicarbonate was added as needed to maintain equivalent alkalinity between treatments.

2.5. Feeding

Atlantic salmon were fed a commercially available diet containing 43% protein and 24% fat (Bio-Oregon, Westbrook, ME, USA). Salmon in each RAS were fed at the same rate for the first three weeks of the study; thereafter, feeding rates were fine-tuned separately per RAS

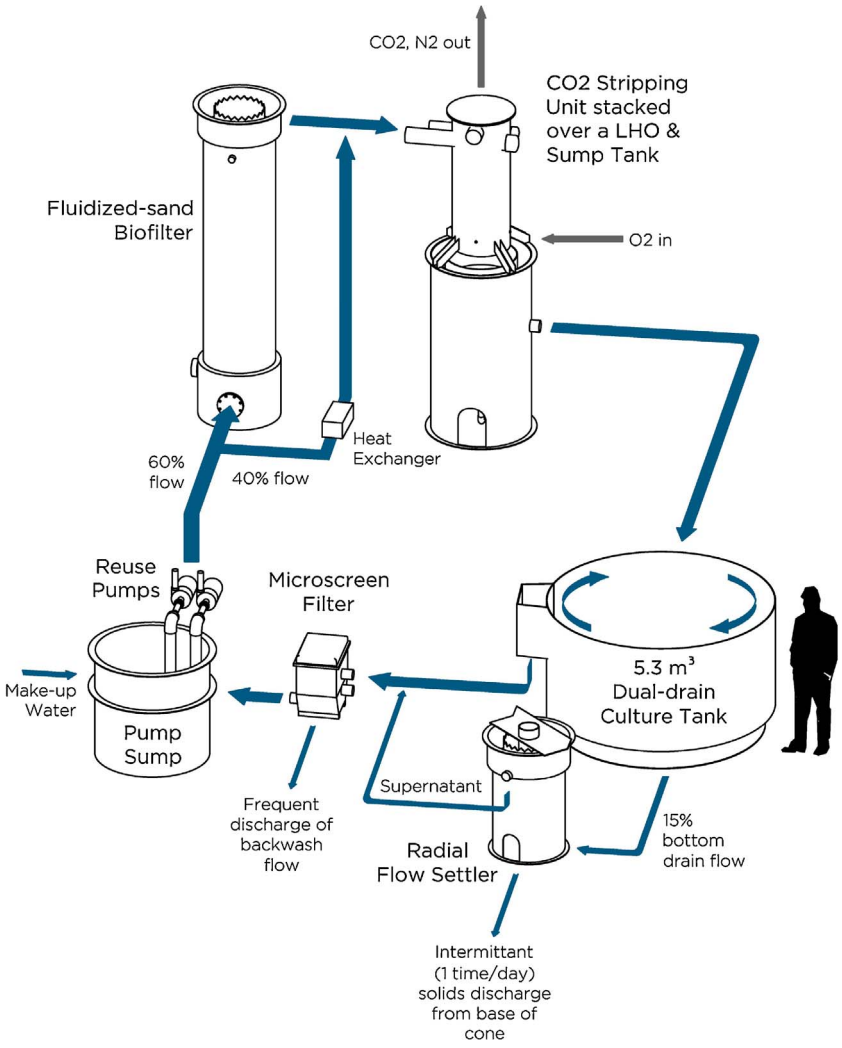

Fig. 1. Process flow drawing of an individual 9.5 m^3 recirculation aquaculture system used in the present study (courtesy Kata Rishel, TCFFI Engineering Services).

based on observations of feeding activity and wasted feed. Fish were fed to apparent satiation using a computer operated feeding system (Freshwater Institute, Shepherdstown, WV, USA), programmed to deliver short feed bursts once an hour via automated feeders (T-drum 2000CE, Arvotec, Huutokoski, Finland). A constant 24-h photoperiod was provided to facilitate around-the-clock feeding.

2.6. Water quality sampling and analyses

Water samples were collected weekly from the side drain of each tank and tested on-site. All water quality parameters measured on-site were analyzed according to methods described in APHA (2012) and HACH (2003, 2015) (Table 1). An array of 27 dissolved metals and nutrients were analyzed by REI Consultants Inc. (Beaver, WV, USA) (Table 1).

2.7. Fish performance sampling

Mortalities were removed and recorded daily to assess cumulative survival. Lengths and weight measurements of a random sample of 60–90 fish were collected approximately every two months, including sampling events to begin and end the study. Sample size (n) (Kitchens, 1998), thermal growth coefficient (TGC), condition factor (CF), and feed conversion ratio (FCR) were calculated using these formulae:

$n = (Z * (\text{stdev.}_g/\text{accepted error}_g))^2$

where Z = 1.65 (relative to a 90% confidence interval)

$TGC = ((\text{End Weight}_g \hat{} (1/3) - \text{Start Weight}_g \hat{} (1/3))/((\text{Days Between} * \text{Avg. Temp.}_{\circ}C) \times 1000)$

$CF = 100{,}000 * \text{Weight}/(\text{Length}_{mm})^3$

FCR = Cumulative Feed Delivered/Fish Biomass Gain

2.8. Fish health assessment

Whole blood gas, plasma chemistry, and tissue histopathology were assessed according to methods described in Good et al. (2016). Fin erosion of anal, caudal, dorsal, pelvic (left and right), and pectoral (left and right) fins was assessed qualitatively on a 3-point scale (low to no damage = 1, moderate = 2, severe = 3) for each fish sampled during length and weight assessments at the beginning and end of the study. General health and physical observations were also noted during each sampling event including the presence of cataracts and *Saprolegnia* spp. infection (fungus).

2.9. Statistical analysis

Parameters that were sampled during multiple events over time from the same location, including most water quality parameters and growth rates were analyzed using a mixed model, where “tank” was included as a random effect to buffer the treatment effect from

Table 1
Water quality parameters evaluated, methodologies, and frequency of testing.

Parameter	Method of Analysis	Frequency of Recording/Testing
Conductivity	YSI 30 Salinity/Conductivity/Temperature Meter	Daily
Dissolved Oxygen	Hach SC100 Controller & LDO® Probe	Daily
ORP	Hach SC100 Controller & Differential ORP Sensor	Daily
Temperature	Hach SC100 Controller & Differential ORP Sensor	Daily
Alkalinity	Hach Method 8203–Sulfuric Acid Digital Titration	4–5 times weekly
Nitrate Nitrogen	Hach Method 8171–Cadmium Reduction	4–5 times weekly
pH	Standard Methods 4500-H^+ B–Electrode	4–5 times weekly
Sulfate	Hach Method 8051 USEPA Methylene Blue	4–5 times weekly
Carbon Dioxide	Hach Method 8223–Sodium Hydroxide Buret Titration	Once weekly
$CBOD_5$	Standard Methods 5210B–5-day test (No prefiltration)	Once weekly
Hardness	Hach Method 8213–Digital Titration using EDTA	Once weekly
Nitrite Nitrogen	Hach Method 8507 USEPA Diazotization	Once weekly
Total Ammonia Nitrogen	Hach Method 8038 USEPA Nessler	Once weekly
Total Suspended Solids	Standard Methods 2540D–Dried at 103–105 °C	Once weekly
True Color	Hach Method 8025–Platinum-Cobalt Standard	Once weekly
Ultraviolet Transmittance	Hach Method 10054–Organic UV Absorbing (UV-254)	Once weekly
Dissolved Metals	Inductively Coupled Plasma Atomic Emission Spectrometry	3 events (Months 2, 4, 6)

individual RAS variability (Ling and Cotter, 2003; Thorarensen et al., 2015). Fin scores and dissolved metals/trace element concentrations were compared between treatments using a *t*-test. Each data set was analyzed for normality using a Shapiro-Wilk test. Non-normal data were transformed for statistical comparison. A probability level of 0.05 was used to determine significance. Statistical analyses were carried out using SYSTAT Version 13 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Fish performance

Atlantic salmon growth curves established for each NO_3-N treatment overlapped throughout the study (Fig. 2). At study's end, salmon weights from the high and low NO_3-N treatments were 1148 ± 22 and 1174 ± 8 g, respectively, and average lengths were 446 ± 3 and 446 ± 1 mm, respectively ($P > 0.05$; Table 2), indicating that growth was unaffected by chronic exposure to the two nitrate treatments. Mean condition factor of salmon from both the high and low NO_3-N treatments was 1.3 ± < 0.1 at the end of the study. There were no differences ($P > 0.05$) between treatments for other bi-monthly performance metrics (Table 2) or between treatments for the grand study means calculated from these data. Mean thermal growth coefficient for the study duration was 1.74 ± 0.02 and 1.76 ± 0.01, for the high and low NO_3-N treatments, respectively. Mean feeding rates for the high and low NO_3-N treatments over the study duration were 0.9 ± 0.02 and 1.0 ± 0.01% of the tank biomass, and FCR was 1.00 ± 0.03 and 0.99 ± 0.01, respectively ($P > 0.05$). In addition, fish density was nearly identical to conclude the study, i.e., 60 kg/m^3 in the high NO_3-N RAS and 61 kg/m^3 in the low NO_3-N RAS. Cumulative Atlantic salmon survival, excluding jumpers and a few culls due to saprolengniasis, was > 99% for both treatments.

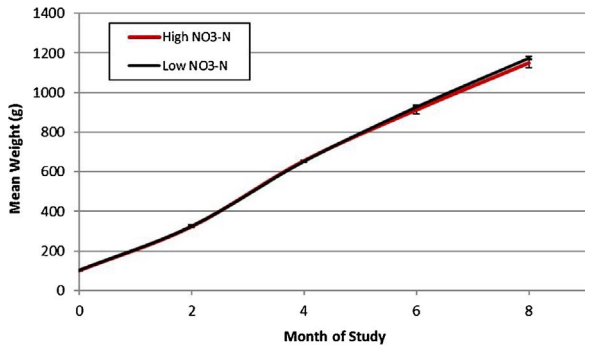

Fig. 2. Mean Atlantic salmon weights (mean ± standard error; n = 3) plotted over the 8-month study for replicate populations exposed to high and low NO_3-N concentrations.

3.2. Fish health

Whole blood gas and plasma chemistry values (Table 3; Good et al., 2016) were consistent with those obtained on-site for healthy Atlantic salmon and were generally within published normal ranges for freshwater salmonids (Stoskopf, 1993; Wedemeyer, 1996). Tissue histopathology findings were statistically similar between treatments (Good et al., 2016). Overall, plasma chemistry and histopathology evaluations indicated normal fish health and no effect of nitrate.

Fin quality declined slightly for salmon from both NO_3-N treatments from beginning to end of study with original scores denoting good quality and little to no fin erosion, and end-of-study scores trending towards moderate erosion (Table 4). However, quality scores for all fin types were unaffected by treatment over the study duration and consistent with acceptable fins per on-site experience culturing other Atlantic salmon cohorts.

Two months into the study, salmon in all RAS were observed to have a mild external *Saprolegnia* spp. infection (saprolegniasis), typically referred to as fungus. The prevalence of infection was slightly lower, albeit not significantly ($P > 0.05$), in the high NO_3-N RAS (13 ± 3% infected) compared to the low NO_3-N RAS (25 ± 6% infected). On average, the degree of saprolegniasis was relatively mild and was generally observed as an abrasion or patchy loss of scales on the either side of the fish body. Salt was added to each RAS following this sampling event to achieve 1.5–2 ppt salinity, which was maintained for three days. Two months later (Month 4 of the study), the incidence of saprolegniasis was substantially reduced in all RAS; only 1% of sampled fish from the high NO_3-N RAS were observed to have any extent of saprolegniasis, while 5% of salmon from the low NO_3-N RAS were noted as being affected. During subsequent sampling events (Months 6 and 8) the incidence of saprolegniasis was ≤1% of the population for both NO_3-N treatments.

Cataracts were not observed during the initial fish sampling event, but became progressively evident in the eyes of salmon from both NO_3-N treatments during the study. At study's end 22 ± 2 and 21 ± 1% of the population from the high and low NO_3-N treatments were observed as having cataracts, indicating that the cause was independent of NO_3-N treatment.

3.3. Maturation

Good et al. (2016) provided detailed results on maturation, reproductive physiology, plasma hormones, and waterborne hormones

Table 2
Bimonthly growth, feeding, and fish performance metrics (mean ± standard error; n = 3) for Atlantic salmon cultured in recirculation aquaculture systems with high (100 mg/L) and low (10 mg/L) nitrate-nitrogen.

	Begin Study		Month 2		Month 4		Month 6		Month 8	
Performance Metrics	High NO_3-N	Low NO_3-N	High NO_3-N	Low NO_3-N	High NO_3-N	Low NO_3-N	High NO_3-N	Low NO_3-N	High NO_3-N	Low NO_3-N
Weight (g)	101 ± 1	102 ± 1	323 ± 2	325 ± 6	654 ± 3	652 ± 5	911 ± 19	927 ± 10	1148 ± 22	1174 ± 8
Length (mm)	212 ± 1	213 ± 1	296 ± 2	297 ± 3	370 ± 1	367 ± 2	417 ± 3	418 ± 2	446 ± 3	446 ± 1
Condition Factor	1.1 ± < 0.1	1.0 ± < 0.1	1.2 ± < 0.1	1.2 ± < 0.1	1.3 ± < 0.1	1.3 ± < 0.1	1.2 ± < 0.1	1.3 ± < 0.1	1.3 ± < 0.1	1.3 ± < 0.1
Density (kg/m^3)	6 ± < 1	7 ± < 1	18 ± < 1	18 ± < 1	36 ± 1	36 ± < 1	49 ± < 1	49 ± 1	60 ± < 1	61 ± < 1
TGC	–	–	2.6 ± < 0.1	2.6 ± < 0.1	2.1 ± < 0.1	2.1 ± 0.1	1.2 ± 0.1	1.3 ± < 0.1	1.0 ± < 0.1	1.1 ± < 0.1
FCR	–	–	0.83 ± 0.02	0.85 ± 0.02	0.97 ± 0.01	1.00 ± 0.02	1.18 ± 0.09	1.10 ± 0.02	1.02 ± 0.04	1.01 ± 0.03
Feeding Rate (% of biomass)	1.0 ± < 0.1	1.0 ± < 0.1	1.6 ± 0.1	1.8 ± < 0.1	0.7 ± < 0.1	0.7 ± < 0.1	0.6 ± < 0.1	0.6 ± < 0.1	0.7 ± < 0.1	0.7 ± < 0.1
Survival (%)	100	100	> 99	> 99	> 99	> 99	> 99	> 99	> 99	> 99

Table 3
Whole blood gas and plasma chemistry results (mean ± standard error; n = 3) at study's end after 8 months of nitrate exposure. Data originally presented in Good et al. (2016).

Parameter	High NO_3-N 100 mg/L	Low NO_3-N 10 mg/L	*P*-value
Sodium (mmol/L)	154 ± 0.62	152 ± 1.01	0.1039
Potassium (mmol/L)	3.10 ± 0.11	3.16 ± 0.11	0.8573
Calcium (mmol/L)	1.67 ± 0.01	1.70 ± 0.02	0.6907
Chloride (mmol/L)[a]	136 ± 0.47	132 ± 0.77	0.0006
Glucose (mg/dL)	88.0 ± 2.07	86.6 ± 1.59	0.3542
Hematocrit (%PCV)	41.0 ± 0.84	41.9 ± 1.36	0.0677
Hemoglobin (g/dL)	13.9 ± 0.29	14.2 ± 0.46	0.0637
pH	7.01 ± 0.02	6.97 ± 0.02	0.2860
pCO_2 (mm Hg)	43.0 ± 1.33	44.0 ± 1.20	0.7087
HCO_3 (mmol/L)[a]	10.9 ± 0.31	10.1 ± 0.33	0.0256
Total CO_2 (mmol/L)	9.46 ± 0.26	9.20 ± 0.35	0.0791
pO_2 (mm Hg)	12.9 ± 1.01	10.5 ± 1.01	0.0967
O_2 saturation (%)	8.47 ± 1.10	5.94 ± 0.93	0.0786
Lactate (mmol/L)	3.30 ± 0.22	3.41 ± 0.24	0.0897

[a] Indicates significant difference between treatments.

Table 4
Fin quality scores (mean ± standard error; n = 3) assessed qualitatively on a 3-point scale (low to no damage = 1, moderate = 2, severe = 3) for Atlantic salmon exposed to high and low NO_3-N treatments at the beginning and end of the study.

	Begin Study		End Study	
Fins	High NO_3-N 100 mg/L	Low NO_3-N 10 mg/L	High NO_3-N 100 mg/L	Low NO_3-N 10 mg/L
Anal	1.0 ± < 0.01	1.0 ± 0.01	1.2 ± 0.03	1.1 ± 0.03
Caudal	1.1 ± 0.02	1.1 ± 0.03	1.5 ± 0.06	1.5 ± 0.05
Dorsal	1.4 ± 0.02	1.3 ± 0.02	1.7 ± 0.02	1.7 ± 0.02
Left Pelvic	1.1 ± 0.02	1.1 ± 0.03	1.5 ± 0.05	1.5 ± 0.05
Right Pelvic	1.0 ± 0.02	1.1 ± 0.03	1.2 ± 0.03	1.2 ± 0.02
Left Pectoral	1.2 ± 0.06	1.1 ± 0.02	1.6 ± 0.06	1.6 ± 0.04
Right Pectoral	1.1 ± 0.03	1.1 ± 0.03	1.5 ± 0.02	1.5 ± 0.03

assessed during the same study. In brief, sexually mature males were highly prevalent in both NO_3-N treatment groups by study's end, but early maturation did not correlate with NO_3-N concentration.

3.4. Water quality

All water quality variables that could be adjusted manually including alkalinity, conductivity, dissolved oxygen, pH, and water temperature were effectively controlled ($P > 0.05$) between treatments (Table 5). Biochemical oxygen demand, water hardness, oxidative reduction potential, total ammonia nitrogen, and total suspended solids levels were also similar between treatments ($P > 0.05$; Table 5). Nitrate-nitrogen (Fig. 3), nitrite-nitrogen, sulfate, carbon dioxide, true color, and UV transmittance were affected by treatment ($P < 0.05$; Table 5). Differences in nitrate-nitrogen and sulfate were expected due to the experimental design. Nitrite-nitrogen and carbon dioxide levels were greater in high NO_3-N RAS, and true color and ultraviolet transmittance were lower ($P < 0.05$); albeit, these differences were relatively small in magnitude.

Of the 27 dissolved metals/trace elements analyzed in the RAS water, 14 were less than the method detection limit (MDL) for both treatments, including: aluminum, arsenic, beryllium, cadmium,

Table 5
Water quality concentrations (mean ± standard error; n = 3) measured in RAS culture tanks for high and low NO_3-N treatments. Units are in mg/L, unless otherwise noted.

	High NO_3-N 100 mg/L	Low NO_3-N 10 mg/L
Alkalinity	239 ± 2	242 ± 2
Carbon Dioxide[a]	4.5 ± 0.3	3.4 ± 0.2
$cBOD_5$	0.76 ± 0.03	0.77 ± 0.04
Conductivity (μS)	1322 ± 8	1320 ± 10
Dissolved Oxygen	9.54 ± 0.08	9.58 ± 0.01
Nitrite Nitrogen[a]	0.013 ± 0.002	0.005 ± 0.001
Hardness	289 ± 7	306 ± 3
Nitrate Nitrogen[a]	94.2 ± 0.7	10.0 ± 0.3
Oxidative Reduction Potential (mV)	296 ± 10	281 ± 5
pH	8.06 ± 0.01	8.10 ± 0.01
Temperature (°C)	14.3 ± 0.01	14.3 ± 0.02
Total Ammonia Nitrogen	0.094 ± 0.002	0.089 ± 0.002
Total Suspended Solids	1.23 ± 0.04	1.18 ± 0.07
True Color (Pt-Co Units)[a]	4.2 ± 0.1	4.7 ± 0.1
Sulfate[a]	23.2 ± 0.1	278 ± 3
UV Transmittance (%)[a]	92.3 ± 0.2	95.9 ± 0.1

Nitrate nitrogen was maintained at 99 ± 1 for the high NO_3-N treatment over the last 7 months of the study.
[a] Indicates significant difference between treatments.

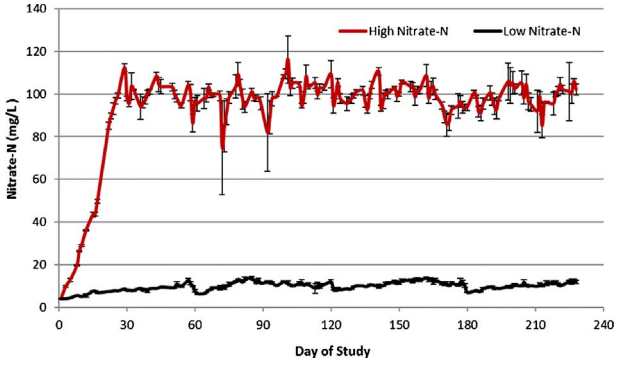

Fig. 3. Nitrate-nitrogen concentrations (mean ± standard error; n = 3) measured over the study duration for high and low NO_3-N treatments.

Table 6
Mean dissolved metals/trace element concentrations (mean ± standard error; n = 3) measured in RAS culture tanks for high and low NO_3-N treatments.

Parameter (mg/L)	High NO_3-N	Low NO_3-N	MDL
Barium	0.17 ± 0.01	0.21 ± 0.03	0.002
Boron[a]	0.079 ± 0.002	0.049 ± 0.003	0.035
Calcium[a]	105 ± 1	111 ± 0.3	0.050
Chloride	17.3 ± 0.3	15.3 ± 0.2	0.100
Iron	0.017 ± 0.003	0.031 ± 0.010	0.010
Magnesium	12.8 ± 0.1	12.4 ± 0.1	0.050
Phosphorous	0.13 ± 0.01	0.16 ± 0.01	0.010
Potassium[a]	12.0 ± 0.2	4.1 ± 0.1	0.050
Silicon	5.00 ± 0.007	5.19 ± 0.002	0.020
Sodium[a]	141 ± 1	161 ± 1	1.000
Strontium	0.95 ± 0.01	1.02 ± 0.03	0.001
Sulfur[a]	8.36 ± 0.03	115 ± 1	0.080
Zinc	0.030 ± 0.003	0.033 ± 0.001	0.003

MDL = Method Detection Limit.
[a] Indicates significant difference between treatments.

chromium, cobalt, copper, lead, manganese, molybdenum, nickel, selenium, titanium, and vanadium. Metals/trace elements consistently measured at > MDL for both treatments included: barium, calcium, chloride, magnesium, phosphorous (total), potassium, silicon, sodium, strontium, sulfur, and zinc (Table 6). Boron and potassium were significantly greater in the high NO_3-N RAS, and calcium, sodium, and sulfur were greater in the low NO_3-N RAS ($P < 0.05$).

4. Discussion

4.1. Fish health and performance

All Atlantic salmon performance metrics assessed during this trial, including weight, length, condition factor, thermal growth coefficient, feed conversion ratio, and survival, were unaffected by NO_3-N concentration. Likewise, assessment of a comprehensive set of health variables, including blood chemistry, tissue histopathology, fin quality, and physical observations, generally indicated normal Atlantic salmon health.

One of the few negative health effects observed during the 8-month trial was a mild *Saprolegnia* spp. infection, which affected fish in all RAS regardless of treatment. The prevalence of saprolegniasis was consistently higher in low NO_3-N RAS from Months 2–4 of the study; albeit, this minor health issue did not seem to be related to treatment and was effectively ameliorated by a 3-day salt treatment. Saprolegniasis is relatively common in traditionally-farmed Atlantic salmon at various life stages (fry – Bruno and Stamps, 1987; eggs – Thoen et al., 2011; various developmental stages including adult – Sandoval-Sierra et al., 2014). Saprolegnia spp. incidence in land-based RAS Atlantic salmon production has not been extensively studied. Davidson et al. (2016a) reported that *Saprolegnia* spp. infection was the primary cause of fish health concerns and minor mortality for on-site, land-based Atlantic salmon production. On-site experience indicates that smoltifying Atlantic salmon are particularly susceptible to *Saprolegnia* spp. infection. The salmon used for this study had recently progressed through smoltification prior to relocation to the experimental RAS. It is likely that the combination of recent smoltification and several handling events to move and sample the fish instigated the *Saprolegnia* spp. infection. Nevertheless, the potential interaction of freshwater RAS conditions and the incidence of *Saprolegnia* spp. infection in conjunction with on-grow rearing of Atlantic salmon smolts and post-smolts in RAS requires further investigation.

Cataracts were also observed during the study but in equal proportions within each treatment group; therefore, nitrate-nitrogen concentration did not correlate with cataract prevalence during the present study. Cataracts are a relatively common problem in wild and traditionally-farmed Atlantic salmon, and a variety of biological, nutritional, and environmental causes have been described (Bjerkås et al., 2004). Bjerkås et al. (1996) found that rapid growth rates correlated with susceptibility to cataract development in Atlantic salmon reared in freshwater. Various water quality effects have also been linked to cataract formation such as rapid changes in water temperature (Bjerkås et al., 2001), fluctuating salinity (Bjerkås and Sveier, 2004), and normoxic and hyperoxic dissolved oxygen conditions (Waagbø et al., 2008). Nitrate has not been implicated as a causative agent of cataracts in Atlantic salmon. Research focused on identification of contributing factors to cataract formation in RAS-produced Atlantic salmon is limited.

Further, unusual swimming behaviors, such as “side-swimming” and rapid swimming velocity were not observed during this study. Davidson et al. (2011) reported increased rainbow trout swimming speeds and a significantly greater prevalence of “side-swimming” in trout exposed to NO_3-N levels of approximately 80–100 mg/L, but these effects were not noted during this trial with Atlantic salmon. In addition, the NO_3-N levels evaluated during this study do not appear to be a primary factor driving early male maturation, as described by Good et al. (2016). Overall, the assemblage of biological data from the present study suggests that chronic exposure to NO_3-N concentrations up to 100 mg/L does not negatively impact post-smolt Atlantic salmon health and performance in freshwater RAS.

4.2. Water quality

When conducting fish toxicity trials, all variables should be precisely controlled except for the parameter of interest (Sprague, 1985). The ability to separate the effects and/or interactions of multiple variables becomes particularly complex if several water quality concentrations exist outside of reported thresholds for safe culture. To the authors’ knowledge, all water quality concentrations measured during the present study were within safe limits for general fish culture (Wedemeyer, 1996) and for salmonids, as reviewed by Davidson et al. (2009, 2011). The authors found it impossible, however, to control every water quality variable among treatments, and a few parameters other than nitrate-nitrogen were significantly different, including boron, calcium, carbon dioxide, nitrite-nitrogen, potassium, sodium, sulfate, dissolved sulfur, true color, and ultraviolet transmittance (Tables 5 and 6). In the case of most of these parameters, the magnitude of difference between treatments was relatively low and the concentrations/levels were diluted. High water flushing rates were intentionally established to maintain water quality concentrations other than nitrate at low levels and thereby focus on the effect of nitrate alone. Davidson et al. (2014) also noted significantly different levels of boron, calcium, nitrite-nitrogen, potassium, sodium, sulfate, sulfur, and ultraviolet transmittance related to treatments when evaluating the effects of NO_3-N concentrations on rainbow trout health and performance. The reoccurrence of these differences indicates that sodium nitrate and sodium sulfate dosing influence these water quality parameters. Davidson et al. (2014) also suggested that elevated nitrite-nitrogen was related to back conversion of available nitrate through passive denitrification. A correlation between sodium nitrate dosing and increased nitrite was also reported by Freitag et al. (2015) during a study evaluating the effects of nitrate on Atlantic salmon endocrine function. Greater sulfate and dissolved sulfur levels were expected in the low NO_3-N RAS, based on the use of sodium sulfate to balance conductivity between treatments, which created similar capacities for ion exchange across the gills.

The findings of this study add to the body of knowledge describing Atlantic salmon tolerance to nitrate concentrations, but should be considered within the context of the experimental conditions. First, this research was conducted using hard, highly alkaline freshwater sourced from a karst-geology aquifer. Hard, alkaline groundwater is relatively common in certain parts of the United States (White, 1988); however,

water supplies in some salmon-producing nations, such as Norway, are inherently soft (Kristensen et al., 2009). Sprague (1985) described alkalinity and hardness as important abiotic factors that can influence the toxicity of pollutants in aquatic environments. Baker et al. (2017) recently found that increasing hardness and ionic strength of solution reduced the toxicity of nitrate 2–10-fold for a variety of aquatic organisms, including rainbow trout fry. This may explain the low toxicity thresholds reported by Kincheloe et al. (1979) who concluded that NO_3-N levels as low as 2 mg/L could impair the reproductive success and ultimate survival of juvenile salmonid species in water with hardness < 40 mg/L as $CaCO_3$. In contrast, the water hardness measured during the present trial was approximately 300 mg/L as $CaCO_3$.

Salinity has also been reported as a factor that could influence the toxicity threshold of certain water quality parameters, but to a lesser extent compared to other abiotic factors (Sprague, 1985). Nonetheless, Camargo et al. (2005) reported that freshwater fish appear to be more sensitive to nitrate toxicity compared to marine fish. The present study was conducted using freshwater (0.7 ppt), except for a 3-day period when salinity was increased to 1.5–2.0 ppt as a treatment for fungus. Existing land-based Atlantic salmon production facilities utilize source water with varying salinities, ranging from those that use freshwater throughout the production cycle to others that use full strength seawater for grow-out (Good and Davidson, 2016). Salmon farmers using land-based RAS may opt to use brackish water or full-strength seawater, depending on source-water availability. As such, the chronic toxicity threshold for nitrate could vary compared to that of freshwater production. In addition, other production settings could have inherently different dissolved oxygen, pH, and water temperatures, which have also been described as factors capable of modifying the toxicity of aqueous compounds (Sprague, 1985).

Lastly, this study was designed to isolate the effect of nitrate through chemical dosing, while diluting other water quality constituents. As such, water flushing rates were higher and feed loading rates were lower compared to typical RAS operating conditions that produce 100 mg/L NO_3-N. For perspective, average NO_3-N levels of 100 mg/L have been achieved in the same experimental RAS with a system HRT of 6–7 days and feed loading rates of 4–5 kg feed per m^3 daily makeup water (Davidson et al., 2009, 2011). Under these operating conditions, other water quality concentrations accumulated and were measured at greater concentrations compared to this trial. During the present study, the average system HRT was 1.7 days and the mean feed loading rate was 0.233 ± 0.003 kg feed/m^3 daily makeup water; therefore, the potential interacting effects of other water quality parameters likely went untested at the expense of isolating nitrate as the parameter of concern. Atlantic salmon have been cultured in the same replicate RAS with a mean feed loading rate of 3.2 kg feed/m^3 daily makeup water and an average NO_3-N concentration of 65 mg/L without apparent negative effects to health or performance (Davidson et al., 2016b), but have not been cultured on-site at 100 mg/L NO_3-N under typical RAS operating conditions.

5. Conclusion

This research indicates that post-smolt Atlantic salmon (0.1–1.7 kg) can be humanely cultured in land-based recirculation aquaculture systems with mean nitrate levels up to 100 mg/L NO_3-N under the described conditions. These findings are important for commercial salmon farms already raising or planning to culture post-smolt Atlantic salmon to larger sizes in land-based RAS using freshwater (Bergheim et al., 2009; Martins et al., 2010; Drengstig et al., 2011; Dalsgaard et al., 2013; Summerfelt et al., 2016) and other farms intending to culture Atlantic salmon to market-size in freshwater RAS (Summerfelt and Christianson, 2014). Knowledge of the tolerance of Atlantic salmon to increasingly higher nitrate levels could lead to reduced water use and lower costs associated with water pumping for RAS operations. In turn, effluent volumes may be reduced and more heat retained in system water, possibly resulting in energy budget savings. The requirement for incorporating denitrification technologies into the water recycle loop would also be lessened which reduces capital investment and operating complexity. This research also provides valuable information to engineers designing RAS for land-based salmon production, as nitrate is often the limiting factor for establishment of water recycle and system dilution rates. Additional research is needed to evaluate the effect of higher nitrate-nitrogen levels, e.g. 100–200 mg/L NO_3-N or greater, on Atlantic salmon health, performance, and welfare using similar conditions. Further investigation should also consider the effect of different water types (e.g. soft water, brackish, or full-strength seawater) on nitrate toxicity to Atlantic salmon to gain a better understanding of the effect of nitrate among various culture conditions.

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Study V

Evaluating the effects of prolonged peracetic acid dosing on water quality and rainbow trout Oncorhynchus mykiss performance in recirculation aquaculture systems

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Evaluating the effects of prolonged peracetic acid dosing on water quality and rainbow trout *Oncorhynchus mykiss* performance in recirculation aquaculture systems

John Davidson[a], Steven Summerfelt[a,1], David L. Straus[b], Kevin K. Schrader[c], Christopher Good[a,*]

[a] The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV, 25443, USA
[b] USDA, Agriculture Research Service, Harry K. Dupree - Stuttgart National Aquaculture Research Center, Stuttgart, AR, 72160, USA
[c] USDA, Agricultural Research Service, Natural Products Utilization Research Unit, University, MS, 38677, USA

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ABSTRACT

Peracetic acid (PAA) is an effective disinfectant/sanitizer for certain industrial applications. PAA has been described as a powerful oxidant capable of producing water quality benefits comparable to those expected with ozone application; however, the water oxidizing capacity of PAA in aquaculture systems and its effects on fish production require further investigation, particularly within recirculation aquaculture systems (RAS). To this end, a trial was conducted using six replicated RAS; three operated with semi-continuous PAA dosing and three without PAA addition, while culturing rainbow trout *Oncorhynchus mykiss*. Three target PAA doses (0.05, 0.10, and 0.30 mg/L) were evaluated at approximately monthly intervals. A water recycle rate > 99% was maintained and system hydraulic retention time averaged 2.7 days. Rainbow trout performance metrics including growth, survival, and feed conversion ratio were not affected by PAA dosing. Water quality was unaffected by PAA for most tested parameters. Oxidative reduction potential increased directly with PAA dose and was greater ($P < 0.05$) in RAS where PAA was added, indicating the potential for ORP to monitor PAA residuals. True color was lower ($P < 0.05$) in RAS with target PAA concentrations of 0.10 and 0.30 mg/L. Off-flavor (geosmin and 2-methylisoborneol) levels in culture water, biofilm, and trout fillets were not affected by PAA dosing under the conditions of this study. Overall, semi-continuous PAA dosing from 0.05-0.30 mg/L was compatible with rainbow trout performance and RAS operation, but did not create water quality improvements like those expected when applying low-dose ozone.

1. Introduction

Peracetic acid (PAA) is an antimicrobial agent that is approved for use as a surface disinfectant or sanitizer for various industrial applications including food and beverage operations, organic livestock and crop production, and medical facilities (USEPA, 1993; Warburton, 2014; United States Food and Drug Administration (USFDA, 2015; United States Department of Agriculture (USDA, 2016). In recent years, PAA has also been used to prevent biofilm formation in the paper/pulp industries and as a disinfectant for wastewater treatment (Kitis, 2004). PAA is sold commercially as an equilibrium mixture of acetic acid, hydrogen peroxide, and water, with percent inclusion of ingredients varying among manufacturers. Recently, PAA has emerged as a promising water sanitizer or disinfectant for use in aquaculture, in part, due to its environmentally friendly attributes. When applied at relatively low concentrations, PAA degrades rapidly in aquaculture systems (Pedersen et al., 2009, 2013; Liu et al., 2014) and doesn't form toxic byproducts that could harm fish or create pollution discharge. Only benign compounds are formed during degradation including acetic acid, oxygen, and water (Wagner et al., 2002; Pfuntner, 2011). At present, PAA is approved in Europe for use in veterinary medicine (Lehmann, 1974) and as a water sanitizer for aquaculture systems (Schäperclaus, 1991); therefore, it can be legally used to prevent and control disease outbreaks in fish production systems in the EU. Research carried out over the past decade indicates that PAA can control a variety of fish pathogens including *Icthyophthirius multifilliis* ("Ich") (Meinelt et al., 2007a, b; Meinelt et al., 2009; Straus and Meinelt, 2009; Sudová et al., 2010; Picón-Camacho et al., 2012), *Saprolegni*a spp. (Marchand et al., 2012; Straus et al., 2012; Good et al., 2017a), *Flavobacterium columnare* – causative agent of columnaris (Marchand et al.,

* Corresponding author.
E-mail address: cgood@conservationfund.org (C. Good).
[1] Current work address: Superior Fresh, W15506 Superior Fresh Drive, Hixton, WI54635.

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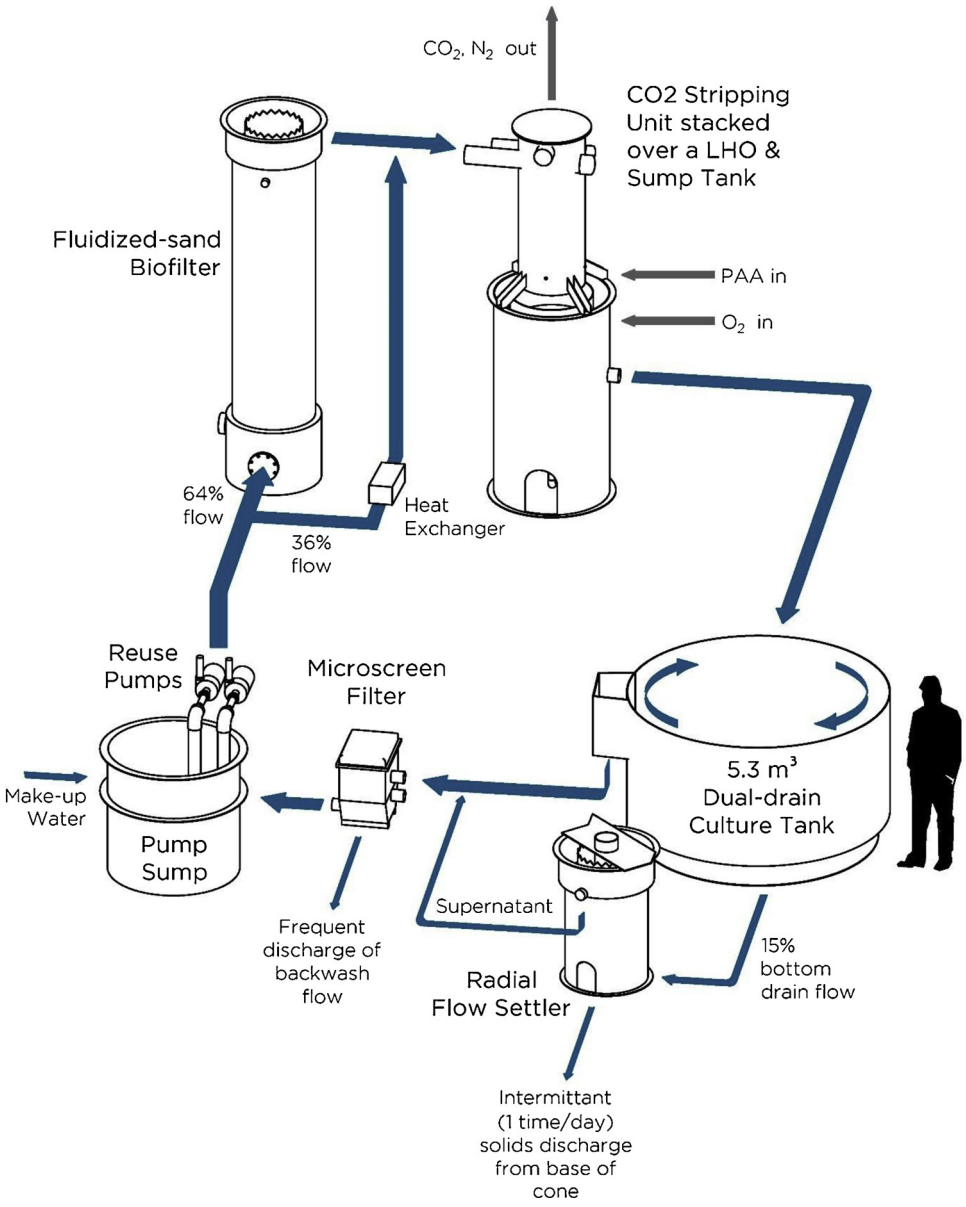

Fig. 1. Water flow, process design, and point of PAA application for an individual experimental reuse system (9.5 m^3) used during the study (courtesy Kata Sharrer, TCFFI Engineering Services).

2012), *Ichthyobodo necatar* (Farmer et al., 2013; Jaafar et al., 2013), *Aeromonas salmonicida* – etiological agent of furunculosis and *Yersinia ruckeri* – causative agent of enteric redmouth disease (Meinelt et al., 2015), and marine microalgae *Tetraselmis chuii* (Liu et al., 2016), among others (Pedersen et al.,2013). However, PAA has not gained approval in the US as a veterinary aid or water sanitizer with fish present. In June 2017, the US Environmental Protection Agency accepted the first registration of a commercial PAA compound (VigorOx SP-15 Antimicrobial Agent, Peroxychem Inc., Philadelphia, PA, USA), which is approved for use in US aquaculture for: (1) sanitizing surfaces of harvesting equipment; and (2) cleaning and disinfecting fish culture tanks when water is drained and fish are absent (Straus et al., 2018).

Most trials demonstrating the effectiveness of PAA for treating or preventing fish disease have been conducted in flow-through systems; however, studies investigating its use in recirculation aquaculture systems (RAS) are limited. Much of the existing research related to the use of PAA in RAS has focused on effects to nitrification and/or the development of standard operating procedures for its application. Pedersen et al. (2009) found that batch addition of PAA to achieve 1 mg/L had minimal effect on nitrification compared to 2 and 3 mg/L PAA, which resulted in significant, prolonged increases of nitrite levels in freshwater RAS culturing rainbow trout *Oncorhynchus mykiss*. A later study determined that increased organic matter content significantly increased PAA decay and noted that biofilm and fish biomass also contributed to its dissipation rate (Pedersen et al., 2013). During another RAS-focused trial, Liu et al. (2017a) evaluated pulse applications of PAA delivered every three to four days over a 5-wk period to achieve a concentration of 2 mg/L PAA in a tank culturing common carp *Cyprinus carpio*. Fish were initially stressed by this procedure as measured by waterborne cortisol but appeared to adapt to subsequent treatments.

There is still much to learn about the use of PAA in RAS and its potential benefits. In particular, a greater understanding of its ability to improve RAS water quality is lacking. The oxidizing capacity of PAA in RAS is of interest because PAA has been described as having properties similar to ozone (Pedersen et al., 2015), a powerful oxidant that is frequently used for RAS water treatment. Ozone improves the culture environment by enhancing water clarity, microflocculating fine solids, adding oxygen, and reducing dissolved metals, nitrite, and organic

concentrations (Summerfelt and Hochheimer, 1997; Davidson et al., 2011; Gonclaves and Gagnon, 2011; Powell and Scolding, 2018). Antimicrobial effects can also be achieved when high ozone doses are applied to RAS water followed by ultraviolet (UV) irradiation (Liltved, 2002; Summerfelt, 2003; Sharrer and Summerfelt, 2007; Summerfelt et al., 2009), and improved growth performance, health, and survival of cultured species have been attributed to the enhanced culture environment created by ozone (Davidson et al., 2011; Good et al., 2011; Powell and Scolding, 2018). However, ozone is relatively expensive and complex to use (Summerfelt and Hochheimer, 1997; Gonclaves and Gagnon, 2011) and can present safety hazards for fish and human health (Gearhart and Summerfelt, 2007); therefore, an alternate water treatment method would be welcomed by the RAS industry.

In addition, research evaluating the effect of PAA on the common off-flavor compounds geosmin and 2-methylisoborneol (MIB) in RAS has not been reported in peer-reviewed literature. The occurrence of off-flavors in RAS-produced finfish products continues to be detrimental to this industry sector (Schrader et al., 2013), as off-flavor can contribute to consumer dissatisfaction, result in a negative perception of aquaculture products, and inhibit future purchasing (Tucker, 2000). Yet, a proven method that immediately mitigates off-flavor in the primary fish production system (grow-out) has not been developed. Most RAS operations employ specific depuration/ "purging" protocols to remove off-flavors from fillets (Davidson et al., 2014a; Lindholm-Lehto and Vielma, 2018); however, this production step results in added capital, operating, and labor costs for the farmer. Given the reported oxidizing capacity and antimicrobial effectiveness of PAA, investigation into its effects on off-flavor-producing bacteria and common off-flavors in RAS is warranted and could provide insight towards a solution to this ongoing problem.

To this end, a study was developed to provide a comprehensive evaluation of the effects of semi-continuously dosed PAA on water quality, rainbow trout performance, and off-flavor compounds in replicate RAS. This work was designed to gain a better understanding of the full benefit of PAA dosing in RAS with intention to add to the body of knowledge on PAA use in aquaculture, inform regulatory decision making related to its use in culture systems with fish present, and to contribute to the development of standard operating procedures for its use in RAS.

2. Methods

2.1. Experimental design

Six replicate RAS, described by Davidson et al. (2009; Fig. 1), were used for the study. Three RAS were semi-continuously dosed with PAA, and the other three RAS did not receive PAA and served as controls. Three target PAA doses (0.05, 0.10, and 0.30 mg/L) were evaluated separately at approximately monthly intervals/dosing periods. The PAA solution used for the trial, VigorOx® SP-15 (PeroxyChem Inc., Philadelphia, PA, USA), consisted of 15–17 % peracetic acid, 9–11 % hydrogen peroxide, 33–38 % acetic acid, and 31–44 % water (PeroxyChem Inc., 2016). Rainbow trout (Troutlodge Inc., Bonney Lake, WA, USA) was used as the test-species.

2.2. Recirculation aquaculture systems

Each 9.5 m^3 RAS recirculated 329 ± 2 L/min (˜87 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). Continuous makeup water flow (2.46 ± 0.04 L/min) originating from a freshwater spring source was added to each pump sump to maintain mean nitrate-nitrogen levels at < 75 mg/L, per recommendation by Davidson et al. (2014b) for onsite rainbow trout production. Makeup water flow rates were calibrated four to five times weekly via bucket testing, and cumulative makeup water addition was measured with digital flow meters installed upstream of float valves which delivered spring water to the systems. The water recycle rate was > 99% on a flow basis, system hydraulic retention time (HRT) averaged 2.7 days (37% of system volume exchanged daily), and mean feed loading rate was 1.38 ± 0.02 kg feed/m^3 of daily makeup water. Tank HRT was approximately 15 min. Sodium bicarbonate (Church & Dwight Co. Inc., Ewing, NJ, USA) was added to each RAS as needed to maintain mean alkalinity levels from 100 to 200 mg/L. Sodium chloride (Diamond Crystal Naturals Solar Salt, Cargill Inc., Minneapolis, MN, USA) was added to each RAS for most of the study to maintain 2–3 ppt salinity as a prophylactic measure against Ich, which was diagnosed, treated, and eliminated via chlorine disinfection at the conclusion of the research trial preceding the present study.

2.3. Peracetic acid dosing

A 208-L (55-gal) drum of VigorOx SP-15 PAA solution was purchased and placed on top of a spill containment pallet. A cooling jacket (Powerblanket, Salt Lake City, UT, USA) receiving a continuous flow of cool (13–14 ° C) spring water was placed around the drum to maintain constant temperature and limit decomposition of the contained solution. A length of stainless-steel conduit was connected and extended through the PAA drum adapter plug to approximately 5 cm from the bottom of the container. Opaque, acid-compatible tubing (Masterflex C-flex L/S #14, Cole Parmer, Vernon Hills, IL, USA) was routed through the conduit to the bottom of the drum and through three pump heads (Item EW-07014-21, Cole Parmer) connected to separate Masterflex L/S peristaltic pumps (Model 07528-10, Cole Parmer) which supplied a semi-continuous dose of PAA to each treatment system. Semi-continuous dosing was achieved by using an on-off pumping cycle (0.5 min on/ 4.5 min off), which was established by integrating a PLC relay (Model SG2-20HR-A, TECO, Taipei, Taiwan) with the peristaltic pump controls. A slow drip of PAA entered the systems at the head space of the LHO distribution plate (Fig. 1).

PAA dosing rate was calculated as follows:

$$\frac{\text{Water Recycle Flow (L/day)} \times \text{Target PAA Concentration (mg/L)}}{10^6 \times 0.15\ (\text{Percent PAA VigorOx SP} - 15)\ \text{day}} = \frac{\text{VigorOx PAA (kg)}}{\text{day}}$$

Daily PAA required (kg/day) was converted to mL/min to establish the dosing rate necessary to achieve the specified target concentration. Dosing rate was validated by collecting drip samples in a graduated cylinder during a stopwatch-timed interval. A room-air monitoring system (Model F12/D Analytical Technology, Inc., Collegeville, PA, USA) was situated nearby the PAA dosing system and wired to trigger an alarm if an unexpected PAA leak caused unsafe off-gas concentrations. This safety measure was adopted due to the American Conference of Governmental and Industrial Hygienists (ACGIH) 2014 establishment of a Short-Term Exposure Limit for airborne PAA of 0.4 ppm (PeroxyChem, Inc., 2016). Three target PAA doses (0.05, 0.10, and 0.30 mg/L) were evaluated separately at approximately monthly intervals/dosing periods. Following the 0.10 mg/L trial, rigorous data collection was temporarily delayed to troubleshoot an unexpected turbidity problem that occurred in RAS from both treatments. During this period, PAA dosing was maintained at a rate that targeted PAA concentrations of 0.10-0.15 mg/L.

2.4. Rainbow trout

Rainbow trout were received as fertilized eyed-eggs, hatched onsite, and cultured in flow-through and partial reuse systems prior to the study. Trout were cultured in the experimental RAS for two and a half

months prior to dosing PAA. To begin the study, each RAS contained approximately 370 rainbow trout (407 ± 6 g), which resulted in an average biomass density of 28.8 ± 0.5 kg/m^3 among replicate RAS. After the 0.10 mg/L PAA dosing period, fish numbers were reduced by approximately 140 fish per RAS to maintain fish densities within acceptable welfare limits defined by the onsite Institutional Animal Care and Use Committee.

2.5. Fish performance sampling

Mortalities were removed and recorded daily to assess cumulative survival. Fish sample size (n) calculated using equations in Bhujel (2008) yielded an impractical number of fish due to the expanded standard deviation expected for trout approaching 2 kg.

n = [(Z * standard deviation)/ accepted error $_g$)]^2; where Z = 1.65 and accepted error = 20 g

Therefore, a correction factor calculation (Martin et al., 1987) was applied to normalize sample size relative to tank population (N).

n* = 1/[(1/n) + (1/N)]

Length and weight measurements of a random sample of 100–110 trout (minimum 27% of the population) from each RAS were collected to begin the study as a baseline, prior to dosing PAA and at the end of each PAA dosing period. Thermal growth coefficient (TGC), condition factor (CF), feed conversion ratio (FCR), and fish survival (%) were calculated using the following formulae:

TGC = ((End Weight ^ (1/3) – Start Weight ^ (1/3))/ ((Days Between * Avg. Temp.) x 1000)

where weight is in grams, length is in mm, and temperature is in º C.

CF = 100,000 * Weight / (Length) ^{3}

FCR = Cumulative Feed Delivered / Fish Biomass Gain

Survival (%) = ((Number Fish to Begin – Cumulative Morts + Culls)/ Number Fish to Begin) *100

2.6. Feeding methods

A constant 24-h photoperiod was provided to facilitate “around-the-clock” feeding and consistent water quality. Rainbow trout in each RAS were fed at the same rate for the first week of the study; thereafter, feeding rates were fine-tuned separately per RAS based on observations of feeding activity and wasted feed. Fish were fed to apparent satiation using a computer operated feeding system (The Conservation Fund Freshwater Institute, Shepherdstown, WV, USA), programmed to deliver short feed bursts once an hour via automated feeders (T-drum 2000CE, Arvotec, Huutokoski, Finland). Feeding rates were reduced accordingly when fish were culled following the 0.10 mg/L PAA dosing trial. A commercially-available 42/16 (protein/fat) trout diet (Zeigler Brothers Inc., Gardners, PA, USA) was fed throughout the study.

2.7. Water quality sampling and analyses

Water samples were collected from the side drain of each RAS, and most parameters were tested onsite according to methods described in APHA (2012) and HACH (2003; 2015) (Table 1). An array of 25 dissolved metals/elements were analyzed by REI Consultants Inc. (Beaver, WV, USA) (Table 1). The effective concentration of PAA stock solution was validated using HACH Company’s application note titled “Determination of Peracetic Acid and Hydrogen Peroxide in Water: Concentration Range of 0.01 to 35% (Titration)”, using a sample volume of 0.2 mL. The PAA concentration of inlet and side drain tank water was determined using HACH Company’s Application Note titled “Determination of Peracetic Acid and Hydrogen Peroxide in Water: Concentration Range of 0.1 to 10 mg/L”, using only the procedure for PAA. Test samples from the ‘Concentration Range of 0.1 to 10 mg/L’ method were analyzed using a DR6000 spectrophotometer (Hach Company, Loveland, CO, USA).

2.8. Off-flavor sampling and analysis

Water, biofilm, and fillet samples were collected at the beginning of the study prior to PAA dosing and near the end of each PAA dosing period for analysis of off-flavor compounds, geosmin and MIB. Glass scintillation vials (20 mL) with foil-lined caps were used for collection of water and biofilm samples. Water samples were collected at the side drain of each RAS by submerging the vials and capping underwater for a complete fill void of air bubbles. Biofilm samples were scraped from the sidewall of culture tanks near the inlet after draining the tank volume by several inches. A small amount of tank water was used to rinse biofilm into each vial. Methods for determination of geosmin and MIB in water and biofilm samples followed Shrader et al. (2013). Specifically, gas chromatograph sample vials were heated at 40° C for 20 min before volatile compounds were absorbed onto a 100-µm polydimethyl siloxane (SPME) fiber (Supelco, Bellfonte, PA, USA). The fiber assembly was shaken for 10 min during the absorption period and desorbed for 2 min at 250° C in the injection port of an Agilent 7890B gas chromatograph-mass spectrometer (GC—MS) (Agilent Technologies, Santa Clara, CA, USA) with a 5977B mass selective detector operated in selected ion monitoring mode. The conditions of the gas chromatograph were as follows: (1) initial oven temperature was 60° C for 0.5 min; (2) then ramp rate of 30° C/min to 100° C; (3) then ramp rate of 20° C/min to 300° C with an isotherm time of 2 min; and (4) the maintenance of flow pressure was at 18 lb/in^2 with helium used as a carrier gas. The molecular ion base peaks were monitored at *m/z* 168, 95, and 135 for MIB and at *m/z* 182, 112, and 126 for geosmin. A DB-5 capillary column (5%-phenyl-methylsiloxane, 30 m, 0.25 mm inside diameter, 0.25-µm film thickness; J&W Scientific, Folsom, CA, USA) was used. The retention time for geosmin was 6.6 min and, for MIB, 5.1 min. Standards for MIB and geosmin were prepared in deionized water at 0.1, 0.5, 1.0, and 2.5 µg/L. The original standards were obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA) and were included at the beginning, middle, and end of each group of samples analyzed using a CombiPal autosampler (LEAP Technologies, Inc., Carrboro, NC, USA).

In addition, three rainbow trout were randomly collected from each RAS near the end of each PAA dosing interval. Trout were humanely euthanized via percussive stunning and filleted. Skinless, right-side fillet portions were packaged in zip-lock freezer bags and frozen prior to shipment for analysis. One 20-g portion from the anterior of each fillet was used to obtain distillate following standard microwave distillation procedures and methods outlined by Lloyd and Grimm (1999). Each distillate sample was analyzed using SPME GC–MS.

2.9. Statistical analysis

Water quality data were analyzed using a mixed models approach that modeled water quality criterion as dependent variables; treatment, time, and treatment x time as independent fixed factors; and RAS/tank as a random effect nested within treatment (Ling and Cotter, 2003; Thorarensen et al., 2015). Analysis of covariance (ANCOVA) with feed loading rate (daily makeup water (m^3)/daily feed (kg)) modeled as a covariate was used to analyze dissolved metals and nutrient concentration data from each PAA dosing trial. Mean off-flavor concentrations and fish performance metrics were analyzed using a Student’s t-test. Each data set was analyzed for normality using a Shapiro-Wilk test. Non-gaussian distributed data sets were analyzed using non-parametric statistics, including the Kruskal Wallis test. A probability level of 0.05 was used to determine significance. Separate analyses were carried out for water quality, performance metrics, and off-flavor for each PAA dosing trial where these data were available. All statistical

Table 1
Water quality parameters evaluated, methodologies, and frequency of testing.

Parameter	Method of Analysis	Frequency of Recording/Testing
Dissolved Oxygen	Hach SC100 Controller & LDO® Probe	Daily
Oxidative Reduction Potential	Hach SC100 Controller & Differential ORP Sensor	Daily
Temperature	Hach SC100 Controller & Differential ORP Sensor	Daily
Salinity	YSI 30 Salinity/Conductivity/Temperature Meter	4-5 times weekly
Specific Conductance	YSI 30 Salinity/Conductivity/Temperature Meter	4-5 times weekly
Alkalinity	Hach Method 8203 - Sulfuric Acid Digital Titration pH endpoint. Accumet #AB150	3 times weekly
pH	Standard Methods 4500-H^+ B – Electrode	3 times weekly
Carbon Dioxide	Hach Method 8223 - Sodium Hydroxide Buret Titration pH endpoint. Accumet #AB150	Once weekly
Biochemical Oxygen Demand	Standard Methods APHA 5210B - 5-day test (No prefiltration) YSI Model 58, YSI BOD probe #5905	Once weekly
Nitrate Nitrogen	Hach Method 8171 - Cadmium Reduction	Once weekly
Nitrite Nitrogen	Hach Method 8507 USEPA Diazotization	Once weekly
Total Ammonia Nitrogen	Hach Method 8038 USEPA Nessler	Once weekly
Total Coliform Bacteria	Hach Method 10,029 - Membrane Filtration, Fisher Isotemp Incubator #516D	Once weekly
Total Heterotrophic Bacteria	Hach Method 8242 - Membrane Filtration, Fischer Isotemp Incubator #516D	Once weekly
Total Phosphorous	Hach Method 8190 – USEPA PhosVer3 with Acid Persulfate Digestion. DRB200 reactor	Once weekly
Total Suspended Solids	Standard Methods APHA 2540D – 1.5 µm filter papers dried at 103-105 ° C. Thelco Oven #6540, Mettler Toledo #AE240 and #PM30K	Once weekly
True Color	Hach Method 8025 - Platinum-Cobalt Standard	Once weekly
Ultraviolet Transmittance	Hach Method 10,054 - Organic UV Absorbing (UV-254)	Once weekly
Dissolved Metals	Inductively Coupled Plasma Atomic Emission Spectrometry	3 events (1 for each PAA dosing rate)

Spectrophotometers DR2700 and DR6000 (Hach Company, Loveland, CO, USA) were used for analysis of nitrate nitrogen, nitrite nitrogen, total ammonia nitrogen, and total phosphorous. Spectrophotometer DR4000 (Hach Company) was used for analysis of true color and UV transmittance.

analyses were carried out using SYSTAT 13 software (2009; San Jose, CA, USA).

3. Results and discussion

3.1. Water quality

3.1.1. Alkalinity

Alkalinity was the only water quality parameter found to be significantly different between treatments during the 0.05 mg/L trial ($P < 0.05$). Mean alkalinity in the PAA-treated and control RAS was 154 ± 1 and 144 ± 2 mg/L, respectively, during this dosing period. Sodium bicarbonate ($NaHCO_3$) was added as needed to maintain alkalinity between 100–200 mg/L; however, $NaHCO_3$ addition was similar between treatments during the 0.05 mg/L trial, i.e., 0.102 ± 0.007 and 0.107 ± 0.003 $NaHCO_3$/ kg feed for the PAA-treated and control RAS, respectively ($P > 0.05$). Although PAA appears to have mildly influenced alkalinity during the 0.05 mg/L trial, a discrepancy of 10 mg/L is not relevant for fish health or biofilter performance (Summerfelt et al., 2015; Boyd et al., 2016). Statistical differences in alkalinity levels were not detected between treatments when evaluating target PAA concentrations of 0.10 and 0.30 mg/L (Table 2).

3.1.2. Oxidative reduction potential

During the 0.05 mg/L trial, mean ORP in the PAA-treated RAS was 248 ± 7 mV compared to 212 ± 13 mV in the control RAS. While a statistical difference in ORP was not identified between treatments during this dosing period, a trend towards significance was evident ($P = 0.072$). When comparing ORP between treatments with time, a highly significant effect was found ($P < 0.001$). There may have been a break-through period of several weeks before PAA residuals resulting from the 0.05 mg/L dose began to fully influence ORP (Fig. 2); thereafter, ORP gradually increased in PAA-treated RAS over the remainder of the dosing period (Fig. 2). The trend for PAA to cause an increase in ORP continued with increasing target concentrations (Table 2; Fig. 2). For example, ORP measured in PAA-treated and control RAS during the 0.10 mg/L trial was 268 ± 12 and 203 ± 8 mV ($P < 0.05$), respectively. Similarly, during the 0.30 mg/L trial, ORP reached 290 ± 2 mV in PAA-treated RAS, while levels in the control RAS were 232 ± 11 mV ($P < 0.05$; Table 2; Fig. 2). This ORP response is like that which is typically observed when applying ozone in RAS (Summerfelt and Hochheimer, 1997; Davidson et al., 2011). During the present study, increasing ORP corresponded with increasing target PAA concentrations, indicating the potential for continuously monitored ORP to track PAA residual concentrations. As such, ORP could be used to monitor and/or control PAA residuals through an integrated on/off control loop with the PAA dosing system, much like the proportional-integral-derivative control strategy used to manage ozone residuals in RAS.

3.1.3. Total suspended solids and bacteria

Peracetic acid did not reduce total suspended solids (TSS) levels in the culture water during any dosing period (Table 2). This result is opposite to the TSS reductions that are expected when applying ozone in fish production systems (Rueter and Johnson, 1995; Summerfelt et al., 1997; Davidson et al., 2011). However, it is important to note that TSS levels measured during the present study were substantially greater and more variable compared to concentrations measured during other onsite trials in the same replicate RAS (Davidson et al., 2011, 2014b). The authors hypothesize that increased TSS levels resulted due to periodic bacterial blooms of an organism identified late in the study as *Flectobacillus roseus* (Larkin et al., 1977; Sheu et al., 2009; Adikesavalu et al., 2015). This bacterium was found to be non-pathogenic to rainbow trout (results presently unpublished) but was seemingly present in large enough numbers to create periodic increases in visual turbidity of the culture water of both treatments. Larkin et al. (1977) reported *Flectobacillus* cell diameters ranging from 0.6 to 1.0 µm and lengths of 1.5–5.0 µm; therefore, at least some of these bacteria would have been captured on the surface of the standard 1.5 µm filter papers used for in-house TSS analysis.

The presence of *F. roseus* created an additional opportunity to evaluate the sanitizing effect of semi-continuous PAA dosing from 0.05-0.30 mg/L. Based on periodic observance of turbid conditions and random spikes in TSS associated with *F. roseus* for both treatments, we conclude that semi-continuous PAA dosing from 0.05-0.30 mg/L did not act as a sanitizer for *F. roseus*. In addition, general heterotrophic bacteria and total coliform counts were not significantly reduced at the tested PAA doses (Table 2). Kitis (2004) reported that a disadvantage of PAA as a disinfectant in the wastewater industry is increased organic content in the effluent caused by the acetic acid component, and the associated potential for microbial regrowth. Semi-continuous dosing was employed during this study as a strategy to limit wide water quality fluctuations in favor of constant conditions; however, this strategy may

Table 2
Water quality concentrations (mean ± standard error) measured in PAA-treated and control RAS (n = 3).

	0.05 mg/L PAA		0.10 mg/L PAA		0.30 mg/L PAA	
	PAA	Control	PAA	Control	PAA	Control
Alkalinity (mg/L as $CaCO_3$)	154 ± 1 *	144 ± 2 *	159 ± 3	154 ± 7	141 ± 5	127 ± 5
Biochemical Oxygen Demand (mg/L)	6.8 ± 2.3	6.8 ± 1.7	10.6 ± 4.0	8.6 ± 3.5	11.7 ± 4.7	11.7 ± 1.7
Carbon Dioxide (mg/L)	9 ± < 1	9 ± < 1	10.7 ± 0.3 *	12.2 ± 0.8 *	14 ± 1	13 ± 1
Dissolved Oxygen (mg/L)	10.2 ± 0.1	9.9 ± 0.1	10.2 ± < 0.1	10.1 ± 0.2	10.2 ± 0.3	10.1 ± < 0.1
pH	7.58 ± 0.02	7.51 ± 0.02	7.54 ± 0.03	7.47 ± 0.06	7.38 ± 0.03	7.39 ± 0.01
Nitrite Nitrogen (mg/L)	0.23 ± 0.04	0.16 ± 0.06	0.19 ± 0.09	0.11 ± 0.03	0.09 ± 0.06	0.05 ± < 0.01
Nitrate Nitrogen (mg/L)	69 ± 3	71 ± 1	64 ± 2	69 ± 3	54 ± 3	64 ± 2
ORP (mV)	248 ± 7	212 ± 13	268 ± 12 *	203 ± 8 *	290 ± 2 *	232 ± 11 *
Salinity (ppt)	2.8 ± 0.1	2.7 ± < 0.1	2.9 ± < 0.1	2.9 ± < 0.1	0.4 ± < 0.1	0.4 ± < 0.1
Specific Conductance (µS)	5.0 x 10^3	5.0 x 10^3	5.2 x 10^3	5.1 x 10^3	3.1 x 10^3	3.1 x 10^3
Total Ammonia Nitrogen (mg/L)	0.58 ± 0.06	0.55 ± 0.02	0.58 ± 0.03	0.64 ± 0.05	0.59 ± 0.03	0.68 ± 0.04
Temperature (° C)	13.7 ± 0.1	13.7 ± 0.1	13.0 ± 0.1	13.0 ± 0.1	13.6 ± 0.1	13.6 ± < 0.1
Total Coliform Bacteria (cfu/100 mL)	2.2 x 10^4	2.9 x 10^4	1.4 x 10^4	7.4 x 10^4	7.4 x 10^3	4.7 x 10^4
Total Heterotrophic Bacteria (cfu/mL)	4.6 x 10^3	3.2 x 10^3	2.5 x 10^3	5.2 x 10^3	4.0 x 10^3	9.6 x 10^2
Total Phosphorous (mg/L)	4.2 ± 0.4	4.1 ± < 0.1	4.0 ± 0.1	4.0 ± < 0.1	3.3 ± 0.3	3.5 ± 0.1
Total Suspended Solids (mg/L)	14.8 ± 9.4	10.9 ± 3.0	16.1 ± 8.0	11.6 ± 4.4	9.3 ± 2.6	7.7 ± 0.7
True Color (Pt Co units)	37 ± 2	40 ± 2	32 ± 2 *	40 ± 2 *	18 ± 1 *	23 ± 2 *
UV Transmittance (%)	69 ± 1	69 ± 1	71 ± 2	69 ± 1	79 ± 1	78 ± 1

* Indicates significant difference between treatments.

have created adaptive conditions for certain microbial populations like that described by Kitis (2004). Likewise, Liu et al. (2017b) reported that continuous application of PAA in flow-through tanks used for rainbow trout culture resulted in excess biofilm formation compared to a pulse application strategy.

3.1.4. True color

Dissolved organic compounds including humic substances originating from soils, sediments, and aquafeeds tend to accumulate in low exchange RAS and likely contribute to the tea-colored water typical of these fish production systems (Christensen et al., 2010; Yamin et al., 2017a). During the 0.10 mg/L PAA trial, true color of the culture water was significantly reduced by PAA dosing ($P = 0.023$). True color in PAA-treated and control RAS was 32 ± 2 and 40 ± 2 Platinum Cobalt (Pt Co) units, respectively, indicating some ability of PAA to oxidize and reduce the dissolved organic compounds responsible for colored water. Water samples analyzed for true color were pre-filtered with 0.45 µm filters to remove solids, which minimized the effect of *F. roseus* on this parameter. True color levels dropped by 50% from approximately 40 to 20 Pt Co units following a reduction of fish numbers and biomass that was carried out after the 0.10 mg/L PAA trial. Due to these changes and the associated reduction in feeding, the concentrations of most water quality constituents, including true color, were reduced.

Overall, these results indicate that PAA, when applied at certain concentrations, has some capacity to oxidize the dissolved organic compounds responsible for tea-colored RAS water. Peracetic acid has been reported to oxidize humic compounds, but at undiluted concentrations used in soil science applications (Schnitzer and Skinner, 1974; Schnitzer and Hindle, 1980). However, the effects of PAA on true color during the present study were not profound, particularly when drawing comparisons to ozone's effect on color. Davidson et al. (2011) demonstrated that application of low-dose ozone (ORP ~ 250 mV) in the same replicate RAS reduced true color by more than 90%, albeit while culturing rainbow trout at greater feed loading rates (3.98 kg feed/m^3 daily makeup water). During another onsite study evaluating the effect of ozone on waterborne hormone levels, ozone reduced true color by 74% from 20 ± 1 to 3.7 ± 0.3 Pt Co units, respectively (Good et al., 2017b), when operating RAS with feed loading rates comparable to the present study. In comparison, true color was reduced by approximately 20 and 22%, respectively, during the 0.10 and 0.30 mg/L PAA trials. Whether or not color reduction and the associated oxidation of dissolved organics and humic substances responsible for colored water is an advantage for Atlantic salmon is unknown. Yamin et al. (2017b) found that common carp exposed to humic substances had lower rates of infection when challenged with *Aeromonas salmonicida*. In addition, several studies have found that humic substances provided a protective

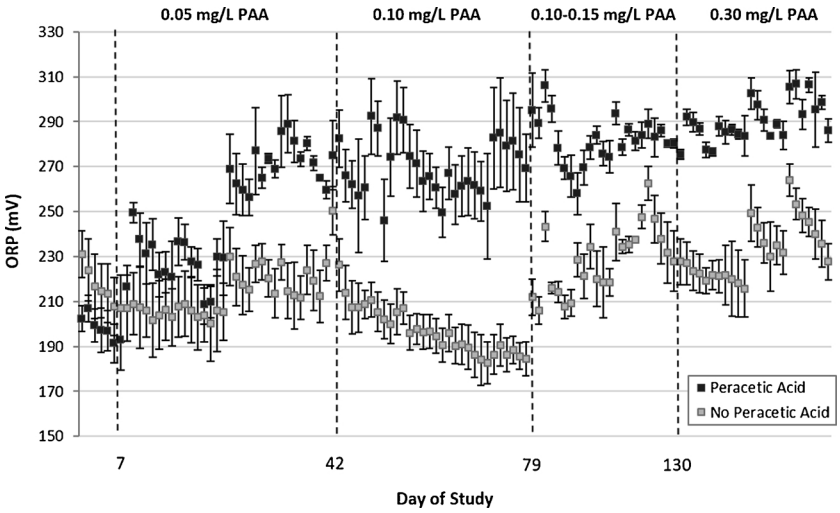

Fig. 2. Oxidative reduction potential (mean ORP (mV) ± standard error) in RAS culture tanks operated with and without peracetic acid dosing over the study duration. Data collection during the 0.10-0.15 mg/L PAA period was limited because of troubleshooting related to culture water turbidity; therefore, time does not reflect exact scale.

effect to the toxicity of dissolved nitrogenous wastes and heavy metals in various fish species (Peuranen et al., 1994; Hammock et al., 2003; Meinelt et al., 2010). On the other hand, clear water void of dissolved organics enhances the ability of fish to see and capture feed, which can result in increased growth (Sigler et al., 1984) and allows the farmer to effectively observe fish (Christensen et al., 2000) health, behavior, and feeding activity. Davidson et al. (2011) reported increased rainbow trout growth in RAS where ozone had significantly reduced color; albeit, other water quality variables were also different between treatments and the growth effect could not be solely attributed to reduced color of the culture water.

3.1.5. Nitrogen

No significant differences in total ammonia nitrogen, nitrite-nitrogen (NO_2-N), or nitrate-nitrogen concentrations were detected during any of the PAA dosing trials (Table 2), indicating that semi-continuous dosing to achieve 0.05-0.30 mg/L PAA did not negatively impact nitrification. A trend for slightly greater mean NO_2-N was evident during all dosing periods, indicating a low-level effect of PAA on nitrification; however, NO_2-N levels remained within safe limits for onsite salmonid production (Davidson et al., 2009). Pedersen et al. (2009) reported that pulse application of 1 mg/L PAA caused minor impacts to nitrification in RAS with submerged biofilters, but PAA levels of 2.0 and 3.0 mg/L resulted in significant and prolonged increases in nitrite. Liu et al. (2017b) also noted that pulse addition of PAA to achieve 1.0 mg/L resulted in partial inhibition of nitrification but found that continuous PAA dosing at 0.2 mg/L did not have a negative impact. The compilation of information regarding the effect of PAA on nitrification suggests that PAA target concentrations from 0 to 1.0 mg/L are compatible with biofilter performance. In considering the effect of PAA dosing on nitrification, the chemical application site is important. During this study, PAA was added at the water distribution chamber of the LHO (Fig. 1) which provided maximum contact time through the water recycle loop for PAA residuals to react and dissipate before reaching the fluidized sand biofilter. A one-time water sampling event during the 0.30 mg/L PAA trial demonstrated that PAA levels dissipated to 0.2 mg/L at the side drain of a fish culture tank. Liu et al. (2017a) used a similar application strategy in RAS by applying PAA at the tank inlet, but with a reduced water flow rate to maximize reaction time, and thereby limit negative impacts on nitrification.

3.1.6. Dissolved metals

Of the 25 dissolved metals/nutrients analyzed in the culture water (Table 3), nine were generally less than the minimum detection limit (MDL) including arsenic, beryllium, cadmium, chromium, cobalt, lead, manganese, molybdenum, nickel, and titanium. During the 0.05 mg/L trial, magnesium, potassium, and sulfur were significantly lower in PAA-dosed RAS. Calcium trended towards significantly lower concentrations in PAA-dosed RAS and iron concentrations were slightly higher ($P = 0.05$) when targeting 0.05 mg/L PAA. No significant differences in dissolved metals concentrations were measured between treatments during the 0.10 mg/L PAA trial; however, dissolved calcium trended towards lower concentrations in RAS where PAA was dosed ($P = 0.05$). Of the dissolved metals evaluated during the 0.30 mg/L PAA trial, boron, iron, and zinc were significantly greater in PAA-dosed RAS ($P < 0.05$).

Overall, the differences in trace metals and nutrients identified between treatments were small in magnitude, i.e., disparities of a fraction of, or only a few mg/L and were not expected to be biologically relevant for fish production. Davidson et al. (2011) found that dissolved copper and zinc were significantly reduced ($P < 0.05$) in the same replicated RAS when applying low-dose ozone (ORP ~ 250 mV). During the same study, dissolved iron was not detected in ozonated RAS compared to the controls when operating with extremely low water exchange rates (HRT > 94 days) and mean feed loading rates of 55.9 kg feed/ m^3 makeup water. Reduction of heavy metals such as copper and zinc due to ozonation was an important finding because these water quality constituents can accumulate in low exchange RAS (Davidson et al., 2009) and are toxic to fish at relatively low concentrations (Spear and Pierce, 1979; United States Environmental Protection Agency (USEPA, 2007; Davidson et al., 2009). During the present study, copper, zinc, and iron were generally unaffected by PAA dosing, except for during the 0.30 mg/L trial where slightly greater concentrations of iron and zinc were measured in the PAA systems. Ultimately, the PAA doses evaluated during this study did not provide an advantage for reduced heavy metals concentrations like that which is expected when applying low-dose ozone.

3.2. Off-flavor

Geosmin and MIB concentrations in water, biofilm, and trout fillets were not affected by PAA during any of the dosing periods ($P > 0.05$). Geosmin concentrations were undetectable (below the instrument detection limit of 1 ng/L) in RAS water and were measured at relatively low concentrations in the biofilm prior to the study, and MIB was not detected (< 1 ng/L) in both the culture water and biofilm (Table 4). Rainbow trout fillets, however, contained substantial concentrations of geosmin and MIB to start, suggesting that trout had bioaccumulated

Table 3
Dissolved metals/trace element concentrations (mean ± standard error) measured in PAA-treated and control RAS (n = 3).

	0.05 mg/L PAA		0.10 mg/L PAA		0.30 mg/L PAA		All Sample Events
Parameters (mg/L)	PAA	Control	PAA	Control	PAA	Control	Makeup Water
Aluminum	0.007 ± 0.001	0.006 ± 0.001	0.009 ± 0.001	0.008 ± < 0.001	< det	< det	0.010 ± 0.003
Barium	0.226 ± 0.025	0.343 ± 0.083	0.261 ± 0.053	0.204 ± 0.007	0.225 ± 0.003	0.213 ± 0.005	0.213 ± 0.004
Boron	0.061 ± 0.007	0.096 ± 0.027	0.075 ± 0.023	0.044 ± 0.002	0.065 ± 0.002 *	0.052 ± 0.002 *	0.040 ± 0.003
Calcium	110 ± 0.3 †	111 ± 0.3 †	121 ± 1 †	125 ± 1 †	117 ± 1	117 ± 1	116 ± 1
Copper	0.031 ± 0.002	0.033 ± 0.001	0.032 ± 0.001	0.031 ± 0.002	0.023 ± 0.002	0.024 ± 0.002	< det
Iron	0.035 ± 0.005 †	0.017 ± 0.002 †	0.053 ± 0.002	0.038 ± 0.012	0.028 ± 0.003 *	< det *	< det
Magnesium	12.6 ± 0.1 *	12.9 ± 0.1 *	13.2 ± 0.1	13.4 ± 0.2	14.3 ± 0.3	14.1 ± 0.2	12.4 ± 0.1
Potassium	19.4 ± 0.2 *	21.2 ± 0.4 *	22.9 ± 0.2	23.6 ± 0.6	11.2 ± 1.0	10.2 ± 0.8	2.2 ± < 0.1
Selenium	0.013 ± 0.002	0.015 ± 0.002	< det	< det	< det	< det	< det
Silicon	5.59 ± 0.02	5.59 ± 0.05	6.05 ± 0.01	6.12 ± 0.02	5.41 ± 0.03	5.34 ± 0.04	5.30 ± 0.19
Sodium	849 ± 7	896 ± 19	832 ± 30	811 ± 9	27 ± 3	26 ± 2	9 ± < 1
Strontium	1.03 ± 0.01	1.04 ± < 0.01	0.99 ± < 0.01	1.01 ± 0.01	1.05 ± 0.01	1.06 ± < 0.00	1.06 ± 0.01
Sulfur	13.1 ± 0.2 *	13.8 ± 0.1 *	17.0 ± 0.1	17.1 ± 0.7	12.6 ± 0.7	12.1 ± 0.4	7.7 ± 0.2
Vanadium	0.011 ± < 0.001	0.011 ± < 0.001	0.003 ± < 0.001	0.003 ± < 0.001	0.003 ± < 0.001	0.003 ± < 0.001	0.006 ± 0.003
Zinc	0.065 ± 0.029	0.143 ± 0.042	0.146 ± 0.042	0.083 ± 0.002	0.119 ± 0.003 *	0.094 ± 0.011 *	0.078 ± 0.005

* Indicates significant difference between treatments ($P < 0.05$).
† Indicates trend towards significance ($P = 0.05$).

Table 4
Geosmin and MIB concentrations (mean ± standard error) in water (ng/L), biofilm (ng/L), and trout fillets (ng/kg) collected at the end of each PAA dosing period from PAA-treated and control RAS (n = 3).

	Baseline		0.05 mg/L PAA		0.10 mg/L PAA		0.30 mg/L PAA	
ng/L; ng/kg	PAA	Control	PAA	Control	PAA	Control	PAA	Control
Geosmin (Water)	< det	< det	21 ± 15	16 ± 3	59 ± 30	54 ± 23	11 ± 5	9 ± 5
Geosmin (Biofilm)	19 ± 4	23 ± 5	2717 ± 2612	3789 ± 2916	394 ± 311	3895 ± 3836	185 ± 133	236 ± 157
Geosmin (Fillets)	1831 ± 604	544 ± 35	3096 ± 1824	4951 ± 1569	8449 ± 4038	2757 ± 775	3546 ± 2057	2431 ± 1182
MIB (Water)	< det	< det	17 ± 10	10 ± 1	8 ± 4	27 ± 23	3 ± 1	2 ± 0
MIB (Biofilm)	< det	< det	51 ± 35	209 ± 137	43 ± 19	186 ± 12	14 ± 7	23 ± 7
MIB (Fillets)	101 ± 11	87 ± 32	343 ± 222	607 ± 79	400 ± 323	1303 ± 1281	55 ± 10	27 ± 5

these off-flavors in a separate production system. During the study, concentrations of geosmin and MIB generally increased in the culture water and biofilm, and tended to persist or, in some cases, increase in fish flesh. The substantial drop in geosmin and MIB in RAS water and biofilm during the 0.30 mg/L trial is interesting but occurred in PAA-treated RAS as well as control systems, indicating that other factors influenced concentrations of these off-flavor compounds (e.g., reduction of daily feed amounts). Ultimately, the present study indicated that semi-continuous dosing of PAA in RAS to achieve target doses of 0.05-0.30 mg/L does not reduce geosmin and MIB concentrations in water, biofilm, or fish flesh, and therefore does not mitigate these types of off-flavor problems.

A challenge with applying oxidants in RAS with the intention to mitigate off-flavor problems is that these compounds must be dosed to produce low residual concentrations that are compatible with fish health and nitrification. As such, oxidant residuals are only present in the water from the point of application (during this study at the LHO) through the culture tank, with nearly full dissipation taking place before the recycle flow reaches the biofilter. The lack of impact of oxidants such as low-dose ozone (Davidson et al., 2011) and PAA on off-flavors in RAS could, in part, be related to their effectiveness being limited to a section of the water recycle loop. Biofilms inside of pipes and unit processes such as the drum filter, heat exchangers, and biofilter are sources of geosmin and MIB-producing bacteria (Schrader and Summerfelt, 2010) that likely remain untreated. In addition, the dosing approach used during the present study may have promoted biofilm growth, which is contrary to conditions that are consistent with reduced concentrations of geosmin and MIB in fish fillets (Davidson et al., 2014b). Research by Liu et al. (2017b) possibly corroborates this theory, as this manuscript reported that continuous, low dose application of PAA enhanced biofilm formation in flow-through tanks stocked with rainbow trout. Conversely, Lindholm-Lehto et al. (2018) recently noted that batch addition of PAA to achieve 2.2 mg/L PAA in RAS raising rainbow trout resulted in a significant reduction of geosmin and MIB, particularly with increased frequency of batch addition. Although PAA does not appear to be a viable solution for eliminating common off-flavor problems in RAS under the conditions of the present study, future work investigating its effect when applied using once daily or periodic batch addition and/or semi-continuously at greater concentrations may be necessary to fully understand its potential for managing common off-flavors.

3.3. Trout performance

Rainbow trout growth curves established for fish from PAA-treated and control RAS overlapped almost identically throughout the study (Fig. 3); therefore, rainbow trout growth was not affected by semi-continuous PAA dosing at the tested target concentrations. During the final dosing period (0.30 mg/L PAA), a small, but insignificant separation in mean fish weights occurred (Fig. 3). Mean rainbow trout weights at study's end for PAA-treated and control RAS were 1911 ± 30 and 1954 ± 11 g. Mean thermal growth coefficient

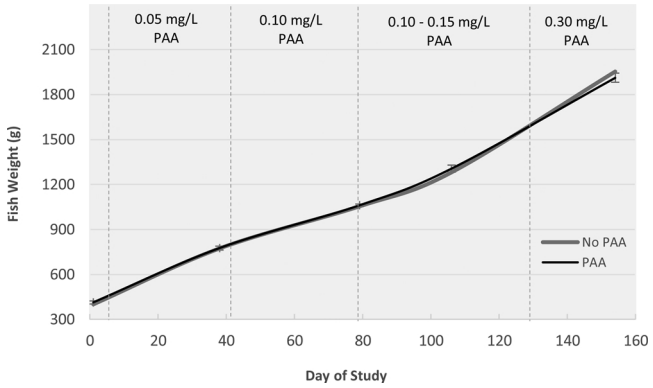

Fig. 3. Rainbow trout growth (mean weight ± standard error) in RAS operated with and without peracetic acid dosing over the study duration.

assessed over the duration of the trial for treatments with and without PAA was 2.41 ± 0.01 and 2.45 ± 0.02, respectively ($P > 0.05$). No significant differences were detected for a variety of other performance responses including feed conversion ratio, condition factor, and fish survival during each PAA dosing period and over the duration of the study (Table 5). Therefore, semi-continuous PAA dosing from 0.05-0.30 mg/L did not negatively affect trout performance and appeared to be compatible with rainbow trout production in RAS under the conditions of this study.

3.4. Costs

Improvements to RAS economics are necessary to enhance the commercial viability of this growing aquaculture sector, including optimization of capital and operating cost efficiencies, fish production capacity, economies of scale, and marketing (Losordo and Westerman, 1994; De Ionno et al., 2006; Liu et al., 2016). However, cost efficiencies specific to individual water treatment technologies have not been extensively studied. The relative costs of PAA application in RAS have not been assessed; however, economic estimates and discussion have been provided for PAA use in different fish production systems and for the wastewater treatment industry. Pedersen and Henriksen (2016) estimated that it would cost a flow-through Danish trout farm (130 L/sec with low organic matter) $20 USD per day for semi-continuous dosing of PAA to achieve prophylactic water treatment, plus the upfront costs for pumps and dosing equipment estimated at $400-500 USD. Kitis (2004) noted the minimal capital investment associated with use of PAA for wastewater treatment, an advantage that likely extends to aquaculture applications. During the present study, the energy and labor costs for effective operation were minimal and the upfront capital costs to setup the PAA dosing system totaled approximately $6000 USD. Capital costs were related to the purchase of peristaltic pumps and associated parts, pump tubing, a spill containment pallet, a room air monitoring system, one 208-L PAA drum, and a drum cooling jacket. These upfront costs may vary depending on water flow to treat and

Table 5
Fish performance metrics (mean ± standard error) to begin the study and at the end of each PAA dosing period for PAA-treated and control RAS (n = 3).

	Baseline		0.05 mg/L PAA		0.10 mg/L PAA		0.30 mg/L PAA	
Fish Performance Metrics	PAA	Control	PAA	Control	PAA	Control	PAA	Control
Fork Length (mm)	289 ± 1	290 ± 1	328 ± 2	330 ± 2	359 ± 1	360 ± 1	425 ± 2	427 ± 5
Weight (g)	414 ± 10	401 ± 6	776 ± 13	773 ± 12	1054 ± 9	1061 ± 8	1911 ± 30	1954 ± 11
Condition Factor	1.9 ± 0.04	1.8 ± 0.02	2.1 ± 0.04	2.1 ± 0.01	2.3 ± 0.01	2.3 ± 0.01	2.4 ± 0.01	2.5 ± 0.02
Feed Conversion Ratio	–	–	1.3 ± 0.02	1.4 ± 0.09	2.2 ± 0.16	1.9 ± 0.08	1.4 ± 0.08	1.5 ± 0.16
% Body Weight Feed	–	–	2.6 ± 0.06	2.5 ± 0.06	2.0 ± 0.07	2.1 ± 0.01	1.5 ± 0.03	1.6 ± 0.02
Biomass Density (kg/m^3)	28.5 ± 0.7	29.1 ± 0.7	52.1 ± 1.5	53.3 ± 0.8	66.3 ± 1.8	62.0 ± 4.8	80.4 ± 2.0	80.6 ± 1.8
Survival (%)	–	–	99.4 ± 0.3	99.1 ± 0.2	96.9 ± 0.5	93.9 ± 3.8	97.8 ± 0.9	98.5 ± 0.4

decisions related to equipment purchasing for worker safety. The 2018 cost for one 208-L drum of VigorOx SP-15 PAA is $950 plus shipping or a 1249-L tote can be purchased for $6150 plus shipping. Consistent with Kitis (2004), the relative cost of the PAA chemical itself is relatively high; however, the potency of PAA has shown to be 100 times that of hydrogen peroxide (Straus et al., 2012). Based on the dosing regimen used during the present study, semi-continuous peracetic acid treatment of a water recycle flow of 329 L/min in one 9.5 m^3 RAS would require the purchase of one 208-L drum every 7–8 months; however, treatment of the water recycle flow of an onsite semi-commercial scale RAS (~4000 L/min recycle flow; 270 m^3 total volume) would require a new PAA drum every 18–19 days or a new 1249-L tote every 3–4 months. During the 0.30 mg/L PAA trial, for which these cost estimates are based, only marginal water quality benefits were observed. As such, fish farmers using RAS most likely would not adopt semi-continuous PAA dosing as a feasible strategy for broad-ranging water quality improvement. However, these cost estimates may be relevant for other PAA application strategies in RAS such as once-daily or periodic batch addition, which has shown promise related to antimicrobial treatment effects (Good et al., 2017a; Liu et al., 2017a, 2017b), or semi-continuous dosing in flow-through systems where prophylactic effects have been reported (Pedersen and Henriksen, 2016). To the authors' knowledge, a detailed cost assessment for ozonation in RAS has not been carried out; therefore, accurate cost comparisons cannot be drawn. Several publications have reported that the equipment and operating costs associated with ozonation are not trivial, particularly when ozone is applied in combination with UV (Summerfelt and Hochheimer, 1997; Gonclaves and Gagnon, 2011), and the use of ozone comes with its own set of safety considerations for fish and human health (Gearhart and Summerfelt, 2007). Nevertheless, the extensive water quality benefits created by ozone (Davidson et al., 2011; Gonclaves and Gagnon, 2011; Powell and Scolding, 2018) likely justify the capital and operating expenses of this technology.

3.5. Conclusions

The findings from this study indicate that semi-continuous PAA dosing at target concentrations of 0.05-0.30 mg/L in research-scale RAS provided marginal water quality improvements, unlike the profound and wide-ranging enhancements to the fish culture environment expected when applying low-dose ozone. Nevertheless, these findings do not negate the potential for PAA to be applied differently in RAS, such as with once-daily or periodic batch addition, an approach which has shown promise for antimicrobial and prophylactic control. A comprehensive assessment of water quality, salmonid performance, and off-flavor compounds may be informative when applying PAA in RAS using batch addition up to 1.0 mg/L or with semi-continuous doses to achieve concentrations > 0.30 mg/L.

This research demonstrated a safe and effective protocol for dosing PAA in RAS of this design that could be replicated elsewhere. The injection point for PAA dosing to each RAS was strategically identified. By adding the PAA drip just above the LHO distribution plate, a mixing effect was provided by water cascading through the carbon dioxide stripping column. This location was also ideal because it followed the fluidized sand biofilter, thereby allowing ample reaction time to limit the effect of PAA residuals on nitrification. The authors recommend this point of PAA application for future studies evaluating PAA use in RAS. In addition, PAA residuals appear to impact oxidative reduction potential readouts like ozone, indicating that ORP could be used as an on/off control when applying PAA semi-continuously or otherwise as an indirect measure for PAA residual concentrations when using alternate dosing methods.

Although semi-continuous dosing of PAA from 0.05-0.30 mg/L did not result in profound water quality improvements, this dose was compatible with fish health and performance, as well as biofilter operation. These results are important when considering PAA's capacity as a water sanitizer or disinfectant and its possible use to improve fish health by controlling pathogens in the water column. Although state of the-art RAS inherently provide robust biosecurity against the introduction of obligate fish pathogens, opportunistic pathogens will occasionally cause disease in RAS under conditions that favor the infectious agent (Wedemeyer, 1996). Given the innocuous nature of low concentration PAA demonstrated during this study, PAA should not be ruled out as a viable chemical to control pathogenic bacteria with fish present in RAS. Few chemicals are compatible as water sanitizers in RAS; therefore, more research is required to understand PAA's antimicrobial effects in RAS at low concentrations and when applied using methods other than those used during the present study.

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Study VI

Integrating activated sludge membrane biological reactors with freshwater RAS: Preliminary evaluation of water use, water quality, and rainbow trout Oncorhynchus mykiss performance

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Integrating activated sludge membrane biological reactors with freshwater RAS: Preliminary evaluation of water use, water quality, and rainbow trout *Oncorhynchus mykiss* performance

John Davidson[a,*], Steven Summerfelt[a,1], Kevin K. Schrader[b], Christopher Good[a]

[a] The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, USA
[b] USDA, Agricultural Research Service, Natural Products Utilization Research Unit, University, MS, 38677, USA

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ABSTRACT

Onsite research indicates that activated sludge membrane biological reactors (MBRs) are an effective waste treatment technology for aquaculture effluents. MBRs produce a filtered permeate that is nearly free of dissolved nutrients, organics, and solids; therefore, this technology could be well-suited for integration within the process control loop of recirculation aquaculture systems (RAS). A four-month study was carried out to evaluate the feasibility of incorporating single-vessel MBRs within freshwater RAS while culturing rainbow trout *Oncorhynchus mykiss*. Triplicate RAS with and without MBRs (controls) were evaluated; *mRAS* and *cRAS*, respectively. System backwash water of *mRAS* was processed and retained within MBRs which allowed increased water recycling, while *cRAS* utilized standard dilution rates to limit nitrate accumulation. On average, *mRAS* required six and a half times less makeup water. Mean daily water replacement of the RAS volume for *mRAS* and *cRAS* was 1.2 ± 0.4 and 7.8 ± 0.5%, respectively ($P < 0.05$). A range of water quality concentrations were significantly greater in *mRAS* including chloride, carbon dioxide, heterotrophic bacteria count, pH, nitrate-nitrogen, total ammonia-nitrogen, total phosphorous, and true color, as well as dissolved concentrations of calcium, copper, magnesium, and sulfur. Alkalinity and ultraviolet transmittance levels were significantly lower in *mRAS*. These culture environment differences did not affect rainbow trout growth, feed conversion, or survival ($P > 0.05$). In addition, concentrations of common off-flavor compounds (geosmin and 2-methylisoborneol) in water and fish flesh were not affected by MBR presence. Improvements for future MBR integration with RAS were realized including optimization of MBR permeate rates, increased RAS water exchange through the MBRs, and infrequent supplementation of a carbon source to enhance denitrification efficiency and alkalinity recovery. Overall, incorporating MBRs within RAS resulted in substantial water savings and was biologically feasible for rainbow trout production.

1. Introduction

Activated sludge membrane biological reactors (MBRs) are widely used for municipal and industrial wastewater treatment to remove nutrients, organics, and solids from concentrated effluents (Gunder, 2001; Van der Bruggen et al., 2003; Jyoti et al., 2013; Ozgun et al., 2013; Hai and Yamamoto, 2011). MBRs utilize a series of fine-pore membranes (typically < 0.2 µm) plumbed in parallel that create a semi-purified filtrate, while associated aerobic and anoxic processes functioning within an activated sludge facilitate nitrification and denitrification, respectively (Hai and Yamamoto, 2011). Onsite research has shown that MBRs are a promising wastewater treatment technology for solids-laden aquaculture discharge. Sharrer et al. (2007, 2010a) demonstrated effective MBR treatment of an aquaculture effluent with the filtrate containing < 3 mg/L total nitrogen (TN), < 0.1 mg/L total phosphorus (TP), < 1 mg/L biochemical oxygen demand (BOD), < 1 mg/L total suspended solids (TSS), and significantly reduced heavy metals concentrations. Several laboratory scale studies utilizing MBRs have also demonstrated efficient treatment of aquaculture wastewater. For example, Visvanathan et al. (2008) found that an MBR treating a synthetic aquaculture effluent eliminated suspended solids and achieved 91.4% TN removal. Additionally, Pulefou et al. (2008) demonstrated 78% removal of TN from aquaculture wastewater using bench scale MBRs. Other studies have shown that membrane-based

* Corresponding author.
E-mail address: jdavidson@conservationfund.org (J. Davidson).
[1] Current address: Superior Fresh, W15506 Superior Fresh Drive, Hixton, WI 54635, USA.

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technologies efficiently remove solids and fine particles from aquaculture process water in the absence of biological treatment with an activated sludge (Viadero and Noblet, 2002; Holan et al., 2014a, 2014b; Wold et al., 2014; Ng et al., 2018).

Activated sludge MBRs have primarily been evaluated as a treatment mechanism for fish farm effluents (Sharrer et al., 2007, 2010a; Pulefou et al., 2008; Visvanathan et al., 2008); however, the pristine permeate produced by these technologies appears to be suitable for return to RAS (Gemende et al., 2008; Visvanathan et al., 2008; Boley et al., 2017). The earliest study to report strong potential for integrating MBRs within RAS was carried out by Gemende et al. (2008) who reported a remarkable reduction in wastewater and residue loads in a pilot scale system. In a recent trial conducted with 1.85 m^3 RAS, Boley et al. (2017) found that integration of a "membrane-denitrification reactor" resulted in improved water quality for common carp *Cyprinus carpio* while significantly reducing water usage.

The potential for MBR-integrated RAS to conserve water is directly related to denitrification capacity, e.g., microbial reduction of nitrate (NO_3^-) to dinitrogen gas (N_2). Adding denitrification within RAS eliminates the necessity to dilute nitrate via flushing, which is commonly accomplished through daily replacement of 5–10% of the system volume (Masser et al., 1999; authors' experience). Regardless of the approach, RAS designs that necessitate NO_3^- control are critical, as there is ample evidence that NO_3^- can accumulate to chronically toxic levels for fish (Hrubec, 1996; Camargo et al., 2005; Hamlin, 2005; Davidson et al., 2011a, 2014; Van Bussel et al., 2012; Schram et al., 2014). At present, denitrification technologies are not extensively utilized within the water recycle loop of commercial RAS; however their effective use has been reported in both freshwater and marine systems (van Rijn et al., 2006; Tal et al., 2009; Müller-Belecke et al., 2013; van Rijn, 2013; Qiu et al., 2016) and for end-of-pipe treatment of aquaculture effluents (Suhr et al., 2013, 2014). Incorporating denitrification within RAS reportedly provides benefits beyond NO_3- reduction including stabilized buffering capacity through alkalinity recovery, prevention of accumulating toxic sulfides, and phosphate uptake when microbial conditions are optimized (Barak and van Rijn, 2000; van Rijn et al., 2006; Tal et al., 2009). When anaerobic digestion is coupled with denitrification, additional advantages may include reduced biosolids waste volumes through sludge digestion, biogas production, and stabilized, non-malodorous sludge with potential for value-added use as a soil amendment (Tal et al., 2009; Mirzoyan et al., 2010). In addition, several studies have reported that anaerobic zones within sludge digestion basins provide conditions for absorption and remediation of the bacterial metabolites geosmin and 2-methylisoborneol (MIB) (Guttman and van Rijn, 2008; Azaria et al., 2017), which can bioaccumulate in fish flesh and cause "earthy" and "musty" off-flavors resulting in unpalatable products and negative consumer perception of aquaculture products (Engle et al., 1995; Tucker, 2000).

Many of the advantages common to anaerobic denitrification systems are expected to translate when incorporating activated sludge MBRs within RAS (Sharrer et al., 2007, 2010a). However, MBR systems have the potential for providing additional water quality benefits via membrane filtration. Onsite studies found that an MBR used to process aquaculture backwash water removed > 99% of solids via submerged membrane separation, producing a filtrate with < 1 mg/L TSS from an activated sludge containing > 16,000 mg/L TSS (Sharrer et al., 2007, 2010a). Exclusion of fine particles has been demonstrated as a distinct advantage of membranes used in municipal wastewater and potable water treatment (Van der Bruggen et al., 2003; Hai and Yamamoto, 2011) that appears to extend to treatment of aquaculture water. For example, Viadero and Noblet (2002) reported > 94% TSS rejection by membranes from an aquaculture system, and Holan et al. (2014b) measured a 38% reduction in colloidal particles and a 77% reduction in turbidity when treating a RAS sidestream with membranes. In addition, MBRs used at municipal wastewater and potable water plants have shown successful reduction and removal of microorganisms, including coliform bacteria, protozoa, viruses, and bacteriophages (Radjenović et al., 2008; Ozgun et al., 2013; Hai et al., 2014; Purnell et al., 2015, 2016). Microbial separation by MBRs was also demonstrated by Sharrer et al. (2007) who reported total coliform and heterotrophic bacteria reduction efficiencies as high as 7.0 $\log_{10}$ and 5.6 $\log_{10}$, respectively, from aquaculture wastewater.

The perceived advantages of integrating MBRs with RAS are primarily based on results from municipal wastewater processing plants and limited studies evaluating MBR treatment of aquaculture effluents. It is uncertain if these benefits can be replicated when incorporating MBRs within relevant scale RAS or if returning the permeate from MBRs to RAS will negatively affect the culture environment for salmonids, particularly in freshwater systems with dramatically reduced water exchange rates and extended hydraulic retention times. To this end, a study was developed to evaluate the feasibility of integrating MBRs within the water treatment loop of RAS while raising rainbow trout *Oncorhynchus mykiss*. A comprehensive assessment was carried out to evaluate the effectiveness of MBR-RAS integration with focus on denitrification efficiency, alkalinity recovery, and accumulation of potentially harmful concentrations of suspended solids, nutrients, and dissolved metals. Engineering metrics such as water usage, system hydraulic retention time, and operational aspects were assessed, and challenges for incorporating MBRs within RAS were ascertained.

2. Methods

2.1. Experimental design

Six replicate RAS (9.5 m^3) originally described by Davidson et al. (2009 were used for the 4-month study. Three RAS included single-vessel MBRs within the water treatment loop (*mRAS;* Fig. 1), while three control RAS without MBRs (*cRAS*) were operated with standard dilution and feed loading rates that limit nitrate accumulation and support acceptable salmonid health and performance (Davidson et al., 2009, 2011b). Each RAS recirculated 336 L/min (89 gpm) of freshwater 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 (Fig. 1). Dilution rate for *cRAS* was dictated by the combined water volume removed as drum filter backwash and radial flow settler discharge, which was sensed and replaced with an equal volume of spring water by a float valve. A similar makeup water strategy was used in *mRAS*; however, backwash water was pumped to MBRs and thereby retained in the process control loop, and a filtered permeate was returned to RAS (Fig. 1). As such, *mRAS* only received makeup water to replace evaporative loss, splashing, minor system overflows, and small volumes removed for wet chemistry analyses. Cumulative makeup water addition was measured in each RAS by magnetic drive flowmeters (Model C700, Elster AMCO Water Inc., Ocala, FL, USA) installed upstream of float valves. Sodium bicarbonate ($NaHCO_3$; Church & Dwight Co. Inc., Ewing, NJ, USA) was periodically added to maintain alkalinity levels that support nitrification (Summerfelt et al., 2015; Boyd et al., 2016). Daily batch addition of $NaHCO_3$ was performed for *cRAS* throughout the study, while *mRAS* were expected to recover alkalinity as a byproduct of denitrification and were only dosed with $NaHCO_3$ during the first month. Rainbow trout was used as the test-species for this research.

2.2. Membrane biological reactors

Three single-vessel MBRs were installed and incorporated within the water treatment loop of respective *mRAS* (Fig. 1). Each cylindrical fiberglass vessel (1.2 m dia. × 2.4 m tall; 2.7 m^3 operating volume) received intermittent backwash from drum filters and radial flow settlers which accumulated inside of the reactors as an activated sludge, i.e., mixed liquor suspended solids or biofloc. A head-pressure driven

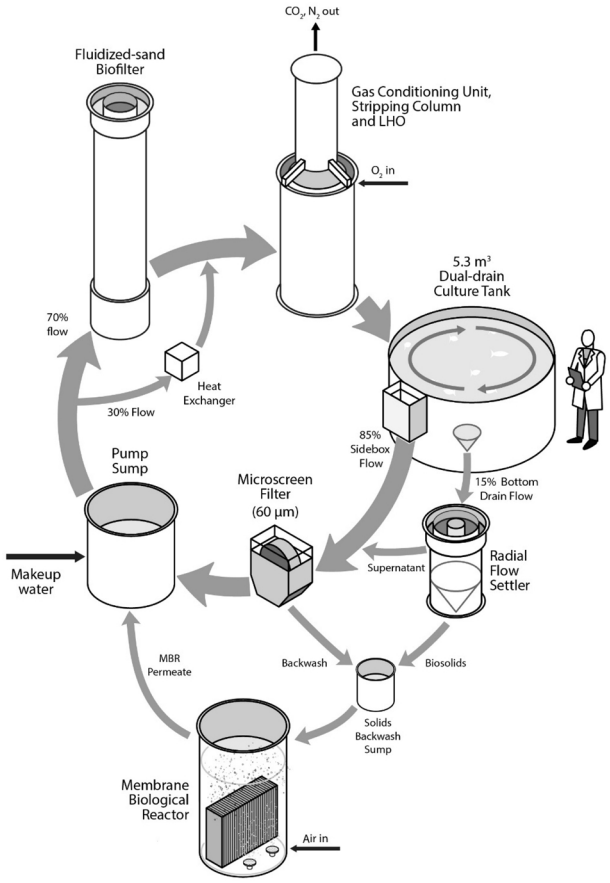

Fig. 1. Water flow and process design for an individual recirculation aquaculture system (9.5 m^3) with integrated MBR used during the study.

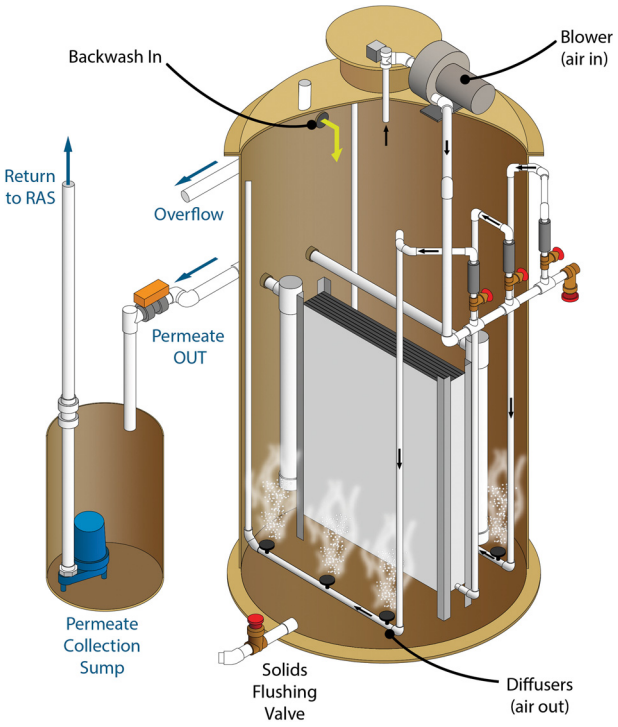

Fig. 2. Cross-sectional schematic of an individual MBR showing membrane module positioning, air delivery system, and inlet and outlet water flow locations and direction.

permeate passed through a flat plate membrane module (MFM100-25, Alfa Laval, Lund, Sweden) contained within each MBR (Fig. 2). Fourteen hollow-sheet membranes (0.2 µm pore size) constructed of polyvinylidenfluoride were stacked in parallel within the module, providing a total membrane surface area of 25 m^2. Transmembrane pressure, the difference between internal vessel water height and permeate valve elevation, was maintained between 60–90 cm (2–3 ft). An electrically actuated permeate valve (Type EA11, Georg Fischer LLC, Irvine, CA, USA) opened when rising water level within the vessel triggered a float switch and closed when the water level receded to the height of a lower positioned float. In addition, a timed 1-min on/10-min off period was integrated with a programmable logic controller (PLC) and the permeate valve to provide a relaxation period for the membranes. Permeate from each MBR collected within an external lift station and was returned to *mRAS* pump sumps (Figs. 1 and 2). Permeate flow rates were measured four to five times per week using a volumetric bucket test.

A centrifugal blower (Airtech Vacuum, Englewood, NJ, USA) delivered compressed air to an aeration manifold that extended to the bottom of each MBR (Fig. 2). Air flow was monitored and adjusted based on pressure gauge readings and was distributed at two locations: 1) orifice piping positioned beneath the membrane module purposed to scour membrane surfaces (7.5–8.0 SCFM), and 2) 3-inch Snap-Cap™ diffusers (Evoqua Water Technologies, Waukesha, WI, USA) located around the inside perimeter of each reactor vessel (5.0 SCFM to each side) (Fig. 2). A three-way valve (Type EA25, Georg Fischer LLC, Irvine, CA, USA), connected at the blower inlet and integrated with the PLC, selected between external ambient air with “high” oxygen content or internal headspace air with “low” oxygen depending on the aerobic conditions of the activated sludge. Air from the enclosed headspace of the MBR was primarily utilized to maintain 0–2 mg/L dissolved oxygen. Oxygen level within the activated sludge was measured with RDO-PRO-X dissolved oxygen sensors (In Situ Inc., Fort Collins, CO, USA) integrated with the blower, an on/off timer (approximately three weeks into the study), and the PLC. On/off duration was adjusted to maintain low oxygen conditions that balanced nitrification and denitrification. Lastly, an overflow pipe, which also acted as an air vent, was provided at a vessel height of approximately 2.6 m, and a flushing valve was provided at the base of each MBR vessel to remove excess solids (Fig. 2).

MBR operation began two months before the study to establish nitrifying and denitrifying microbial populations. During this acclimation period, RAS backwash was directed to the MBRs; however, permeate was not returned to the RAS. In addition, MBRs were seeded with FritzZyme Turbostart™ nitrifying bacteria (Fritz Aquatics, Mesquite, TX, USA), and granulated sugar and expired fish feed were added to provide carbon and nitrogen, respectively.

2.3. Rainbow trout

Rainbow trout were received as fingerlings from the United States Department of Agriculture – Agricultural Research Service (USDA-ARS), National Center for Cool and Coldwater Aquaculture (Leetown, WV, USA) and cultured in separate flow-through and partial reuse systems prior to the study. To begin, each RAS was stocked with approximately 650 randomly selected rainbow trout (103 ± 1 g) providing an initial biomass density of 13 kg/m^3 per RAS. Mortalities were removed and recorded daily to assess cumulative survival. Length and weight measurements of a random sample of 60 trout from each RAS were collected to begin the study. Fish were resampled three times at approximately 40-day intervals. Sample size increased by approximately ten fish for each sampling event to account for expanding size deviation of the populations. Fish sample size (n) was calculated using equations in Bhujel (2008) as follows:

$$n = [(Z * \text{standard deviation})/\text{accepted error}_g)]^2; \text{ where } Z = 1.65$$

Thermal growth coefficient (TGC), feed conversion ratio (FCR), and fish survival (%) were calculated using the following formulae:

TGC = (End Weight ^ (1/3)) – Start Weight ^ (1/3))/((Days Between * Avg. Temp.) x 1000)

where weight is in grams, length is in mm, and temperature is in º C.

FCR = Cumulative Feed Delivered/Fish Biomass Gain

Survival (%) = ((Initial Number of Fish – Cumulative Mortalities & Culls)/Initial Number of Fish) * 100

Fin erosion of the anal, caudal, dorsal, and left and right pelvic and pectoral fins was assessed qualitatively on a 4-point scale (no damage = 1, minor damage = 2, moderate damage = 3, severe damage = 4) for fish sampled at the beginning and end of the study as a measure of welfare.

2.4. Feeding methods

Fish were fed to apparent satiation using a computer operated system (The Conservation Fund Freshwater Institute (TCFFI), Shepherdstown, WV, USA) programmed to deliver short feed bursts once an hour via automated feeders (T-drum 2000CE, Arvotec, Huutokoski, Finland). Feeding rates were fine-tuned separately per RAS based on observations of feeding activity and wasted feed. A constant 24-h photoperiod was provided to facilitate “around-the-clock” feeding and consistent water quality. A commercially available 45/24 (protein/fat) trout/steelhead diet (Biotrout™, Bio-Oregon, Westbrook, ME, USA) was fed throughout the study.

2.5. Water quality sampling and analyses

Water samples were collected from RAS side drains, MBR permeate, and makeup water and tested onsite according to methods described by APHA (2012) and HACH Company (2003, 2015) (Table 1). Eleven select dissolved metals/elements were analyzed based on positive detection during previous studies in the same replicate RAS (Davidson et al., 2011a, 2014). Metals analysis was carried out by REI Consultants Inc. (Beaver, WV, USA) on water samples collected before the study while systems were operating with similar flushing rates and monthly during the study from RAS side drains, MBR permeates, and makeup water (Table 1). In addition, activated sludge samples from each MBR were collected weekly for TSS analysis via the solids flushing valve (Fig. 2), and solids-laden discharge flows from each RAS were collected weekly to characterize wastewater pumped to the MBRs. Wastewater samples were collected from two discharge flows from each RAS including: (1) the drum filter backwash and (2) the concentrated flow flushed from radial flow settlers. Samples were analyzed for BOD, TN, TP, and TSS, and resulting concentrations were used for determination of mass balances and waste production metrics. Measurements required for mass balance assessment included volumes flushed from each discharge location (L) and feed delivered (kg). The volume flushed from radial flow settlers was determined by collecting the flow in a tared bucket and subsequently weighing the solution. Daily drum filter backwash volume was assessed by magnetic drive digital flowmeters (Model C700, Elster AMCO Water Inc., Ocala, FL, USA) installed on the spray water side of the drum filters. Feed was weighed into calibrated feeders. Mass balance calculations used to determine effluent waste mass per kg feed were performed as follows:

$$\text{Mass (kg waste/kg feed)} = \frac{(C_{out})(\text{mg})}{\text{L}} * \frac{\text{Total Discharge Volume (L)}}{\text{Total Feed (kg)}} * \frac{\text{kg}}{10^6\text{mg}}$$

where C_{out} = effluent concentration. Total waste per kg feed contributed to the MBRs was calculated by summing the waste mass contained in the effluents of each RAS. Combined backwash concentration was calculated based on the percent daily wastewater volumes of the drum filter backwash and radial flow settler flushing, respectively, where approximately 97.7% of the wastewater pumped to the MBR was provided by drum filter backwash and approximately 2.3% was provided by settleable solids flushed from the radial flow settler. Daily waste concentrations provided by the radial flow settler were normalized with the assumption that solids were collected at an equal rate around-the-clock in accordance with 24-h feeding.

Table 1
Water quality parameters evaluated, methodologies, and frequency of testing.

Parameter	Method of Analysis	Frequency of Recording/ Testing
Dissolved Oxygen	Hach SC100 Controller & LDO® Probe	Daily
Oxidative Reduction Potential	Hach SC100 Controller & Differential ORP Sensor	Daily
Temperature	Hach SC100 Controller & Differential ORP Sensor	Daily
Specific Conductance	YSI 30 Salinity/Conductivity/Temperature Meter	3-4 times weekly
Alkalinity	Hach Method 8203 - Sulfuric Acid Digital Titration pH endpoint Accumet #AB150	2-3 times weekly
pH	Standard Methods 4500-H^+ B – Electrode	2-3 times weekly
Biochemical Oxygen Demand	Standard Methods APHA 5210B - 5-day test (No prefiltration) YSI Model 58, YSI BOD probe #5905	Once weekly
Carbon Dioxide	Hach Method 8223 - Sodium Hydroxide Burette Titration pH endpoint Accumet #AB150	Once weekly
Nitrate Nitrogen	Hach Method 8171 - Cadmium Reduction	Once weekly
Nitrite Nitrogen	Hach Method 8507 USEPA Diazotization	Once weekly
Total Ammonia Nitrogen	Hach Method 8038 USEPA Nessler	Once weekly
Total Heterotrophic Bacteria	Hach Method 8242 - Membrane Filtration, Fischer Isotemp Incubator #516D	Once weekly
Total Phosphorous	Hach Method 8190 – USEPA PhosVer3 with Acid Persulfate Digestion. DRB200 reactor and Hach Method 10127 (Molybdovanadate w/ Acid Persulfate Digestion)	Once weekly
Total Nitrogen	Hach Method 10071 (Persulfate Digestion) Low Range 0.5- 25 mg/L as N and Hach Method 10072 (Persulfate Digestion) High Range 2-150 mg/L as N	Once weekly
Total Suspended Solids	Standard Methods APHA 2540D - Dried at 103-105 ° C. Thelco Oven #6540, Mettler Toledo #AE240 and #PM30K	Once weekly
True Color	Hach Method 8025 - Platinum-Cobalt Standard	Once weekly
UV Transmittance	Hach Method 10054 - Organic UV Absorbing (UV-254)	Once weekly
Chloride	Hach Method 8113 – Mercuric Thiocyanate	Monthly - 4 events
Dissolved Metals	Inductively Coupled Plasma Atomic Emission Spectrometry	Monthly - 4 events

Spectrophotometers DR2700 and DR6000 (Hach Company, Loveland, CO, USA) were used for analysis of nitrate nitrogen, nitrite nitrogen, total ammonia nitrogen, and total phosphorous. Spectrophotometer DR4000 (Hach Company) was used for analysis of true color and UV transmittance.

2.6. Off-flavor sampling and analysis

Water and trout fillet samples were collected at the beginning, middle, and end of the study for analysis of geosmin and MIB. Glass scintillation vials (20 mL) with foil-lined caps were used to collect water from RAS side drains, MBR permeate, and makeup water sites. Methods for the determination of geosmin and MIB in water followed procedures outlined in Davidson et al. (2019), modified from Lloyd et al. (1998). In addition, three rainbow trout from each RAS were randomly netted, euthanized via percussive stunning, and filleted on the same days that water samples were collected. Skinless, right-side fillet portions were packaged in zip-lock freezer bags and frozen prior to shipment for analysis. A standard portion from the anterior of each fillet was used to obtain distillate following microwave distillation procedures and methods outlined by Lloyd and Grimm (1999). Each distillate sample was analyzed using solid phase microextraction-gas chromatography-mass spectrometry. The instrumental detection limits for geosmin and MIB were 1 ng/L.

2.7. Statistical analysis

Water quality data were analyzed using a restricted maximum likelihood (REML) mixed model test that assigned water quality criterion as dependent variables; treatment, time, and treatment x time as independent fixed factors; and RAS/tank as a random effect nested within treatment (Ling and Cotter, 2003; Thorarensen et al., 2015). Analysis of covariance (ANCOVA) with feed loading rate (daily makeup water (m^3)/ daily feed (kg)) modeled as a covariate was used to analyze dissolved metals and nutrient concentration data. Mean off-flavor concentrations and fish performance metrics were analyzed using a Student's t-test. Each data set was analyzed for normality using a Shapiro-Wilk test. Non-gaussian distributed data were analyzed using non-parametric statistics, including the Kruskal Wallis test. A probability level of 0.05 was used to determine significance. Statistical analyses were carried out using SYSTAT 13 software (2009).

3. Results and discussion

3.1. Water usage

Overall, *mRAS* required six and a half times less makeup water (Table 2) due to retention of system backwash that otherwise would have been flushed to external waste treatment systems, as described by Sharrer et al. (2010b). For example, mean system hydraulic retention time (HRT) for *mRAS* and *cRAS* was 104 ± 31 and 13 ± 1 days, respectively ($P < 0.05$; Table 2). Ultimately, *mRAS* required only 1.2 ± 0.4% daily water exchange compared to 7.8 ± 0.5% daily water replacement necessitated by *cRAS* ($P < 0.05$; Table 2). The 6.5-fold difference in water savings achieved by *mRAS* could have been greater if average MBR permeate flow rates were maintained closer to the expected design criteria (~5 L/min). Permeate flow rates gradually declined during the study and varied considerably among replicate MBRs, i.e. 1.5, 3.6, and 0.4 L/min for MBRs 1, 2, and 3, respectively. At times, declining permeate flows (particularly for MBR 3) resulted in an imbalance with inlet flow rates to the MBRs, thereby causing minor system overflows and increased dilution. Under these circumstances, the membrane relaxation period was adjusted, or, in the worst-case scenario for MBR 3, eliminated in favor of continuous permeate production to balance water exchange between the MBR and the RAS. Average inlet (RAS backwash) flows were relatively consistent among replicates, i.e. 720 ± 67 L/day or ~0.5 L/min resulting in an MBR vessel hydraulic retention time of 3.8 ± 0.3 days, and 13.4 ± 1.1 days for the MBRs to process the entire RAS volume.

Table 2
Average water flushing metrics (mean ± standard error) for RAS operated with and without MBRs over the study duration.

Water Flushing Metrics	*mRAS*	*cRAS*
Daily Water Use (Liters/day)	115 ± 40*	744 ± 44
Daily Water Use (m^3/day)	0.115 ± 0.040*	0.744 ± 0.044
System Hydraulic Retention Time (days)	104 ± 31*	13 ± 1
Daily Feed Loading Rate (kg feed/m^3 makeup water)	39.3 ± 11.6	5.2 ± 0.3
Daily System Water Exchange Rate (%)	1.2 ± 0.4*	7.8 ± 0.5

* Indicates significant difference between treatments ($P < 0.05$).

The water savings resulting from MBR integration with RAS appear to be a significant advantage and could have important implications for the growing RAS industry. Traditional RAS technologies already reduce the water footprint for fish production; however, the need for additional water savings has recently been augmented by the increasing scale of new or planned commercial RAS facilities (5000 to > 30,000 mt/yr) (Intrafish, 2018). For perspective, it was formerly estimated that a 1000 mt/yr RAS salmon farm requires up to 3000 m^3 makeup water/day (Liu et al., 2016), but commercial farms with expanded production goals will require significantly greater water volumes, which may not be sustainable given the high demand for clean water resources. However, successful integration of MBRs with RAS could result in a 10-fold reduction in water usage which would offset the expanding water footprint of commercial farms. Ultimately, this reduced water requirement could result in a range of advantages for RAS facilities including reduced waste discharge, less capital investment for waste treatment, improved effluent quality to meet increasingly stringent discharge limits, and increased flexibility for siting facilities where water resources are scarce and/or near major seafood markets. While diminished water use may result in advantages for the RAS industry, this aspect must also be considered with respect to its effect on the fish culture environment and compatibility with fish health and performance.

3.2. General water quality

A range of water quality parameters were measured at significantly greater mean concentrations in *mRAS* including: alkalinity, carbon dioxide, chloride, heterotrophic bacteria counts, pH, nitrate-nitrogen (NO_3-N), total ammonia nitrogen, total phosphorous, true color (Table 3), and dissolved concentrations of calcium, copper, magnesium, and sulfur (Table 4). Mean alkalinity and ultraviolet (UV) transmittance levels were significantly lower in *mRAS*. Important water quality criteria including dissolved oxygen and water temperature were controlled to maintain equal levels between treatments (Table 3). The accumulation of water quality constituents in *mRAS* (Table 3) was not surprising given the substantially longer hydraulic retention times and limited dilution associated with these systems. However, the elevated water quality concentrations in *mRAS* were generally suitable for rainbow trout health and performance, as will be discussed in more detail in subsequent sections.

3.3. Dissolved metals/elements

Despite using six and a half times less water, the concentrations of selectively analyzed dissolved metals/elements in *mRAS* did not reflect this flushing difference (Table 4). Biosorption of metals in MBR activated sludge and conditions influencing their uptake (pH, temperature, TSS concentration) was described by Sharrer et al. (2010a) relative to treatment of an aquaculture effluent and has been documented for other activated sludge waste treatment systems (Ong et al., 2010; Dhokpande et al., 2014).Of note during the present study, dissolved copper was just two times greater in *mRAS* and dissolved zinc levels were statistically similar between treatments (Table 4) indicating that these metals were being sequestered within the activated sludge of the MBR. Consideration of copper and zinc levels is important because

Table 3
Water quality concentrations (mean ± standard error) measured in the culture water of RAS with and without MBRs, as well as MBR permeate (n = 3) and makeup water.

Parameter	*mRAS*	*cRAS*	MBR Permeate	Makeup Water
Alkalinity (mg/L)	83 ± 5*	161 ± 2	476 ± 32	270 ± 5
Biochemical Oxygen Demand (mg/L)	7.3 ± 0.5	6.3 ± 0.5	1.5 ± 0.1	0.5 ± 0.1
Carbon Dioxide (mg/L)	26 ± 2*	9 ± 1	–	42 ± 1
Chloride (mg/L)	50 ± 1*	26 ± 1	48 ± 10	17 ± 1
Dissolved Oxygen (mg/L)	9.9 ± 0.01	9.9 ± 0.05	2.0 ± 0.1	–
pH	7.0 ± 0.04*	7.6 ± 0.3	7.5 ± 0.1	7.1 ± 0.02
Nitrite Nitrogen (mg/L)	0.042 ± 0.005	0.041 ± 0.002	0.818 ± 0.250	0.0011 ± 0.0002
Nitrate Nitrogen (mg/L)	201 ± 11*	117 ± 3	87 ± 12	2.5 ± 0.1
Specific Conductance (μS)	2,237 ± 84*	1,500 ± 31	–	–
Total Ammonia Nitrogen (mg/L)	0.93 ± 0.06*	0.49 ± 0.02	1.09 ± 0.23	0.018 ± 0.002
Temperature (° C)	13.8 ± 0.1	13.9 ± 0.1	13.7 ± 0.1	–
Total Heterotrophic Bacteria (cfu/mL)	130 ± 39*	42 ± 14	895 ± 829	9 ± 4
Total Phosphorous (mg/L)	14.6 ± 0.5*	5.0 ± 0.2	13.8 ± 1.2	0.026 ± 0.005
Total Suspended Solids (mg/L)	11.0 ± 0.5	9.0 ± 1.0	0.3 ± 0.02	0.5 ± 0.1
True Color (Pt-Co units)	96 ± 7*	52 ± 1	104 ± 5	4 ± 1
UV Transmittance (%)	29 ± 2*	58 ± 1	28 ± 2	98 ± 0.2

– Indicates data was not collected.
* Indicates significant difference between treatments.

these metals are commonly included as essential micronutrients in aquafeeds. When provided in excess of the biological requirement of fish, copper and zinc are excreted into the water and can accumulate to potentially toxic levels in RAS (Wedemeyer, 1996; Davidson et al., 2009). Although the concentrations of dissolved calcium, copper, magnesium, and sulfur were significantly higher in *mRAS*, none of these concentrations appear to be of critical concern for fish health (Wedemeyer, 1996; Davidson et al., 2009; Spear and Pierce, 1979; United States Environmental Protection Agency (USEPA, 2007). However, the dissolved potassium (K+) concentration measured in *mRAS* may deserve further attention. Davidson et al. (2011a) identified accumulating K + as one of several possible variables responsible for chronic health effects of rainbow trout cultured in low exchange RAS. During the present study, K + was largely influenced by feed loading rate and bordered significance ($P = 0.06$) between treatments. Limited research evaluating the toxicity of K+ -based compounds such as potassium permanganate to fish has been reported, as summarized by Davidson et al. (2011a); however, to the authors' knowledge, a safe ionic threshold for rainbow trout has not been established. Due to the paucity of literature regarding K + toxicity and the approximate 3.5-fold increase of K + in *mRAS*, the effects of this accumulating constituent on rainbow trout may require further research.

3.4. Denitrification

Mean NO_3-N increased steadily in the culture water of both treatments over the first 75 days of the trial (Fig. 3), following the trend for increasing daily feed as the fish grew. During this period, a gradual increase in NO_3-N was also reflected in the MBR permeate (Fig. 3) indicating that complete denitrification was not achieved within the activated sludge of the MBRs. Therefore, on days 75 and 79, 1.4 kg of sugar (~1.5% of the daily feed amount over the remainder of the trial) was added to each MBR as a supplemental carbon source. Approximately one week after sugar addition, mean NO_3-N level in the MBR permeate was reduced by ~80%, and NO_3-N in the *mRAS* culture water stabilized (Fig. 3). NO_3-N levels in *cRAS* continued to increase over the

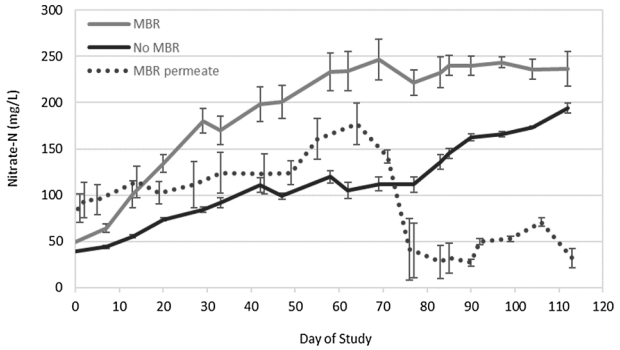

Fig. 3. Nitrate-nitrogen concentrations (mean weight ± standard error; n = 3) measured weekly in RAS operated with and without MBRs and within the MBR permeate.

Table 4
Dissolved metals/trace element concentrations (mean ± standard error) measured in RAS with and without MBRs and MBR permeate (n = 3), as well as makeup water. Baseline represents metals data collected from each RAS prior to MBR integration when flushing rates were equivalent.

	Baseline		Study Means				
Parameter (mg/L)	*mRAS*	*cRAS*	*mRAS*		*cRAS*	MBR Permeate	Makeup Water
Barium	0.186 ± 0.010	0.188 ± 0.008	0.175 ± 0.018		0.187 ± 0.013	0.090 ± 0.003	0.155 ± 0.024
Boron	0.056 ± 0.013	0.040 ± 0.003	0.069 ± 0.006		0.043 ± 0.003	0.060 ± 0.004	< det
Calcium	108 ± 0.3	107 ± 0.3	197 ± 13	*	104 ± 0.3	184 ± 10	109 ± 2
Copper	0.011 ± 0.001	0.011 ± 0.001	0.040 ± 0.001	*	0.022 ± 0.002	0.019 ± 0.003	< det
Iron	< det	< det	0.029 ± 0.002		0.018 ± 0.002	0.033 ± 0.007	< det
Magnesium	12.7 ± 0.03	12.6 ± 0.1	19.4 ± 0.6	*	13.0 ± 0.1	19.0 ± 0.5	11.5 ± 0.1
Potassium	7.8 ± 0.3	7.3 ± 0.4	35.7 ± 4.3		10.8 ± 0.4	33.8 ± 3.9	2.2 ± 0.1
Sodium	9.9 ± 0.03	9.7 ± 0.1	190 ± 22		161 ± 7	161 ± 7	7.7 ± 0.1
Strontium	0.99 ± 0.01	0.99 ± 0.01	1.05 ± 0.04		0.98 ± 0.003	1.00 ± 0.03	1.04 ± 0.02
Sulfur	10.1 ± 0.1	9.9 ± 0.1	50.3 ± 0.6	*	20.5 ± 0.6	46.6 ± 3.1	7.7 ± 0.1
Zinc	0.064 ± 0.001	0.064 ± 0.001	0.089 ± 0.008		0.070 ± 0.005	0.052 ± 0.0003	0.030 ± 0.024

*Indicates significant difference between treatments ($P < 0.05$).

study duration (Fig. 3) due to gradually increasing feed loading rate (kg feed/ m^3 makeup water/day).

In order to normalize and compare NO_3-N dilution between treatments, a ratio of daily water use to accumulated NO_3-N in the culture water was calculated. Over the study duration, *mRAS* used an average of 115 L of makeup water/day and maintained a mean concentration of 201 mg/L NO_3-N, resulting in 0.6 L of daily water use per accumulated mg/L NO_3-N. Meanwhile, *cRAS* used an average of 744 L of makeup water/day while maintaining a mean NO_3-N concentration of 117 mg/L, resulting in 6.4 L of daily water use per accumulated mg/L NO_3-N. Ultimately, *cRAS* required ten times more water to maintain NO_3-N levels at measured concentrations.

As previously mentioned, only partial denitrification was achieved in *mRAS* over the first 75 days of the trial, but denitrification capacity was boosted after sugar was added as a carbon source, indicating that backwash solids were not providing enough carbon to drive denitrification. Letelier-Gordo et al. (2015) found that settleable fecal solids discharged from flow-through systems culturing rainbow trout provided enough carbon to remove 86–156% of system nitrogen when treating the wastewater with anaerobic batch reactors. However, the same study found that lower protein:energy ratios (P:E) of aquafeeds resulted in improved hydrolysis and fermentation of fish fecal solids, thereby providing more readily available carbon for denitrification. When comparing the relatively high energy trout diet used during the present study to the diets studied by Letelier-Gordo et al. (2015), this diet was characterized as having a mid-level P:E (~19) with slightly less capacity for carbon solubilization. This may explain the requirement for minor and infrequent addition of an external carbon source (sugar) to boost denitrification. However, it is difficult to fully explain the denitrification dynamics within the MBRs because many factors beyond diet composition (Letelier-Gordo et al., 2015, 2017) influence denitrification efficiency including: microbial consortium, carbon/nitrogen ratio, type and nature of endogenous and external carbon sources, water quality (particularly oxygen and pH), and system design and operation (van Rijn, 1996; van Rijn et al., 2006).

Nevertheless, improved denitrification efficiency is a priority for future studies evaluating integration of MBRs with RAS. Nitrogen removal efficiency achieved by the MBRs during the present study was just 65.4% (Table 5), and mean NO_3-N levels in *mRAS* exceeded 200 mg/L. No significant effects to fish health or performance were noted under these conditions; however, the lack of negative effects of these elevated NO_3-N levels on rainbow trout was relatively surprising. Previous onsite research suggested that rainbow trout begin to exhibit chronic exposure symptoms (“side swimming” and rapid swimming velocity) when NO_3-N accumulates to approximately 100 mg/L (Davidson et al., 2014). Nevertheless, Camargo et al. (2005) noted that nitrate toxicity in fish varies based on a variety of conditions including exposure time, life stage, and interacting water quality parameters. In addition, a different genetic lot of rainbow trout was used during this trial compared to Davidson et al. (2014).

3.5. Alkalinity recovery

Mean alkalinity in *mRAS* and *cRAS* was 83 ± 5 and 161 ± 2 mg/L, respectively ($P < 0.05$). Sodium bicarbonate ($NaHCO_3$) was added as needed to maintain alkalinity in *cRAS* near 150 mg/L but was only added to *mRAS* during the first three weeks of the trial. Thereafter, $NaHCO_3$ addition in *mRAS* was discontinued to ascertain whether denitrification would effectively recover alkalinity. Trends depicted in Fig. 4 show the decline in alkalinity in *mRAS* after discontinuing $NaHCO_3$ addition. After sugar was added, alkalinity levels peaked in the MBR permeate (Fig. 4) coinciding with the reduction of nitrate created by improved denitrification efficacy (Fig. 3). When excluding the first three weeks of $NaHCO_3$ addition for *mRAS*, MBRs completely eliminated the requirement for $NaHCO_3$ due to alkalinity recovery, while *cRAS* required 0.21 kg $NaHCO_3$/kg feed. It should be noted that this is not a fully balanced comparison, because alkalinity levels were maintained at approximately double the concentration in *cRAS*. Reduced or eliminated addition of an alkalinity buffer results in operational cost savings. During the three-month period when $NaHCO_3$ addition was discontinued in *mRAS*, approximately $175 or $ 0.17/kg feed was spent on $NaHCO_3$ used in *cRAS*.

Although mean alkalinity levels were significantly lower in *mRAS,* the concentrations were within a suitable range for maintenance of nitrification. For instance, Summerfelt et al. (2015) reported 70 mg/L alkalinity as an acceptable target to balance nitrification and pH stability in RAS intended for Atlantic salmon *Salmo salar* smolt production. It is important to note, however, that the reduced alkalinity levels measured in *mRAS* consequently resulted in decreased pH and increased CO_2 levels in the culture water (Table 3). CO_2 levels in *mRAS* were variable and titration analyses may have been periodically affected by accumulating organic acids. Based on acid-base CO_2 calculations (Standard Method 22, 4500-CO2 D; American Public Health Association (APHA, 2012), average CO_2 in the *mRAS* was approximately 20 mg/L versus 26 mg/L measured via titration analysis (Table 3). Regardless, Good et al. (2010) found that chronic exposure to similar CO_2 concentrations did not negatively impact rainbow trout health and performance in the same replicate RAS.

3.6. Phosphorous removal

Another possible benefit of utilizing MBRs within RAS is the potential for phosphorous (P) reduction as a function of denitrification. Barak and van Rijn (2000) reported that phosphate removal in a prototype RAS was mediated by denitrifying bacteria that utilize nitrate instead of oxygen as the electron donor for P uptake. The same authors concluded that denitrification was a feasible method to control P accumulation in RAS. In addition, Sharrer et al. (2010a) reported > 99% P removal using a multi-vessel (anaerobic/aerobic) activated sludge MBR to treat aquaculture wastewater. During the present study average P removal efficiency across the MBRs was only 60%, mirroring the deficiency in nitrogen removal (Table 5). EPA (1993) recommended a BOD:TP ratio of 20-25:1 to achieve effective P removal and resulting effluent TP < 1 mg/L in anaerobic-aerobic reactor waste treatment systems to facilitate selection of phosphorous-storing microorganisms. However, the BOD:TP ratio of the backwash water provided at the inlet to the MBRs during the present study was approximately 14.5:1 and was therefore below the desired ratio, which may have influenced TP removal efficiency. Nevertheless, TP levels in the MBR permeate gradually declined over the study, indicating that improved conditions for denitrifying microorganisms such as carbon supplementation may have enhanced TP uptake and removal. The phosphorous levels measured during the present study were not toxic to fish (Kim et al., 2013), but optimized P removal efficiency is still important for reducing nutrient discharge.

3.7. Membrane filtration

Mean TSS concentrations in the culture water of *mRAS* and *cRAS* were statistically similar (Table 3). Remarkably, the MBR permeate contained an average TSS concentration of 0.3 ± 0.02 mg/L, despite being drawn from an activated sludge with TSS > 5000 mg/L (Table 5). For perspective, average TSS in the MBR permeate was slightly lower than TSS levels measured in spring/makeup water (0.5 ± 0.1 mg/L; Table 3). Average TSS removal efficiency across the MBRs was 99.7%, similar to the removal efficiencies reported by Sharrer et al. (2007, 2010a) when using MBRs to treat aquaculture wastewater.

Likewise, heterotrophic bacteria were generally excluded by membrane filtration (Fig. 5); however, this effect was not evident in the mean value in the MBR permeate, e.g., 895 ± 829 counts/mL (Table 3). At the beginning of study, when MBR permeate was first

Table 5
Waste characterization of MBR Inlet (drum filter backwash + radial flow settler flushing flows), MBR Outlet (permeate), and associated MBR waste removal efficiencies.

	MBR Sample Site	Waste Parameter	Measurement Metric	*mrAS*
Drum Filter Backwash	Inlet		mg/L	184 ± 4
Radial Flow Settler	Inlet		mg/L	8,536 ± 741
Combined Backwash	Inlet	BOD	mg/L	499 ± 66
Waste/kg feed	Inlet		kg /kg feed	0.064 ± 0.003
Permeate	Outlet		mg/L	1.5 ± 0.1
MBR Removal Efficiency	–		**%**	**99.7**
Drum Filter Backwash	Inlet		mg/L	229 ± 12
Radial Flow Settler	Inlet		mg/L	868 ± 46
Combined Backwash	Inlet	TN	mg/L	254 ± 26
Waste/kg feed	Inlet		kg /kg feed	0.037 ± 0.003
Permeate	Outlet		mg/L	~88[a]
MBR Removal Efficiency	–		**%**	**65.4**
Drum Filter Backwash	Inlet		mg/L	19.4 ± 0.5
Radial Flow Settler	Inlet		mg/L	448 ± 32
Combined Backwash	Inlet	TP	mg/L	34.5 ± 4.4
Waste/kg feed	Inlet		kg /kg feed	0.005 ± 0.001
Permeate	Outlet		mg/L	13.8 ± 1.2
MBR Removal Efficiency	–		**%**	**60.0**
Drum Filter Backwash	Inlet		mg/L	419 ± 3
Radial Flow Settler	Inlet		mg/L	24,297 ± 741
Combined Backwash	Inlet	TSS	mg/L	1,239 ± 152
Waste/kg feed	Inlet		kg /kg feed	0.177 ± 0.011
MBR Activated Sludge	MBR Vessel		mg/L	5,573 ± 1,044
Permeate (MBR Out)	Outlet		mg/L	0.3 ± 0.02
MBR Removal Efficiency	–		**%**	**99.9**

[a] TN was not measured in the MBR permeate. Approximate value provided based on nitrate-nitrogen concentration which accounted for > 98% of TN.

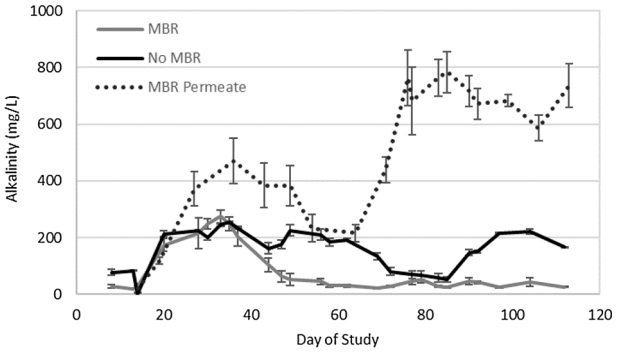

Fig. 4. Alkalinity concentrations (mean weight ± standard error; n = 3) measured weekly in RAS operated with and without MBRs and within the MBR permeate.

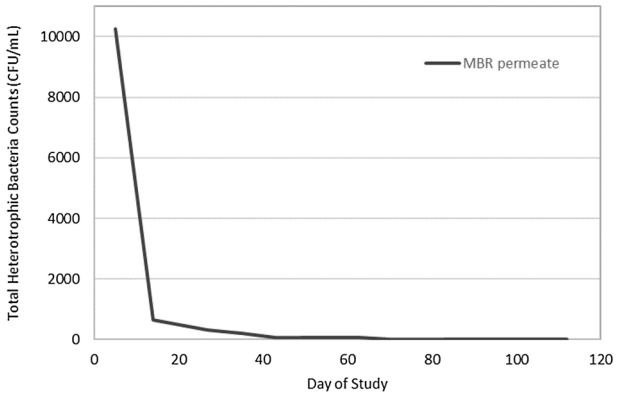

Fig. 5. Mean heterotrophic bacteria counts (cfu/mL) measured weekly in the MBR permeate returning to *mRAS*.

returned to *mRAS*, there was a two-week period when residual bacteria may have been sloughing from the permeate piping. However, total heterotrophic bacteria count in the MBR permeate declined rapidly and remained low thereafter (Fig. 5). During the final month, only one colony forming unit was observed in the MBR permeate at each weekly sampling point (Fig. 5). Despite the low bacteria count measured in the returning permeate, average bacteria counts were still significantly higher in the *mRAS* culture water (Table 3); albeit, these values were relatively low compared to those reported for other onsite research trials (Davidson et al., 2009, 2011a). Nevertheless, the mild increase in bacteria within *mRAS* suggests that optimization of biochemical processes within the activated sludge of the MBRs may also be required to limit nutrients that support microbial growth. If the membrane component of MBRs can consistently exclude bacteria in the returning permeate, and if MBRs can limit nutrient accumulation, then additional water treatment such as UV lighting can likely be excluded from the RAS design.

Membrane filtration did not limit true color of the permeate or *mRAS* culture water, resulting in an amber or tea-stained color of the fish culture environment. Dissolved organic compounds including humic substances originating from soils, sediments, and aquafeeds tend to accumulate in low exchange RAS and contribute to the colored water typical of these fish production systems (Christensen et al., 2000; Yamin et al., 2017). Onsite research (Davidson et al., 2011b) carried out in the same replicate RAS demonstrated that ozone controlled via oxidation reduction potential (250–300 mV) reduced true color by 74 and 90%, respectively, to < 5 Pt-Co units (similar to that of spring/makeup water; Table 3). Adding ozone in MBR-integrated RAS would likely remove most of the color created by dissolved organics and could result

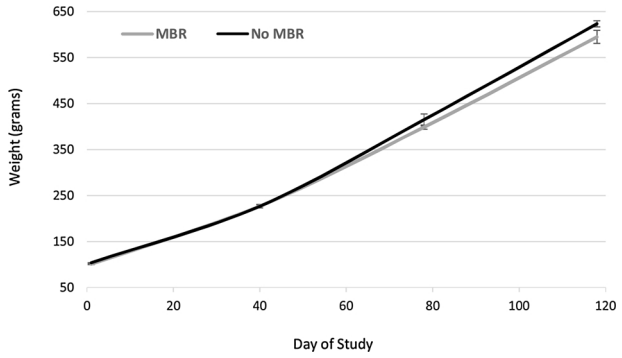

Fig. 6. Rainbow trout growth (mean weight ± standard error; n = 3) in RAS operated with and without MBRs during the four-month study.

in other improvements including increased UV transmittance and reduced TSS, BOD, and dissolved metals concentrations (Davidson et al., 2011b). A corresponding increase in rainbow trout growth was reported along with the improved water quality conditions instigated by ozone (Davidson et al., 2011b). The authors plan to utilize ozone in combination with MBR integration with RAS during future studies.

3.8. Fish performance

The culture environment differences created by integration of MBRs with RAS did not negatively affect rainbow trout growth (Fig. 6) or survival. At the end of the trial, mean rainbow trout weights in *mRAS* and *cRAS* were 595 ± 14 and 623 ± 6 g, respectively ($P > 0.05$), resulting in final biomass densities of 71 ± 2 and 74 ± 1 kg/m^3. Likewise, no differences were detected between treatments for average thermal growth coefficient, i.e., 2.31 ± 0.06 and 2.37 ± 0.06 for *mRAS* and *cRAS*, respectively. Although no significant differences in mean weight were detected between treatments, a mild separation in growth curves was evident over the last month of the study when rainbow trout reared in *cRAS* began to grow slightly faster. In addition to growth performance, cumulative rainbow trout survival (97.9 ± 0.1 in *mRAS* and 98.3 ± 0.2% in *cRAS*) was not affected, and feed conversion ratio (1.07 ± 0.03 and 1.09 ± 0.01 for *mRAS* and *cRAS*, respectively) was not influenced by the presence of MBRs.

Fin scores were recorded as a qualitative measure of fish health and welfare. To begin, cumulative fin scores were 2.1 ± 0.1 for fish in *mRAS* and *cRAS*, indicating minor fin damage. By study's end, fin quality had improved slightly for both treatments and remained statistically similar between *mRAS* and *cRAS*, 1.7 ± 0.03 and 1.6 ± 0.04, respectively. However, the left pelvic fin showed significantly greater damage for fish reared in *mRAS* by study's end with a mean score of 2.8 ± 0.1 versus 2.2 ± 0.1 in *cRAS*. Interestingly, the left pelvic fin score for *mRAS* fish was identical to that recorded at the beginning of the trial, while the left pelvic fin of *cRAS* fish exhibited minor healing. The authors surmise that the left pelvic fin is exposed to biting and fish-to-fish interaction due to continuous counter-clockwise swimming direction, while the right pelvic fin, which scored 1.4 ± 0.1 and 1.3 ± 0.02 is generally protected by the tank wall. The reason for a healing effect of the pelvic fin in *cRAS* is unknown but could be related to differences in water quality reported between treatments.

3.9. Waste characterization

Waste concentrations, MBR removal efficiencies, and waste mass per kg feed contained in the RAS backwash water are described in Table 5 as reference for future MBR-RAS applications. For clarity, it is important to note that the reported waste mass per kg feed treated by the MBRs does not comprise the full mass balance due to the scope of the study. A portion of wastes not accounted for in the mass balance was recycled and maintained within the RAS culture water. As a comparative example, Davidson and Summerfelt (2005) reported 21.6–22.7% TSS production per kg fish feed when using a complete mass balance, and Timmons et al. (2001) reported a general value of 25% TSS/ kg feed. In this study, 17.7% of the feed mass was contributed to the MBR from solids collected by the drum filter and radial flow settler (Table 5), while an additional (but unaccounted for) solids contribution per kg feed remained in suspension within the RAS water, and a small but negligible solids fraction was returned to the RAS in the MBR permeate (Table 3). Explanation of waste production per unit feed for BOD, TN, and TP can be expounded upon similarly. When considering the full mass balance, it is also important to note that the MBRs also appeared to digest biosolids, because manual flushing, aside from the removal of small sample volumes for TSS evaluation, was not required to control the TSS concentration of the activated sludge.

3.10. Off-flavor

Geosmin and MIB concentrations in RAS water and rainbow trout fillets were not affected by the presence or absence of MBRs (Table 6). These common off-flavors were generally measured at levels below or bordering the instrument detection limit of 1 ng/L in MBR permeate samples and RAS water from both treatments (Table 6). The low to non-detectable geosmin and MIB concentrations in permeate and RAS water seem contrary to the gradual increase in off-flavor concentrations measured in rainbow trout fillets; however, it is important to note that these data points represent "snapshots" in time. While off-flavor levels in the culture water were low during these sampling events, there may have been periods between samples when geosmin and MIB concentrations increased, thereby contributing to bioaccumulation of these compounds in rainbow trout flesh. Nevertheless, the final geosmin and

Table 6
Geosmin and MIB concentrations (mean ± standard error) measured in water (ng/L) and rainbow trout fillets (ng/kg) collected from RAS with and without MBRs (n = 3).

		Baseline		Mid-Study		End Study	
Sample	Off-Flavor Compound	MBR	Control	MBR	Control	MBR	Control
Side Drain Water (ng/L)	Geosmin	1 ± 0.3	< det[a]	< det	2 ± 1	2 ± 1	1 ± 1
MBR Permeate (ng/L)	Geosmin	1 ± 1	–	1 ± 0.3	–	< det	–
Makeup Water (ng/L)	Geosmin	< det	< det	< det	< det	< det	< det
Side Drain Water (ng/L)	MIB	2 ± 1	2 ± 2	< det	< det	< det	< det
MBR Permeate (ng/L)	MIB	2 ± 1	–	< det	–	1 ± 1	–
Makeup Water (ng/L)	MIB	< det	< det	< det	< det	< det	< det
Trout Fillets (ng/kg)	Geosmin	52 ± 4	50 ± 11	117 ± 20	72 ± 7	353 ± 105	160 ± 34
Trout Fillets (ng/kg)	MIB	44 ± 9	46 ± 13	48 ± 13	207 ± 159	118 ± 33	103 ± 22

[a] Below the instrumental detection limit of 1 ng/L.

MIB levels measured in rainbow trout fillets were below the reported human sensory detection thresholds in trout of 900 ng/kg (Robertson et al., 2005) and 550 ng/kg (Persson, 1980), respectively.

Organic-rich, anaerobic conditions like those common within MBRs have shown potential for uptake and remediation of geosmin and MIB (Guttman and van Rijn, 2008). The low to non-detectable geosmin and MIB levels measured in the MBR permeate (Table 6) may reflect this outcome; however, these off-flavor levels were also generally low in RAS water. The location in RAS from which inlet spray water for the drum filters is drawn may be an important consideration for geosmin and MIB reduction by MBRs. Ideally, inlet water to the MBRs should be taken from a RAS location where the highest off-flavor levels are expected to be produced. For example, Schrader and Summerfelt (2010) found that aerobic zones such as the microscreen drum filter and inside of heat exchangers are likely sources of the filamentous bacteria (actinomycetes) that produce these compounds. During this study, drum filter spray water, which subsequently became inlet water to the MBRs, was removed from the RAS after the drum filter but prior to the heat exchangers. Additionally, measurements of geosmin and MIB concentrations in the solids-laden backwash entering the MBRs would provide a better understanding of the adsorption potential of the activated sludge for these off-flavor compounds. As such, the findings from this study are inconclusive regarding geosmin and MIB uptake across MBRs, and additional research is required to determine the effectiveness of MBRs for remediation of these common off-flavor compounds.

3.11. MBR operation and optimization

3.11.1. Permeate flux rate

While positive outcomes for MBR integration with RAS were achieved, a range of deficiencies were also realized that require optimization and improvement. For example, average permeate flow rates produced by MBRs were less than design expectations. Reduced permeate flux resulted in several consequences including imbalance of MBR inlet and outlet flows, periodic overflow of MBR vessel contents which required subsequent water replacement, and reduced exchange of RAS water through the MBR which likely limited reduction of NO_3-N in the fish culture tank. Membrane fouling has been described as a limitation of MBRs in industrial and municipal waste treatment applications and is an important consideration for the MBR design and operation (Gkotsis et al., 2014; Iorhemen et al., 2016). A detailed review of membrane cleaning and biofouling mitigation techniques was provided by Gkotsis et al. (2014) including chemical (hypochlorite or acid treatment) and physical cleaning methods (backflushing, aeration/air-scouring, membrane relaxation), coagulant addition, and ultrasound, among other less common procedures. Specific recommendations for controlling membrane biofouling are generally provided by the manufacturer. During this study, one membrane cleaning attempt was carried out for MBR 3, which consistently demonstrated the slowest permeate rate. A 0.5% muriatic acid (31% hydrochloric acid) solution was pumped back to the membranes through the permeate outlet pipe using a peristaltic pump. The next day, after purging the pipes, a 0.5% sodium hypochlorite solution was applied using the same technique. These cleaning attempts resulted in minimal improvement to the permeate flow rate of MBR 3. Due to the lack of success with these procedures and the relatively short study duration, we opted not to carry out additional membrane cleaning and relied solely on air scouring for biofouling control. Manufacturer recommendation for membrane cleaning frequency was every two to three months depending on conditions within the MBR. A standard operating procedure for cleaning will need to be adopted for future studies and is recommended for facilities utilizing MBRs. In addition, greater control over the permeate flow could be achieved by installing a suction pump on the outlet side of the membranes (Ueda et al., 1997).

Optimization of permeate flux rate would likely drive other improvements for MBR integration with RAS. For example, increased permeate flow would enhance NO_3-N reduction in RAS due to increased exchange of the RAS water volume through the MBR. Based on a mass balance assessment, the daily flow rate from the RAS to the MBRs should be doubled to decrease the steady-state NO_3-N level to 100 mg/L, which has been reported as the upper limit for rainbow trout (Davidson et al., 2014). This may require additional water flushing than provided by the natural backwash cycle of RAS. Under these circumstances, a small stream (0.5–1 L/min) could be metered into the backwash sump as extra inlet water to the MBRs.

3.11.2. Aeration and mixing

The system design and original operating protocols assumed that dissolved oxygen within the activated sludge would be rapidly depleted by microorganisms resulting in mildly aerobic conditions. An option to utilize external air or internal headspace air of the MBR for aeration and mixing was provided via a three-way valve at the inlet of respective blowers. Early in the study, it was quickly determined that continuous aeration using either air option was not a feasible method for maintaining low oxygen conditions that support denitrification. As such, an on/off relay timer was installed and integrated with the blowers to limit oxygenation of the activated sludge. The on/off ratio was periodically reduced to balance aerobic conditions that simultaneously supported nitrification and denitrification. Ultimately, blowers were generally operated using a 1 min on/20 min off cycle. Potential drawbacks of this approach were reduced frequency of air scouring of the membrane surfaces and diminished mixing of the activated sludge. These operational changes could have contributed to biofouling of the membrane surfaces and the decline in permeate flux. When utilizing on/off aeration, a separate mixing pump could be utilized to keep the mixed liquor solids in perpetual suspension.

3.11.3. MBR vessel configuration

Previous onsite research with MBRs (Sharrer et al., 2007, 2010a) utilized multi-compartment systems, in which the membrane and activated sludge processes were separated within different vessels. The shift to single-vessel MBRs for this study was based on simplicity of design and operation to minimize complexity for fish farmers using RAS and to lessen costs and capital investment for equipment and fiberglass. In multi-vessel MBRs anoxic and aerobic processes are separated, which may allow greater balance between microbial populations responsible for nitrification and denitrification. A multi-vessel configuration also provides easier access to the membranes for cleaning procedures and membrane removal and replacement; therefore, future research with MBR-RAS applications should consider this alternate design.

4. Conclusions

Overall, results from this preliminary trial indicate that integrating MBRs within the water treatment loop of freshwater RAS is biologically feasible for rainbow trout production despite several deficiencies in MBR performance and operation. This research indicates that MBRs are a viable option for reclaiming and processing RAS backwash water directly within the RAS as opposed to the standard practice of utilizing external waste treatment technologies. As such, MBR integration with RAS could reduce the infrastructure requirement for water supply and waste treatment. Other related advantages may include reduced waste discharge volume, improved effluent quality to meet increasingly stringent point source discharge standards, and general water conservation. Water savings is likely the most important implication for including MBRs as a unit process within RAS, as the reduced water requirement increases flexibility for siting facilities where water resources are scarce and/or near local markets and may allow commercial producers to expand fish production capacity with a smaller water footprint requirement. Additional research is needed to evaluate MBR integration with RAS while addressing improvements realized during this research such as optimizing MBR permeate rates, increasing RAS

water exchange through the MBRs, and using a relatively small but critical supplemental carbon source to enhance denitrification efficiency.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Study VII

Effects of ozone on post-smolt Atlantic salmon Salmo salar performance, health, and maturation in freshwater recirculation aquaculture systems

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Effects of ozone on post-smolt Atlantic salmon (*Salmo salar*) performance, health, and maturation in freshwater recirculation aquaculture systems

John Davidson [a,*], Steven Summerfelt [a,1], Åsa Maria O. Espmark [b], Vasco C. Mota [b], David Marancik [c], Ryan L. Earley [d], Anthony Snead [d], Christopher Good [a]

[a] The Conservation Fund Freshwater Institute, 1098 Turner Road, Shepherdstown, WV 25443, USA
[b] Nofima AS, Tromsø, NO 9291, Norway
[c] Department of Pathobiology, St. George's University, School of Veterinary Medicine, Grenada, West Indies
[d] Department of Biological Sciences, University of Alabama, 300 Hackberry Lane, Tuscaloosa, AL 35401, USA

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ABSTRACT

Steroid hormones accumulate in recirculation aquaculture systems (RAS) and may influence the reproductive physiology of farmed fish. Ozone reduces hormone concentrations in freshwater RAS used to rear Atlantic salmon, but its effect on reproductive development is unknown. Accordingly, an 8-month trial was carried out to evaluate the growth, health, and maturation of post-smolt Atlantic salmon (296 ± 4 g initial weight) reared in six replicated freshwater RAS (9.5 m^3 total volume) operated with or without ozone (N = 3/treatment). Residual ozone was controlled with an oxidation reduction potential (ORP) of 300–320 mV, and mean water temperature was maintained at 14.7 °C. Atlantic salmon growth was generally faster in ozonated RAS. Salmon from RAS with and without ozone weighed 2156 ± 101 and 1810 ± 15 g, respectively, by the end of the study. Caudal, anal, and pelvic fin damage was greater ($P < 0.05$) for salmon in ozonated RAS early in the trial but improved thereafter. No statistical differences in gill, skin, and skeletal muscle histopathology were observed between treatments at the end of the study. Waterborne estradiol, testosterone, and 11-ketotestosterone levels were periodically lower ($P < 0.05$) in ozonated RAS, but maturing salmon were more prevalent in these systems. At the end of the trial, percent maturation of salmon populations reared in RAS with and without ozone was 63 ± 7 and 48 ± 1%, respectively; however, maturity appeared to be related to fish size. Improved water quality was observed in ozonated RAS including reduced dissolved copper, iron, and zinc levels, total heterotrophic bacteria counts, and true color, and increased ultraviolet transmittance, which may have supported improved Atlantic salmon growth. Overall, ozone did not inhibit the onset or prevalence of Atlantic salmon maturation, but significant improvements in water quality and salmon growth performance resulted from its use.

1. Introduction

Many Atlantic salmon farms are now producing smolts and post-smolts using land-based recirculating aquaculture systems (RAS) (Bergheim et al., 2009; Dalsgaard et al., 2013), and a number of companies are producing or planning to produce market-size Atlantic salmon in RAS (Summerfelt and Christianson, 2014; Intrafish, 2018). Nevertheless, commercial development of a RAS industry for Atlantic salmon is still at an early stage, and precocious maturation has emerged as a challenge, particularly in mixed sex populations grown to market-size (Davidson et al. 2016; Good and Davidson, 2016). Atlantic salmon producers generally view early maturation as a significant problem due to coinciding physiological changes that include decreased growth and feed conversion efficiency (McClure et al., 2007), increased sensitivity to opportunistic infection (St-Hilaire et al., 1998; Taranger et al., 2010), and reduced flesh quality (Aksnes et al., 1986; Michie, 2001; Davidson et al. 2016; Davidson et al., 2017). These biological and product quality impacts generally equate to economic losses for Atlantic salmon farmers (McClure et al., 2007; Good and Davidson, 2016); therefore, early maturation should be reduced or eliminated in RAS-produced salmon to improve the economic viability of this aquaculture sector.

The onset and development of salmon maturation, however, is a

* Corresponding author.
E-mail address: jdavidson@conservationfund.org (J. Davidson).
[1] Present address: *Superior Fresh, W15506 Superior Fresh Drive, Hixton, WI 54635.*

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complex, multifactorial process that is influenced by a range of environmental (e.g., photoperiod, water temperature) and biological variables (e.g., feed intake, growth performance, condition factor, lipid reserves, and genetics) (McClure et al., 2007; Taranger et al., 2010; Good and Davidson, 2016). Therefore, causes for increased maturation in RAS are still under investigation. To add to the complexity, when RAS are operated with limited water exchange, dissolved nutrients and compounds accumulate in the culture water (Davidson et al., 2009; Martins et al., 2009), including some which can impact the endocrine system of fish, such as nitrate (Freitag et al., 2015; Good et al., 2017a; Kellock et al., 2018). Sex steroids are also produced by fish and can be excreted into water (Vermeirssen and Scott, 1996; Ellis et al., 2005; Sorensen et al., 2005). Recent trials have shown that steroid hormones including testosterone (T), 11-ketotestosternone (11-KT), estradiol (E2) (Good et al., 2014), and cortisol (Mota et al., 2014) can accumulate in RAS. Evidence of uptake and sensing of waterborne T by rainbow trout *Oncorhynchus mykiss* cultured in RAS has also been reported (Budworth and Senger, 1993), and Mota et al. (2014) suggested that sex steroid levels measured in RAS are within the olfactory sensitivity range of some fish species. Further, Leet et al. (2011) reported that exposure to exogenous hormones in natural environments can: i) disrupt biochemical and endocrine processes essential to reproduction, ii) alter gene expression related to sex determination and sexual differentiation, and iii) cause masculinization, femininization, intersex, and skewed sex ratio effects in fish populations. In addition, waterborne hormones are commonly administered via immersion to early life stage fish as a method to influence sexual differentiation and reversal (Piferrer and Donaldson, 1994; Hoga et al., 2018). Considering this body of research and the role that endogenous sex steroids play in fish maturation (e.g., Schulz and Miura, 2002; Taranger et al., 2010; Tokarz et al., 2015), it is reasonable to suspect that waterborne hormones could influence the endocrine function and onset of maturation in RAS-produced Atlantic salmon.

Within this framework, it is important to investigate water treatment technologies that could reduce hormone concentrations in RAS. For example, ozone, a commonly used water-oxidizing technology that imparts water quality improvements in RAS (Summerfelt and Hochheimer, 1997; Summerfelt, 2003; Davidson et al., 2011; Gonclaves and Gagnon, 2011; Powell and Scolding, 2018), reportedly reduces or eliminates specific waterborne hormones in non-aquaculture applications (Westerhoff et al., 2005; Broséus et al., 2009; Kawasaki et al., 2009). Moreover, Good et al. (2017b) found that ozone application to maintain 290–300 mV ORP reduced waterborne E2 and resulted in generally lower concentrations of T and 11-KT in a freshwater RAS stocked with a mix of immature and mature post-smolt Atlantic salmon (>1.2 kg). Similar research evaluating the potential for ozone to reduce or eliminate maturation in smaller, immature Atlantic salmon, putatively via reduction of waterborne steroid hormones, is therefore a worthwhile follow-up study to improve our understanding of salmon maturation in RAS.

To this end, a study was carried out to evaluate the effect of operating replicate RAS with and without low-dose ozone on the incidence of early maturation in post-smolt Atlantic salmon (<300 g initial weight), and to provide a comprehensive assessment of ozone's effect on salmon performance, health, and welfare. The authors hypothesized that the use of ozone would: i) reduce waterborne hormone concentrations, leading to reduced prevalence of early maturation, and ii) promote Atlantic salmon growth as a function of water quality improvements.

2. Materials and methods

2.1. Atlantic salmon

Mixed-sex Atlantic salmon were received as fertilized eyed eggs from Stofnfiskur (Hafnarfjörður, Iceland) and hatched onsite within a Heath-tray-style RAS incubation system. Following yolk sac absorption, juvenile salmon were transferred to a flow-through system with 24-h LED lighting where they were grown to 70–80 g. At this time, half of the fish were switched to 12-h:12-h light/dark (LD) to simulate an early winter and to induce smoltification per industry standard procedures, while the other half of the population remained on 24-h light (L). Photoperiod evaluation was included due to: i) the importance of this variable for maturation signalling, and ii) conflicting photoperiod × maturation results reported elsewhere (Fjelldal et al., 2011; Good et al., 2016; Hines et al., 2019). Following the 52-day artificial winter photoperiod, the adipose fin of salmon exposed to 24-h L was clipped for future identification, and fish were maintained for one additional month in a partial reuse system described by Summerfelt et al. (2004). The entire pre-study culture period was carried out using freshwater maintained at 12.5–14.5 °C. Thereafter, 500 salmon (250 fish from each photoperiod) were stocked within the six replicate RAS used for the trial (Fig. 1). To begin the study, mean Atlantic salmon weight among replicate RAS was 296 ± 4 g and initial biomass density was 28 kg/m^3. A 2-wk acclimation period was provided to allow fish to adjust to the new environment before adding ozone.

2.2. Recirculation aquaculture systems

Six replicate RAS operated with or without ozone ($N = 3$/treatment) were used for the 8-month study (Fig. 1) (Davidson et al., 2009). Each RAS (9.5 m^3 total volume) recirculated 340 L/min of freshwater through a 5.3 m^3 dual drain culture tank, radial flow settler, microscreen drum filter, fluidized sand biofilter, geothermal heat exchanger, gas conditioning column, and a low-head oxygenator (LHO) (Fig. 1). Three replicated RAS received ozone produced from a pure oxygen feed gas by a Model G22 generator (Pacific Ozone Technology, Benicia, CA, USA). Ozone gas (9–10% O_3 measured by Ozone Monitor M4654, Teledyne Instruments, San Diego, CA, USA) was added within the air space beneath the LHO water distribution plate (Fig. 1). To prevent ozone residuals from reaching unsafe levels, oxidation reduction potential (ORP) was monitored using a digital sensor (Model DRD1R5, Hach Company, Loveland, CO, USA) located near the tank inlet. SC100 Universal Controllers (Hach Company) provided proportional-integral-derivative control of ozone generator output to maintain target ORP levels at 300–320 mV.

RAS were operated with mean hydraulic retention times (HRT) of 14.9 ± 0.9 days (~7% of system water exchange/day) and feed loading rates of 3.6 ± 0.1 kg feed/m^3 of makeup water per day. RAS dilution rate was dictated by the discharged wastewater volume, which was sensed and replaced with new water via a float valve. Makeup water addition was measured in each RAS by a magnetic drive flowmeter (Model C700, Elster AMCO Water Inc., Ocala, FL, USA). Sodium bicarbonate ($NaHCO_3$; Church & Dwight Co. Inc., Ewing, NJ, USA) was periodically added to maintain alkalinity levels that support nitrification (Boyd et al., 2016). Lastly, a 12:12 LD photoperiod was provided throughout the trial, but approximately 5 lx was maintained during the "dark" period to facilitate 24-h feeding and semi-constant water quality conditions.

2.3. Feeding

Salmon were fed to apparent satiation using a computer operated system (TCFFI, Shepherdstown, WV, USA) programmed to deliver short feed bursts once per hour via automated feeders (T-drum 2000 CE, Arvotec, Huutokoski, Finland). Feeding rates were fine-tuned separately per RAS based on observations of feeding activity and wasted feed. Uneaten feed was collected four to five days per week from the cone bottom of radial flow settlers, rinsed to remove fecal material, and weighed in order to gain a general comparison of unconsumed feed amounts between treatments. A commercially available 44/29 (protein/fat - %) salmon diet (EWOS Dynamic Red ™, Cargill, Wayzata, MN, USA) was fed throughout the study.

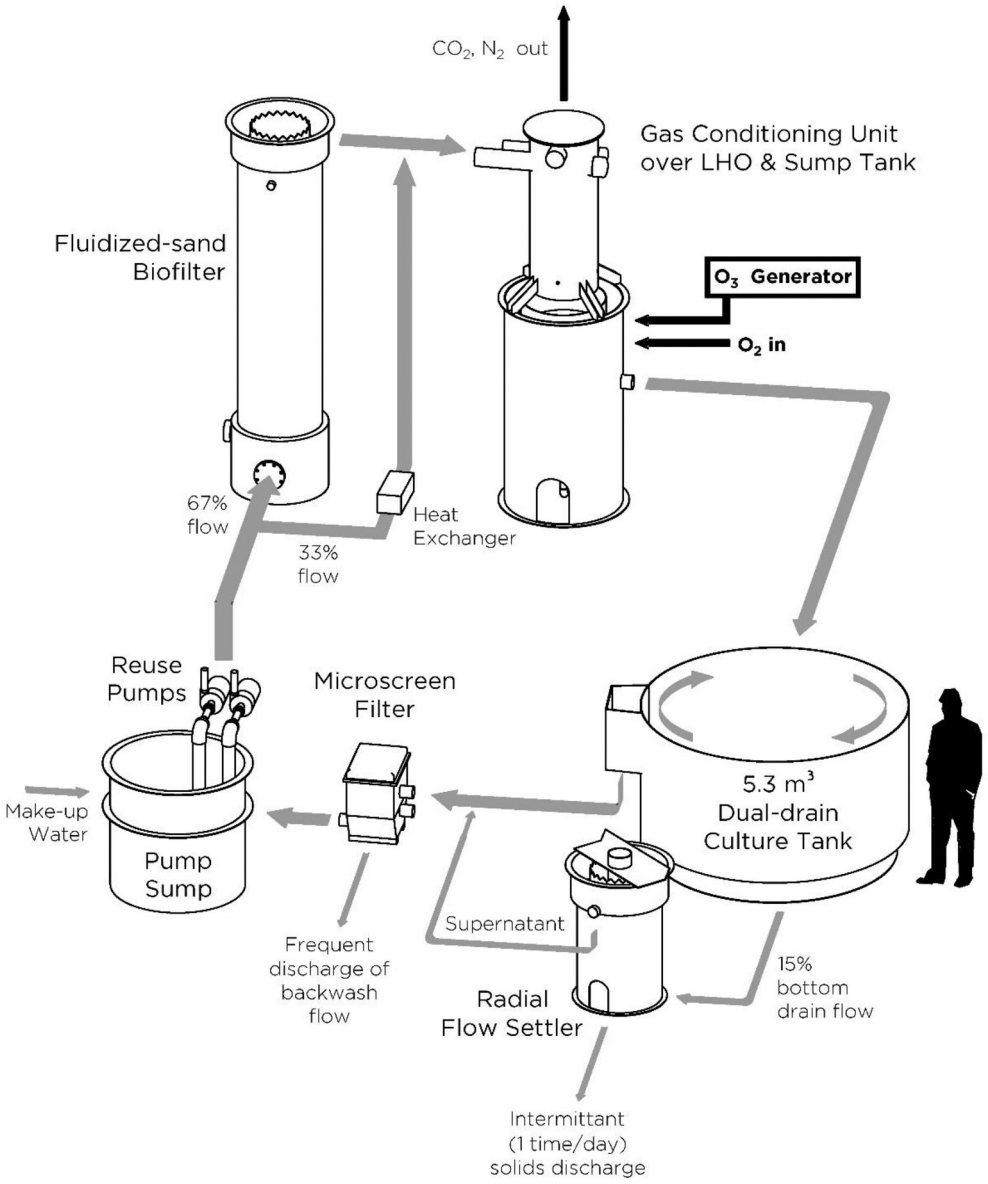

Fig. 1. Water flow and process design for an individual recirculation aquaculture system.

2.4. Fish sampling

Length and weight measurements of a random sample of 60 fish per RAS (~30 per photoperiod group) were collected to begin the study and thereafter at approximately 2-month intervals. Fish sample size was calculated using equations from Bhujel (2008) and Martin et al. (1987). Maturity status was also noted for all sampled fish where sexually mature salmon were identified by morphology characteristics, i.e., bronze coloration and prominent kype in males and ovipositor in females. External welfare indicators including eye cataracts, operculum, skin, snout, and fin damage were also scored for each fish ($n = 60$/RAS) according to guidelines established by Noble et al. (2018). Cataracts were scored with the naked eye using a 0–4 scale where absence was denoted 0 and severe cataracts covering >75% of the eye lens was scored as 4. All other welfare metrics were scored using a 0–3 scale where lack of damage was denoted 0 and severe damage/erosion was scored as 3. Welfare scores of fish sampled from each RAS were averaged and a grand mean was calculated for each treatment ($N = 3$). In accordance with onsite IACUC guidelines and maintenance of fish welfare, fish from each RAS were randomly culled midway through the trial to reduce the population by 50% and to maintain maximum fish density at <100 kg/m^3. Additionally, gonadosomatic index (GSI) percentage was assessed in a subsample of fish from each RAS ($n = 5$ - Month 2; $n = 30$ - Months 4, 6, 8) after fish began to demonstrate morphology consistent with early maturation. GSI (%) was calculated as follows: (gonad weight/ total body weight) * 100. Maturity was denoted for fish with GSI ≥ 1.0%.

Thermal growth coefficient (TGC), feed conversion ratio (FCR), and fish survival (%) were calculated bimonthly and/or cumulatively using the following formulae:

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

where weight is in grams, length is in mm, and temperature is in °C.

$$\mathrm{FCR} = \text{Cumulative Feed Delivered} / \text{Biomass Gain (BG)}$$

where BG = ((mean weight × number of fish $^{\text{after}}$) – (mean weight × number of fish $^{\text{before}}$)).

$$\text{Survival}\ (\%) = ((\text{Initial Number of Fish–Cumulative Mortalities\&Culls}) / \text{Initial Number of Fish})*100$$

2.5. Histopathology

Histopathology was carried out on five randomly selected fish per RAS at the completion of the study through assessments of gill tissue collected from the second arch, left side and a 0.5 × 0.5 cm section of skin tissue collected along the lateral line, ventral to the dorsal fin. All sampled fish were euthanized prior to tissue collection with 200 mg/L tricaine methanesulfonate. Representative samples of gill, skin, and underlying skeletal muscle were carefully removed using stainless steel scissors and forceps and preserved in 10% buffered formalin. Tissues were then processed routinely, sectioned at 4 μm, and stained with hematoxylin and eosin. Slides were examined blindly by a single pathologist using light microscopy and observed tissue alterations were semi-quantitatively scored on a 0-3-point scale based on cellular and extracellular changes and inflammatory infiltrates (0 representing no tissue change, and 3 representing severe changes observed). Specific pathological outcomes examined and scored included mononuclear cell infiltrates, eosinophilic granular cell infiltrates, goblet cell density, epithelial hyperplasia, lamellar adhesion and fusion (gill only), and cellular necrosis.

2.6. Water quality sampling and analyses

Water samples were collected from RAS tanks and makeup water and tested onsite using methods described by APHA (2012) and HACH Company (2003, 2015) (Table 1). Eleven select dissolved metals/elements were analyzed based on positive detection during previous studies in the same replicate RAS (Davidson et al., 2011, 2014). Metals analysis was carried out by REI Consultants Inc. (Beaver, WV, USA) on water samples collected once every two months.

2.7. Waterborne hormone analysis

Water for hormone analysis was collected from RAS tanks and makeup water after salmon from both treatments began to exhibit increasing morphologic signs of maturity. Samples were collected in 500 mL high density polyethylene bottles on study days 136, 164, 197, and 245, placed in freezer storage at -20 °C, and shipped in bulk to the University of Alabama after the study concluded. Waterborne hormones were extracted and assayed using enzyme-immunoassay (EIA) kits (Cayman Chemicals Inc., Ann Arbor, MI, USA) for T, 11-KT, E2 and cortisol in the same manner as described in Good et al. (2017b). To validate the EIA kits and determine appropriate dilution factors for each sample, 30 μL from each resuspended hormone sample of a particular type (i.e., tank or influent) was combined into a pool, which was then diluted from 1:1 (undiluted) to 1:32 (cortisol), 1:64 (11-KT 'influent') or 1:128 (T, 11-KT 'tank', E2) to generate serial dilution curves. All serial dilution curves were parallel to the standard curve, as assessed via the slope comparisons test (Zar, 1996): cortisol – tank: $t_9 = 0.021$, $p = 0.98$; cortisol – influent: $t_9 = 0.02$, $p = 0.99$; 11-KT – tank: $t_{12} = 0.016$, $p = 0.99$; 11-KT – influent: $t_{11} = 0.188$, $p = 0.85$; T – tank: $t_{12} = 0.022$, $p = 0.98$; T – influent: $t_{12} = 0.685$, $p = 0.51$; E2 – tank: $t_{10} = 0.121$, $p = 0.91$; E2 – influent: $t_{10} = 0.275$, $p = 0.79$. Samples were diluted as necessary to ensure that the concentrations would fall on the linear phase of the standard curve; these dilutions were: cortisol (tank and influent) – 1:4; E2 (tank and influent), T (influent), and 11-KT (influent) – 1:1 (i.e., no dilution); T (tank) and 11-KT (tank) – 1:10. Samples were run on two (cortisol, T, 11-KT) or three (E2) 96-well plates with pooled hormone extracts run in duplicate at the beginning and end of each plate to calculate intra- and inter-assay coefficients of variation, all of which were below 11% (intra-assay, cortisol – plate 1, plate 2: 4.4%, 4.3%; 11-KT: 4.2%, 4.1%; T: 1.4%, 9.9%; E2: 3.5%, 4.9%, 5.6%; inter-assay, cortisol: 3.5%; 11-KT: 5.8%; T: 10.6%, E2: 5.7%).

Table 1
Water quality parameters evaluated, methodologies, and frequency of testing.

Parameter	Method of Analysis	Frequency of Recording/ Testing
Dissolved Oxygen	Hach SC100 Controller & LDO® Probe	Daily
Oxidation Reduction Potential	Hach SC100 Controller & Differential ORP Sensor	Daily
Temperature	Hach SC100 Controller & Differential ORP Sensor	Daily
Specific Conductance	YSI 30 Salinity/Conductivity/ Temperature Meter	3–4 times weekly
Alkalinity	Hach Method 8203 - Sulfuric Acid Digital Titration pH endpoint Accumet #AB150	2–3 times weekly
pH	Standard Methods 4500-H^+ B – Electrode	2–3 times weekly
Biochemical Oxygen Demand	Standard Methods APHA 5210B - 5-day test (No prefiltration) YSI Model 58, YSI BOD probe #5905	Once weekly
Carbon Dioxide	Hach Method 8223 - Sodium Hydroxide Burette Titration pH endpoint Accumet #AB150	Once weekly
Dissolved Ozone	Hach Method 8311 (0.01–1.5 mg/L as O_3)	
Nitrate Nitrogen	Hach Method 8171 - Cadmium Reduction	Once weekly
Nitrite Nitrogen	Hach Method 8507 USEPA Diazotization	Once weekly
Total Ammonia Nitrogen	Hach Method 8038 USEPA Nessler	Once weekly
Total Heterotrophic Bacteria	Hach Method 8242 - Membrane Filtration, Fischer Isotemp Incubator #516D	Once weekly
Total Phosphorus	Hach Method 8190 – USEPA PhosVer3 with Acid Persulfate Digestion. DRB200 reactor and Hach Method 10,127 (Molybdovanadate w/ Acid Persulfate Digestion)	Once weekly
Total Suspended Solids	Standard Methods APHA 2540D - Dried at 103–105 ° C. Thelco Oven #6540, Mettler Toledo #AE240 and #PM30K	Once weekly
True Color	Hach Method 8025 - Platinum-Cobalt Standard	Once weekly
UV Transmittance	Hach Method 10,054 - Organic UV Absorbing (UV-254)	Once weekly
Dissolved Metals	Inductively Coupled Plasma Atomic Emission Spectrometry	Monthly - 4 events

-Spectrophotometers DR2700 and DR6000 (Hach Company, Loveland, CO, USA) were used for analysis of dissolved ozone, nitrate nitrogen, nitrite nitrogen, total ammonia nitrogen, and total phosphorus. Spectrophotometer DR4000 (Hach Company) was used for analysis of true color and UV transmittance.

2.8. Statistical analysis

Water quality data were analyzed using a restricted maximum likelihood mixed models test that assigned water quality criterion as dependent variables; treatment, time, and treatment × time as independent fixed factors; and RAS/tank as a random effect nested within treatment (Ling and Cotter, 2003; Thorarensen et al., 2015). Data transformation and/or removal of outliers was carried out as needed when analyzing water chemistry data. Fish performance, feeding, welfare, maturity metrics, dissolved metals, and waterborne hormone concentrations were analyzed using a two-sample Student's *t*-test (means comparison), or in the case of non-Gaussian distributed data, a Kruskal Wallis test. Two-factor ANOVA was utilized to evaluate side by side and interactive effects of primary treatment (ozone v. no ozone) and pre-study photoperiods. Ordered logit regression was carried out for scored histopathology data for each sampling point and tissue lesion type. A probability level of 0.05 was used to determine significance for all tests. Statistical analyses were carried out using SYSTAT 13 software

(2009) except for analysis of histopathology and hormones data, which were assessed using STATA v. 16.1 (StataCorp, College Station, TX, USA).

3. Results and discussion

3.1. Water quality

Important water quality criteria including alkalinity, dissolved oxygen, pH, and water temperature were controlled between treatments (Table 2). A range of other water quality variables were measured at significantly different concentrations between ozonated and non-ozonated RAS including ORP, total heterotrophic bacteria count (THBC), true color, and ultraviolet transmittance (UVT) (Table 2), as well as dissolved metals including copper, iron, and zinc (Table 3). Of these parameters, true color, THBC, copper, iron, and zinc levels were lower in ozonated RAS, while UVT and ORP were greater (Tables 2, 3), reflecting similar water quality improvements that have been observed onsite in ozonated RAS (Davidson et al., 2011; Good et al., 2017b). The implications of water quality differences to Atlantic salmon growth, health, and welfare are selectively discussed in the following sections.

3.2. Atlantic salmon growth and survival

First evidence of separation in Atlantic salmon growth curves was observed after two months as indicated by greater mean weights of sampled fish in ozonated (750 ± 9 g) versus non-ozonated RAS (637 ± 9 g) (Fig. 2). This trend continued throughout the study with statistical comparison indicating either higher mean weights in ozonated RAS or a borderline treatment effect (Fig. 2; Table 4). Resulting *P*-values at Months 2, 4, 6, and 8 were 0.001, 0.074, 0.011, and 0.073, respectively, where variance of means within treatment shifted the statistical outcome at Months 4 and 8. Metrics that considered fish weight such as fish biomass and density followed similar statistical trends (Table 4). Average TGC calculated across the study for salmon cultured in ozonated and non-ozonated RAS was 1.75 ± 0.04 and 1.57 ± 0.03, respectively ($P < 0.05$). However, bimonthly analysis indicated that TGC was greater for ozonated RAS during the first two months but similar between treatments thereafter (Table 4), suggesting that the brunt of the growth effect was dictated early in the study. By the end of the trial, salmon cultured in RAS with and without ozone weighed 2156 ± 101 and 1810 ± 15 g, respectively (Fig. 2; Table 4). Although growth was significantly impacted by treatment, survival was not. Cumulative Atlantic salmon survival in RAS with and without ozone was 98.7 ± 0.5 and 98.8 ± 0.2%, respectively.

In an attempt to discover a combination of variables that limit early maturation of post-smolt Atlantic salmon in RAS, fish exposed to two pre-study photoperiods were tracked throughout the study. It should be noted that salmon previously subjected to 12:12 LD entered the experiment at a significantly smaller mean weight (268 ± 4 g) compared to fish initially reared under continuous, 24-h L (330 ± 10 g). Likewise, Imsland et al. (2014) reported faster growth of juvenile Atlantic salmon subjected to continuous light versus a simulated natural photoperiod. During the present study, a significant growth effect related to pre-study photoperiod was observed at each sampling point except for the final event, indicating that salmon originally exposed to 12:12 LD exhibited compensatory growth (Fig. 3). Overall, however, the growth curves of salmon exposed to each pre-study photoperiod reflected the primary treatment effect, where fish growth was faster in ozonated RAS (Fig. 3). No interactive effects between ozone and pre-study photoperiod treatment were observed.

The reason for enhanced Atlantic salmon growth in systems with ozone is unclear. Davidson et al. (2011) reported a similar positive effect of low-dose ozone on rainbow trout growth when true color, heterotrophic bacteria counts, and dissolved copper were reduced and UVT was increased, among other improvements including reduced biochemical oxygen demand and total suspended solids. Therefore, it is reasonable to hypothesize that cumulative improvements to the culture environment instigated by ozone led to increased growth of post-smolt Atlantic salmon during the present study. In a review of literature on ozone application in aquaculture systems, Powell and Scolding (2018) speculated that the mechanisms for improved fish growth driven by ozone could be explained relative to reduced energetic costs of fish acclimating to water chemistry that might otherwise be suboptimal without ozone addition. Nevertheless, dramatic environmental improvements specifically related to water clarity should be considered. Of the water quality differences typically observed in onsite RAS when operating with and without ozone, true color was 13 times lower in ozonated RAS during the Davidson et al. (2011) trial and 22 times lower during the present study (Table 2, Fig. 4). In addition, UVT increased by approximately 27% as a result of ozonation during both trials. Clear water with reduced turbidity reportedly enhances the ability of salmonids to see and capture feed and can lead to increased growth (Sigler et al., 1984). A similar effect may apply to feed capture in experimental-scale tanks where feed remains suspended in the water for a short time.

Table 2
Water quality concentrations (mean ± standard error; mg/L unless otherwise noted) measured in RAS with and without ozone (N = 3) and makeup water.

Parameter	Ozone		No Ozone	Makeup Water
Carbonaceous Biochemical Oxygen Demand	1.6 ± 0.1		1.4 ± 0.1	0.4 ± 0.1
Carbon Dioxide	7.4 ± 0.4		6.5 ± 0.3	46 ± 2
Dissolved Oxygen	10.3 ± 0.12		10.2 ± 0.02	–
pH	7.62 ± 0.03		7.64 ± 0.04	7.30 ± 0.05
Nitrite Nitrogen	0.017 ± 0.009		0.022 ± 0.004	0.002 ± 0.000
Nitrate Nitrogen	105 ± 3		95 ± 3	2.5 ± 0.1
Oxidation Reduction Potential (mV)	307 ± 1	*	260 ± 5	–
Specific Conductance (μS)	1355 ± 20		1302 ± 15	–
Total Ammonia Nitrogen	0.194 ± 0.023		0.211 ± 0.010	0.018 ± 0.002
Temperature (°C)	14.7 ± 0.04		14.7 ± 0.05	–
Total Alkalinity	162 ± 8		178 ± 2	275 ± 4
Total Heterotrophic Bacteria (cfu/1 mL)	36 ± 7	*	135 ± 17	14 ± 3
Total Phosphorus (mg/L)	1.32 ± 0.14		0.88 ± 0.08	0.03 ± 0.01
Total Suspended Solids	2.1 ± 0.2		1.5 ± 0.1	0.5 ± 0.1
True Color (Pt-Co units)	2.1 ± 0.4	*	47 ± 2	1.1 ± 0.4
UV Transmittance (%)	87 ± 1	*	63 ± 1	98 ± 0.2

- Indicates data was not collected.
* Indicates significant difference between treatments ($P < 0.05$).

Table 3
Dissolved metals/trace element concentrations (mean ± standard error; N = 3) measured in RAS with and without ozone (N = 3) and makeup water.

Parameter (mg/L)	Ozone		No Ozone	Makeup Water
Calcium	107 ± 0.4		106 ± 0.5	110 ± 1.5
Copper	0.0072 ± 0.0004	*	0.0225 ± 0.0010	< det
Iron	0.012 ± 0.001	*	0.019 ± 0.002	< det
Magnesium	12.8 ± 0.01		12.8 ± 0.08	11.0 ± 0.29
Potassium	10.3 ± 0.3		10.1 ± 0.3	2.2 ± 0.1
Sodium	152 ± 7		145 ± 3	7.6 ± 0.2
Strontium	0.917 ± 0.004		0.913 ± 0.007	0.939 ± 0.022
Sulfur	15.3 ± 0.1		14.8 ± 0.1	6.2 ± 0.2
Zinc	0.052 ± 0.003	*	0.063 ± 0.001	0.062 ± 0.011

- Dissolved iron levels were generally above the minimum detection limit but below the practical quantitation limit.
* Indicates significant difference between treatments ($P < 0.05$).

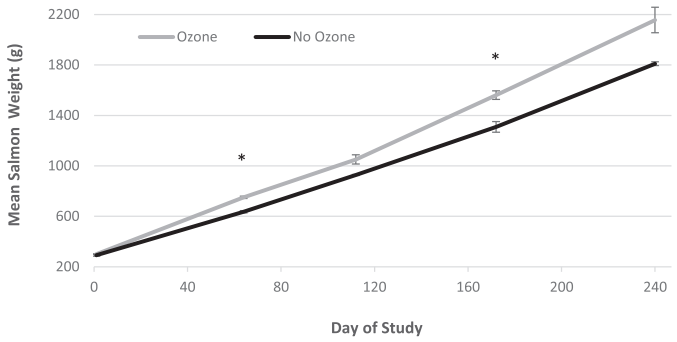

Fig. 2. Atlantic salmon weights (mean ± standard error; $N = 3$) in RAS with and without ozone over the study duration. * Indicates significant difference between treatments.

Table 4
Bimonthly growth performance, feeding, and fish production metrics (mean ± standard error; N = 3) for Atlantic salmon cultured in RAS with and without ozone.

Treatment	Response Variable	Bimonthly Fish Production, Feeding, and Performance Results			
		2	4	6	8
Ozone	Fish Weight (g)	750 ± 9 *	1051 ± 36	1561 ± 35 *	2156 ± 101
No Ozone		637 ± 9	928 ± 4	1309 ± 43	1810 ± 15
Ozone	TGC	2.6 ± 0.05 *	1.4 ± 0.21	1.6 ± 0.19	1.3 ± 0.08
No Ozone		2.1 ± 0.02	1.5 ± 0.05	1.3 ± 0.13	1.4 ± 0.17
Ozone	Fish Biomass (kg)	368 ± 5 *	511 ± 17	381 ± 10 *	454 ± 24
No Ozone		311 ± 4	448 ± 2	311 ± 4	381 ± 6
Ozone	Biomass Density	69 ± 1 *	96 ± 3	72 ± 2 *	86 ± 5
No Ozone	(kg/m^3)	59 ± 1	85 ± 0.3	61 ± 2	72 ± 1
Ozone	Feed Delivered (kg)	180 ± 5 *	162 ± 5 *	116 ± 14	128 ± 15
No Ozone		156 ± 3	128 ± 1	98 ± 4	96 ± 2
Ozone	Wasted Feed (kg)	1.32 ± 0.24 *	3.35 ± 0.43	3.85 ± 0.48	3.77 ± 0.08
No Ozone		2.23 ± 0.18	2.66 ± 0.06	4.42 ± 0.13	4.07 ± 0.31
Ozone	FCR	0.81 ± 0.02 *	1.16 ± 0.34	0.92 ± 0.01	1.04 ± 0.17
No Ozone		0.93 ± 0.02	0.91 ± 0.03	1.07 ± 0.23	0.91 ± 0.10

-TGC, feed delivered, wasted feed, and FCR calculated with representative data generated over 2-month intervals.
* Indicates significant difference between treatments ($P < 0.05$).

Post-study evaluation of feed sinking rates indicated that feed was suspended in the water column of the 1.2-m deep tanks for <10 s and flushed from the tank in approximately 30 s. Under these conditions, nominal inhibition of fish sight could impact feed capture.

Regardless of the exact environmental and/or physiological cause for increased Atlantic salmon growth in ozonated RAS, improved growth in the absence of significant maturation would likely facilitate economic benefits at a commercial farm due to reduced production time and associated expenditures related to energy, oxygen use, and labor. An economic analysis evaluating the capital and energy costs of operating ozone systems along with costs related to duration of the fish production cycle should be carried out to fully understand the tradeoffs.

3.3. Feed conversion

During the first two months of the study, salmon FCR was lower in ozonated RAS (0.81 ± 0.02) versus non-ozonated RAS (0.93 ± 0.02). Given that FCR calculations considered all feed inputs, this difference was likely driven by contrasting wasted feed amounts between treatments. This assertion is supported by periodic wet weight measurements of uneaten feed collected from radial flow settlers indicating nearly double the wasted feed in non-ozonated RAS during this period (Table 4). Per the previous discussion regarding feed capture response, it is interesting to note that the greatest true color measurements in non-ozonated RAS (Months 0–2) coincided with observations of increased wasted feed (Fig. 4). As the study progressed, fish production personnel effectively adjusted daily feed amounts according to wasted feed observations; therefore, differences in mean FCRs were not observed at other sampling intervals. As a result, cumulative FCR for salmon produced in RAS with and without ozone was similar between treatments over the study duration, i.e., 0.98 ± 0.05 and 0.95 ± 0.03, respectively.

3.4. Histopathology

Gill and skin tissue sections appeared in overall good health with only minor, subclinical histopathologic findings. No statistical associations were determined between observed lesions (presence and severity) and RAS ozonation treatment. The most common findings within gill tissue were mild eosinophilic granular cell and mononuclear cell infiltrates, increased goblet cell density, and rare epithelial hyperplasia and single cell necrosis; however, along with skin sections, cellular changes appeared uniform between all groups ($P > 0.05$). Previously, Good et al. (2011) reported increased gill epithelial hyperplasia and hypertrophy in rainbow trout exposed to ozonation (ORP set point = 250 mV) for four months in replicated RAS, compared to unexposed controls; however, these findings were not observed in the present study. Similar on-site research with Atlantic salmon (Good et al., 2017b) did not include histopathology evaluation; however, recent research carried out by Stiller et al. (2020) determined that approximately 40% of post-smolt Atlantic salmon (100 g mean weight) demonstrated gill epithelial lifting, hypertrophy, hyperplasia, and clubbing when exposed to ozone residuals resulting in 250 mV ORP for 10 days in flow-through brackish water. Stiller et al. (2020) also demonstrated that the prevalence of these lesion types, as well as gill lamellar fusion and necrosis, increased as ORP increased up to 500 mV. The absence of similar findings in the present study could be related to environment (i.e., freshwater RAS versus brackish flow-through), study fish (i.e., higher initial weight in the present study), or timing of tissue sampling (i.e., initial lesions associated with ozonated RAS could have resolved by the time of sampling).

3.5. External welfare indicators

No differences were observed between treatments for the following

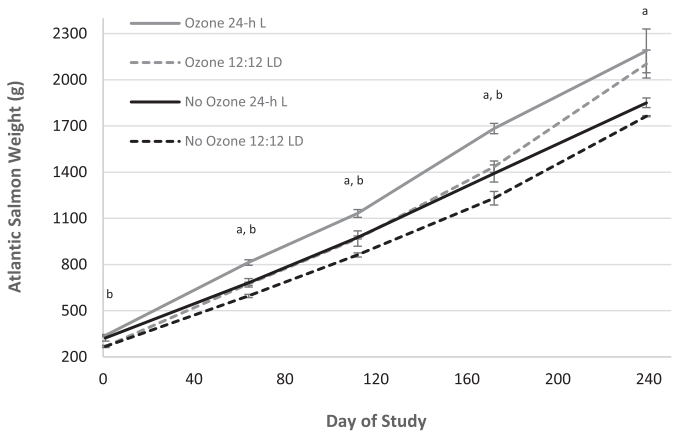

Fig. 3. Weights (mean ± standard error; N = 3) of Atlantic salmon exposed to two pre-study photoperiods (24-h light and 12:12 light/dark) from RAS with and without ozone over the study duration. [a] - Indicates significant effect of primary treatment (ozone v. no ozone). [b] - Indicates significant effect of pre-study photoperiod.

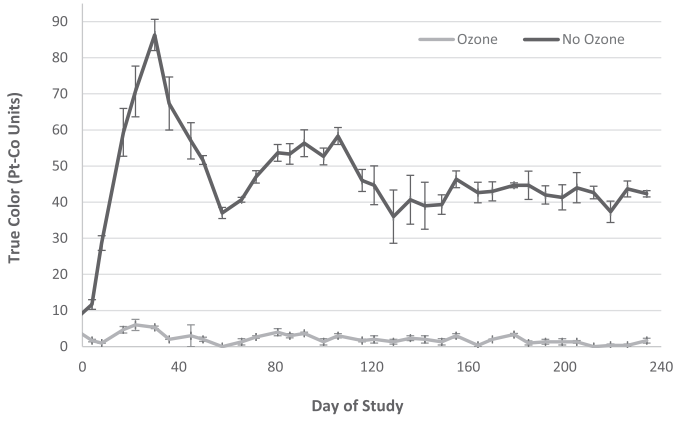

Fig. 4. True color (mean ± standard error; N = 3) in RAS with and without ozone over the study duration.

external welfare indicators: left and right eye cataracts, lesions, operculum damage, skin hemorrhages, and snout damage (Table 5). Mean welfare scores for these parameters were generally <1 indicating that most fish lacked these damage indicators (Noble et al., 2018). Scale loss, which can serve as a gateway for opportunistic infection, was greatest at Month 2, i.e. 1.5 ± 0.21 and 1.8 ± 0.20 for salmon from ozonated and non-ozonated RAS, respectively (Table 5). These slightly elevated scores may have been related to netting and relocating fish to begin the trial. Scale loss was significantly greater for salmon from non-ozonated RAS at Months 6 and 8, but the magnitude of differences was small and likely not of biological importance. Scale loss gradually improved for both treatments over the study duration (Table 5).

Fin damage defined by splitting of fin rays, tissue loss, and secondary issues such as opportunistic infection and hemorrhaging is common in farmed salmonids including Atlantic salmon (Turnbull et al., 1998; Ellis et al., 2002) and is therefore used as a welfare indicator (Stien et al., 2013; Noble et al., 2018). During the present study, fin damage scores were greatest for the caudal fin of salmon from both treatments which is consistent with observations from other studies. For example, Turnbull et al. (1998) found that farmed Atlantic salmon parr attacked the caudal and dorsal fins of conspecifics as a method of competitive aggression more frequently than other fins or areas of the body. Contrary to the findings of Turnbull et al. (1998), dorsal fin scores from the present study were low (Table 5); however, it is important to emphasize that scores were based on observations of active damage. Dorsal fins were damaged prior to the study but had healed, creating thickened nodular tissue that was less prone to further damage. Several important differences in fin scores were observed between treatments, however. For instance, salmon from ozonated RAS had greater damage of the caudal, anal, left and right pelvic fins at Month 2 (Table 5). Greater caudal fin damage was also observed for salmon from ozonated RAS at Month 4. With the exception of the caudal fin, however, fin scores for salmon cultured in ozonated RAS declined after Month 2 indicating a healing effect, while scores for fish from non-ozonated RAS gradually increased (Table 5). The only observation of greater fin damage noted for salmon from non-ozonated RAS was related to the left pectoral fin at Month 6.

During a previous study evaluating the effect of ozone on rainbow trout performance, health, and welfare, Good et al. (2011) did not observe significant dorsal or caudal fin damage; however, fin indices were only evaluated at the end of the study and an ORP setpoint of 250 mV was utilized (Good et al., 2011) versus the 300–320 mV range used during this trial. Although, the maximum fin scores noted during the present study only indicated minor damage, these slightly elevated scores still motivate practical considerations. For example, ozone was applied at the onset of the trial when RAS water contained relatively low levels of accumulating compounds. This approach was purposeful and related to the premise that constant reduction of waterborne hormones via ozonation (Good et al., 2017b) may limit early maturation. It stands to reason, however, that low-level ozone residuals present in the water early in the trial mildly affected salmon fin quality while other accumulating compounds were unavailable for ozone to oxidize. This theory is supported by dissolved ozone levels measured at Month 1 which averaged 0.02–0.03 mg/L, that then went undetected over the

Table 5
Fin damage and external welfare scores (mean ± standard error; N = 3) for Atlantic salmon from RAS with and without ozone.

Treatment	Welfare Variable	External Welfare Scores			
		2	4	6	8
Ozone	Dorsal Fin	0.1 ± 0.08	0.0 ± 0.03	0.0 ± 0.03	0.0 ± 0.03
No Ozone		0.1 ± 0.06	0.1 ± 0.03	0.0 ± 0.03	0.0 ± 0.03
Ozone	Caudal Fin	1.3 ± 0.07 *	1.5 ± 0.06 *	1.1 ± 0.23	1.6 ± 0.24
No Ozone		0.5 ± 0.00	0.8 ± 0.03	0.8 ± 0.02	1.1 ± 0.06
Ozone	Anal Fin	1.1 ± 0.06 *	0.9 ± 0.10	0.8 ± 0.09	0.9 ± 0.06
No Ozone		0.6 ± 0.03	0.8 ± 0.07	0.6 ± 0.05	0.8 ± 0.07
Ozone	Left Pelvic Fin	1.3 ± 0.06 *	1.2 ± 0.06	1.0 ± 0.14	1.0 ± 0.18
No Ozone		0.9 ± 0.07	1.2 ± 0.09	0.9 ± 0.06	1.1 ± 0.00
Ozone	Right Pelvic Fin	1.2 ± 0.09 *	0.9 ± 0.12	0.7 ± 0.11	0.9 ± 0.12
No Ozone		0.7 ± 0.03	1.1 ± 0.03	0.9 ± 0.13	1.1 ± 0.03
Ozone	Left Pectoral Fin	0.9 ± 0.10	0.7 ± 0.03	0.6 ± 0.07	0.7 ± 0.07
No Ozone		0.6 ± 0.07	0.9 ± 0.10	0.9 ± 0.07 *	1.0 ± 0.12
Ozone	Right Pectoral Fin	1.1 ± 0.12	0.7 ± 0.06	0.7 ± 0.12	1.0 ± 0.07
No Ozone		0.9 ± 0.07	1.0 ± 0.10	1.2 ± 0.12	1.2 ± 0.06
Ozone	Left Eye Cataract	0.1 ± 0.04	0.3 ± 0.06	0.3 ± 0.09	0.5 ± 0.17
No Ozone		0.1 ± 0.04	0.2 ± 0.01	0.2 ± 0.02	0.3 ± 0.07
Ozone	Right Eye Cataract	0.1 ± 0.03	0.4 ± 0.05	0.5 ± 0.12	0.8 ± 0.20
No Ozone		0.2 ± 0.05	0.4 ± 0.07	0.6 ± 0.11	0.5 ± 0.10
Ozone	Scale Loss	1.5 ± 0.21	0.8 ± 0.09	0.6 ± 0.09	0.5 ± 0.02
No Ozone		1.8 ± 0.20	0.9 ± 0.09	0.9 ± 0.05†	0.7 ± 0.06 *
Ozone	Snout Damage	0.3 ± 0.07	0.5 ± 0.14	0.3 ± 0.10	0.4 ± 0.07
No Ozone		0.2 ± 0.00	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.04

Cataract scores (0–4 scale); All other welfare scores (0–3 scale).
* Indicates difference between treatments ($P < 0.05$). Notations beside significantly greater values.
† Indicates $P = 0.05$.

remainder of the study. These ozone levels are within the boundaries of the upper threshold (0.008–0.06 mg/L O_3) at which fish reportedly begin to experience somatic damage (Bullock et al., 1997). As such, a RAS facility might consider forgoing the use of ozone during the early months of system operation when tank water is relatively clear. In addition, although ORP was primarily maintained at 300–320 mV in ozonated RAS, ORP peaked beyond this range several times when solenoid valves responsible for controlling ozone delivery failed (Fig. 5). These short-term events cannot be ruled out as the cause for fin damage observed in salmon from the ozonated RAS.

3.6. Waterborne hormones

Testosterone, E2, and 11-KT concentrations were greater in RAS from both the ozone and no ozone treatments compared to the makeup water (Fig. 6), indicating that these sex steroids were produced and excreted by fish and subsequently accumulated in RAS. Cortisol levels in RAS tanks and influent makeup water were similar at each sampling point, indicating the likelihood of low-level cortisol contribution by the supply water. Albeit, other research has shown that factors such as reduced water usage, acute stressors, and water quality can also induce excretion and accumulation of cortisol in RAS water (Mota et al., 2017a; Mota et al., 2017b). Trends for waterborne T and 11-KT concentrations to be lower in ozonated RAS were evident (Fig. 6), with statistical differences noted at study days 197 & 245 (T) and 164 & 245 (11-KT). The general trend of increasing T and 11-KT in both ozonated and non-ozonated RAS points to increased fish production of these hormones as male maturation levels increased (Fig. 6); however, despite increasing levels of female maturation (Table 6) the same trend in waterborne E2 was not observed in non-ozonated RAS. Instead, generally consistent E2 concentrations were quantified across all sampling events (Fig. 6). As previously observed by Good et al. (2017b), E2 appears to be relatively sensitive to ozonation per the significantly lower levels observed in ozonated RAS at study days 136, 164, and 197. The final sampling at study day 245, which corresponded with elevated female maturation in both treatments (Table 6), demonstrated no significant difference in E2 concentrations, due to the relative increase in waterborne E2 in the ozone treatment group. Overall, these findings are consistent with previous trials carried out in the same replicate RAS. For example, Good et al. (2014) also reported mild accumulation of soluble T, 11-KT, and E2 in RAS, but while rearing initially larger (931 g) and more mature Atlantic salmon without ozone. Additionally, Good et al. (2017b) found that E2 was reduced by ozonation, while T and 11-KT levels were generally lower in ozonated RAS; albeit, not at every sampling point.

3.7. Atlantic salmon maturation

Reduction of waterborne hormone levels brought about by ozone did

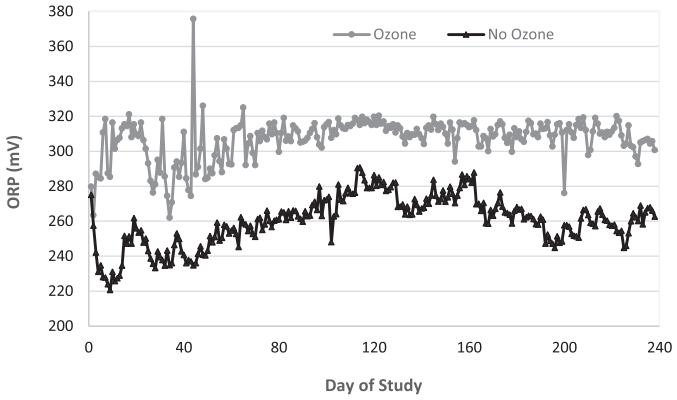

Fig. 5. Mean daily oxidation reduction potential (mV) in RAS with and without ozone (N = 3) over the study duration.

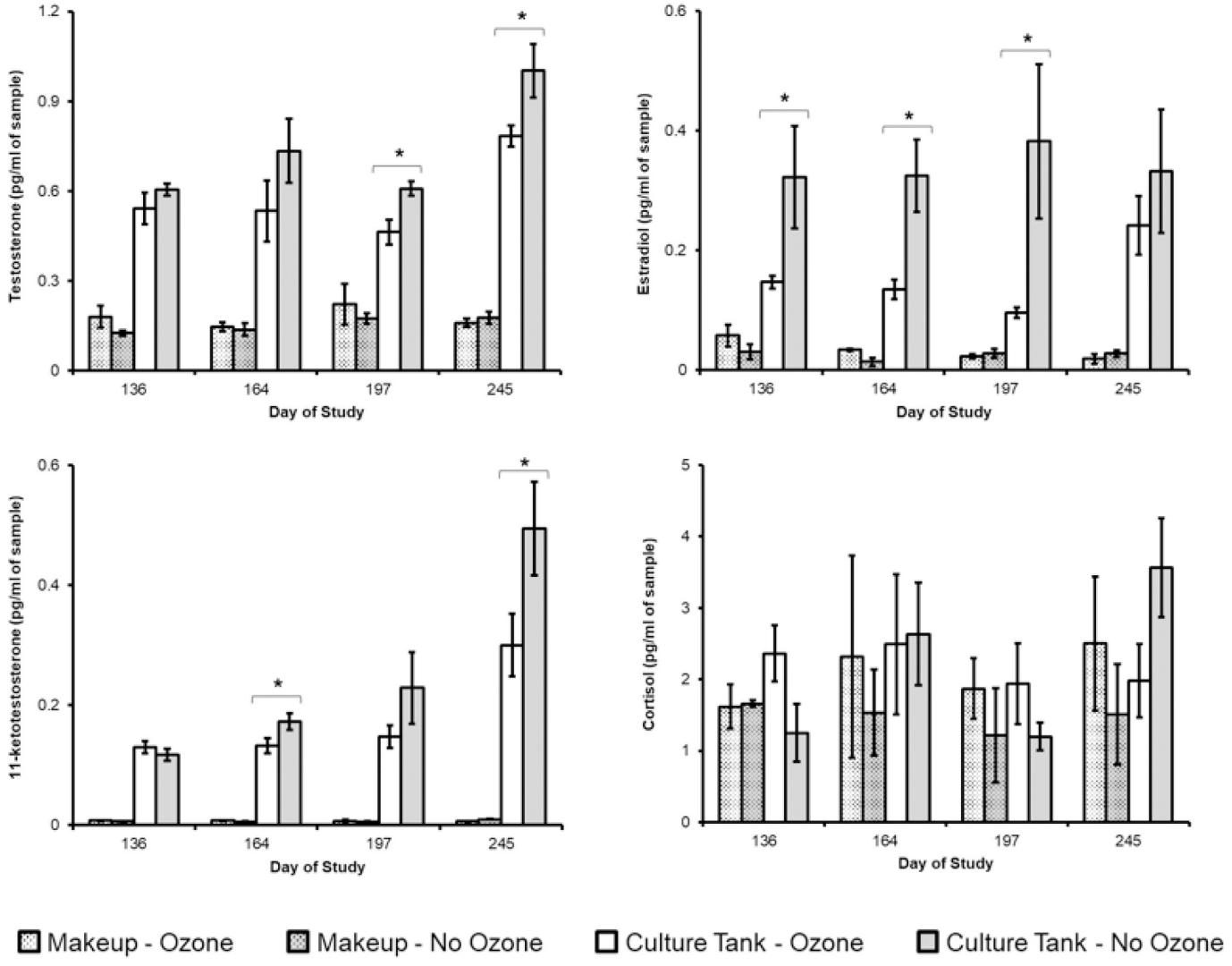

Fig. 6. Waterborne hormone levels (mean ± standard error; N = 3) in pg/mL of water sample, including testosterone, estradiol, 11-ketotestosterone, and cortisol in RAS with and without ozone at four sampling points spanning study days 136–245. Asterisks represent significant ($P < 0.05$) differences in hormones concentrations between ozonated and non-ozonated culture tank water samples.

Table 6
Atlantic salmon maturation percentages (mean ± standard error; N = 3) from bimonthly samples collected over the study duration.

Treatment	Variable	Number Sampled Fish/ RAS	Bimonthly Maturation Indices and Percentages				
			0	2	4	6	8
Ozone	Mean Population Weight (g)	60	300 ± 3	750 ± 9 *	1051 ± 36	1561 ± 35 *	2156 ± 101
No Ozone			292 ± 8	637 ± 9	928 ± 4	1309 ± 43	1810 ± 15
Ozone	Fish with External Maturation Indicators (%)	60	24.8 ± 4.5	17.3 ± 3.4	26.3 ± 3.8	45.0 ± 8.7	55.6 ± 6.9
No Ozone			21.9 ± 0.3	22.5 ± 5.5	31.3 ± 1.9	39.7 ± 5.5	41.1 ± 1.1
Ozone	Gonadosomatic Index (%)	30	–	1.6 ± 1.0 *	1.8 ± 0.3	2.6 ± 0.7	6.6 ± 0.1*
No Ozone			–	0.2 ± 0.01	1.5 ± 0.5	2.3 ± 0.1	4.1 ± 0.4
Ozone	Maturation (%) Gonadosamatic Index ≥1.0	30	–	13.0 ± 7.0 *	28.9 ± 2.8	41.9 ± 9.3	63.0 ± 7.0
No Ozone			–	0.0	18.9 ± 5.9	33.0 ± 2.0	48.0 ± 1.0
Ozone	Male Maturation (%) (GSI ≥ 1.0)	~14	–	–	57.9	63.4	69.0
No Ozone			–	–	36.4	54.9	52.9
Ozone	Female Maturation (%) (GSI ≥ 1.0)	~16	–	–	5.6	24.0	58.3
No Ozone			–	–	2.1	14.5	43.8
Ozone	Maturation (%) 12:12 Pre-study Photo (GSI ≥ 1.0)		–	–	30.6	38.8	62.7
No Ozone		~15	–	–	24.8	33.6	50.0
Ozone	Maturation (%) 24-h Pre-study Photo (GSI ≥ 1.0)		–	–	26.2	46.0	65.1
No Ozone		~15	–	–	17.1	32.8	45.8

- Indicates respective metrics were not evaluated at given sampling interval.
* Indicates difference between treatments ($P < 0.05$).

not inhibit maturation. Atlantic salmon cultured in RAS with and without ozone exhibited high rates of early maturity (Table 6), and mature male and female salmon were observed in both treatments at the end of the study (Table 6). However, salmon cultured in ozonated RAS exhibited higher gonadosomatic index at Months 2 and 8 (Table 6) compared to fish from non-ozonated RAS. When separating maturation data to evaluate effects of photoperiod across the two ozonation treatments, no significant effects of photoperiod were observed, but a statistical effect of ozone treatment was identified at the end of the trial for subjective and objective (related to GSI) maturity assessments (Fig. 7). Both of these data sets showed that salmon cultured in ozonated RAS demonstrated a higher incidence of early maturation at the end of the trial (Fig. 7). When considering cumulative maturity data, salmon in ozonated and non-ozonated RAS exhibited 63.0 ± 7.0% and 48.0 ± 1.0% maturity, respectively. Be that as it may, maturation differences observed between treatments appear to be related to fish growth. For example, when average maturation percentage was plotted with coinciding mean weight (Fig. 8), trendlines overlapped closely between ozone and no ozone treatments, suggesting that gonadal development was partly dictated or coincidental to fish size, and that slower growing salmon eventually would reach the same state of maturity. In hindsight, it would have been valuable to assess GSI at every sampling point to understand the exact timing of gonadal development. A small sample of five fish per RAS collected at Month 2 indicated that salmon in RAS operated with and without ozone had GSI of 1.6 ± 1.0% and 0.2 ± 0.01% (Table 6) suggesting that gonadal development began sooner in faster growing salmon cultured in ozonated RAS. Several studies have shown that increased Atlantic salmon growth rate is partly related to the onset of maturation, often overlapping with other variables (e.g., photoperiod and temperature) that direct reproductive development (e.g., Adams and Thorpe, 1989; Taranger et al., 2010; Fjelldal et al., 2011; Imsland et al., 2014).

In the context of understanding maturation onset, it is important to note that 20–25% of Atlantic salmon used for this study demonstrated morphology consistent with early maturation (e.g., bronze skin coloration and early kype formation) at a mean weight ≤ 300 g (Table 6). Anecdotally, this indicates that environmental cues experienced by fish before the study may have provided the directive for reproductive development. With this in mind, the environmental conditions of land-based systems used for early rearing may deserve more attention relative to maturation onset. The early rearing regime typically employed for Atlantic salmon cohorts at TCFFI consists of RAS incubation at 7–8 °C, flow-through fry culture at 12.5–14 °C, and intermediate production in a partial reuse system at 12.0–14.5 °C. Interestingly, Fjelldal et al. (2011) demonstrated that a combination of increasing water temperature and continuous light can trigger early maturation in male Atlantic salmon during and immediately after smoltification. Specifically, early male maturation was pronounced when parr were cultured at 16.0 °C with continuous, 24-h light compared to fish reared at 5 and 10 °C under various photoperiods (Fjelldal et al., 2011). In addition, Imsland et al. (2014) found that long-term rearing of Atlantic salmon under continuous light, but lower water temperature (8.3 vs. 12.7 °C) balanced growth while limiting early maturation. The pre- and in-study photoperiods that fish were exposed to during this trial did not inhibit maturation. It may also be important to note that the mean and maximum water temperatures for both RAS treatments were 14.7 °C and 16.2 °C, respectively.

4. Conclusions

Overall, ozone did not inhibit the prevalence of Atlantic salmon maturation in freshwater RAS despite notable reductions in waterborne

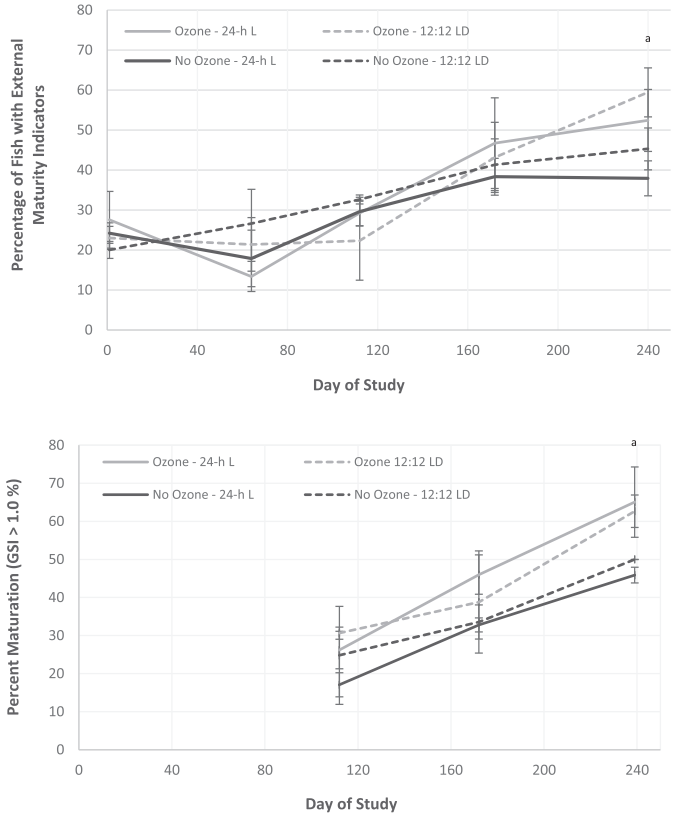

Fig. 7. Subjective maturity assessment (top) based on morphology indicators and objective maturity assessment (bottom) based on gonadosomatic index evaluation where salmon with GSI > 1.0% were considered mature. Percent maturation data presented as mean ± standard error; $N = 3$. Data provided for combinations of ozonation and pre-study photoperiod treatments at sampling points across the study duration. [a] - Indicates significant effect of primary treatment (ozone v. no ozone).

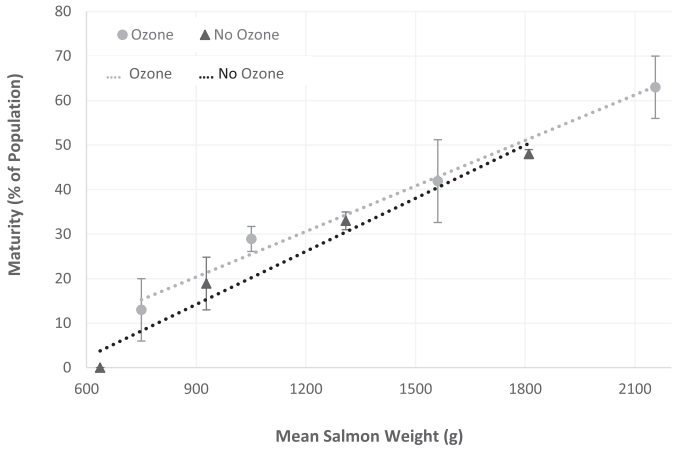

Fig. 8. Mean salmon weight plotted with coinciding percent maturity of Atlantic salmon populations reared in RAS with and without ozone ($N = 3$; mean ± standard error).

hormone levels. Additional research is therefore needed to determine an effective combination of environmental and/or biological conditions that reduce or eliminate early Atlantic salmon maturation in RAS. Given that a small percentage of fish exhibited morphology consistent with early maturation to begin the study, perhaps it would be interesting to evaluate the effect of ozone when rearing Atlantic salmon at a smaller size and earlier life stage, assuredly before the fish have received cues that signal a path towards maturation. As mentioned, more research is also needed to evaluate the potential effect of water temperature on early Atlantic salmon maturation, particularly given the warmer thermal conditions that are inherent of RAS. Lastly, notwithstanding the maturation findings, ozone had a positive effect on post-smolt Atlantic salmon growth that would likely reduce the duration of market-size salmon production in land-based RAS thereby leading to reduced production costs. If the maturation problem in RAS can be solved through establishment of an optimal set of environmental and biological conditions, then the use of ozone could be advantageous for RAS-based production of post-smolt Atlantic salmon.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest to report.

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