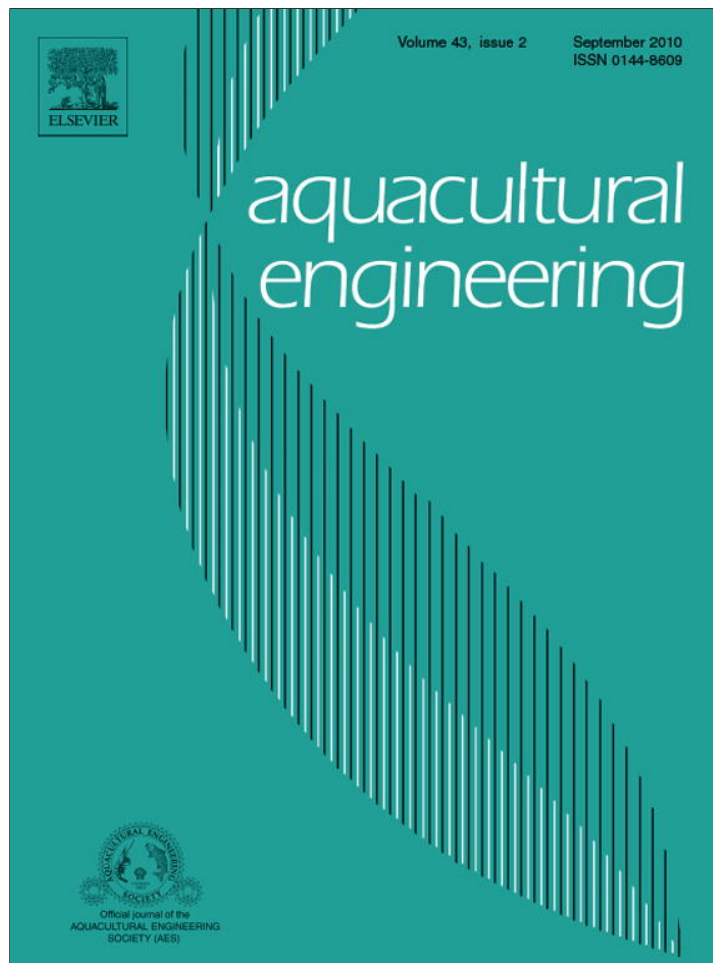


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Evaluation of ozonation on levels of the off-flavor compounds geosmin and 2-methylisoborneol in water and rainbow trout *Oncorhynchus mykiss* from recirculating aquaculture systems

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ABSTRACT

Common “off-flavors” in fish cultured in recirculating aquaculture systems (RAS) are “earthy” and “musty” due to the presence of the off-flavor metabolites geosmin and 2-methylisoborneol (MIB), respectively. Previously, ozone addition has been applied to RAS at relatively low doses to break refractory organic molecules (i.e., reducing color), microfloculate fine particulate matter (i.e., increasing solids removal), and oxidize nitrite to nitrate, but the effect of ozone addition at these dosing rates on levels of off-flavor compounds was unknown. Ozonation has been used in municipal drinking water facilities to reduce the levels of these compounds, to improve water taste, and to subsequently reduce consumer complaints. In this study, the effects of ozone addition to the inlet water of the RAS culture tanks on levels of geosmin and MIB in the culture water and fish flesh were evaluated. Water and rainbow trout (*Oncorhynchus mykiss*) samples were obtained twice after maximum feed rates were reached during a 6-month grow-out period. Results indicated that ozone addition to maintain an oxidation reduction potential of 248 mV (<1 µg/L of ozone residual) did not significantly reduce levels of these off-flavor compounds in the water and fish flesh, though it did significantly improve culture tank water quality. Higher dosages of ozone might be beneficial in removing geosmin and MIB in the RAS water, but the inclusion of ultraviolet (UV) irradiation would be required to prevent mortality associated with ozone toxicity.

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1. Introduction

The production of fish in recirculating aquaculture systems (RAS) continues to be hampered by problems with environmentally derived “off-flavor” that is best described as an “earthy” and/or “musty” taste of the fillet. Recent studies have determined that the presence of the odorous compounds geosmin and 2-methylisoborneol (MIB) in the flesh of RAS-cultured fish is responsible for these off-flavors (Schrader et al., 2005; Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010). Over the past several decades, various municipal drinking water facilities in the United States have used ozonation to remove geosmin and MIB from the water via oxidation. In water, ozone (O₃) can oxidize geosmin and MIB either directly or indirectly by the formation of hydroxyl radicals (OH[•]) from the interaction of O₃ with hydroxide ions (OH⁻). The OH[•] is a more powerful oxidant than the O₃ molecule. Hydrogen peroxide (H₂O₂) will also oxidize geosmin and

MIB via formation of OH[•] and OH⁻ in the presence of metal ions (e.g., iron and copper), also referred to as the Fenton reaction. A number of factors can impact the efficiency of ozone addition in the removal of geosmin and MIB including dosage (Koch et al., 1992; Westerhoff et al., 2006), water temperature (Westerhoff et al., 2006), organic matter content of the water (Bruce et al., 2002; Ho et al., 2004), pH (Westerhoff et al., 2006), and alkalinity (Ho et al., 2004). In addition, geosmin oxidizes faster than MIB (Westerhoff et al., 2006). The resistance of MIB to oxidation by O₃ may be due to greater steric hindrance based upon its chemical structure compared to geosmin (Ho et al., 2004).

The use of ozonation in RAS has recently been studied to determine the requirements to achieve adequate disinfection of recirculating water (Summerfelt et al., 2009). In addition, previous studies have determined that ozone addition in RAS will improve water quality by inducing microflocculation of fine particles [i.e., improving total suspended solids (TSS) capture and reducing TSS concentration] and oxidizing nitrite (i.e., reducing nitrite concentration) and undesirable organic molecules (e.g., reducing non-biodegradable and refractory compounds that stain the water) (Brazil, 1996; Summerfelt et al., 1997, 2009;

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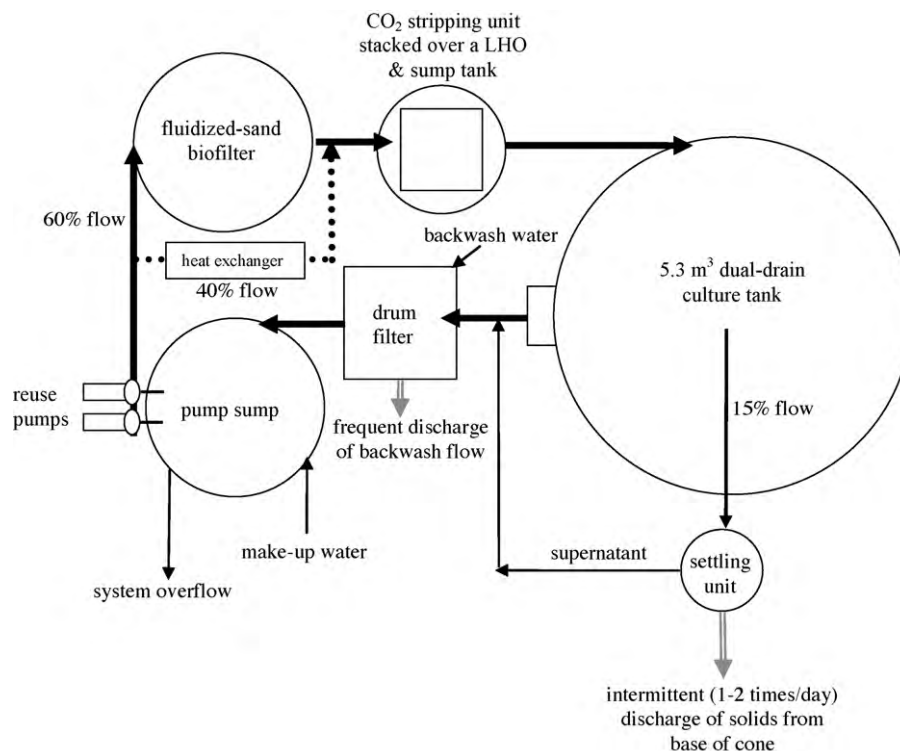


Fig. 1. Process flow drawing of the recirculating salmonid grow-out system (after Summerfelt et al., 2009). The main drain on the culture tank is located on the bottom of the tank and connects to the settling unit.

Christensen et al., 2000; Krumins et al., 2001a,b; Summerfelt, 2003). Recently, Davidson et al. (unpublished results) found that ozone can reduce heavy metals concentrations in low exchange RAS, specifically copper and zinc. Davidson et al. (unpublished results) also determined that rainbow trout growth was significantly greater within low exchange RAS operated with ozone, and Bullock et al. (1997) reported that ozonation of the recirculating water prevented recurring episodes of bacterial gill disease in rainbow trout (*Oncorhynchus mykiss*) without the use of chemotherapeutic treatment. However, there have not been any previous studies to determine the effects of ozone addition on levels of geosmin and MIB that may be present in the RAS water and in RAS-cultured fish.

During an ongoing study designed to assess the effects of ozonation on a suite of water quality parameters (e.g., biological oxygen demand, TSS, color, UV transmittance, metals, total ammonia nitrogen, nitrite nitrogen, total organic carbon, dissolved organic carbon, and heterotrophic bacteria) within low exchange RAS, a supplemental study was performed to monitor levels of geosmin and MIB in the water entering the culture tanks (post-ozonation) and in the flesh of RAS-cultured rainbow trout. Results were evaluated to determine if ozonation utilized at low levels (and without any available UV irradiation to remove ozone) in RAS fish culture would help mitigate earthy-musty off-flavor problems that may occur in RAS-cultured fish.

2. Materials and methods

2.1. Recirculating aquaculture systems

Six individual RAS located at The Conservation Fund's Freshwater Institute, Shepherdstown, WV, were used in this study. Each RAS unit was comprised of a fluidized-sand biofilter, a forced-ventilated cascade aeration column, a low head oxygenation (LHO) unit where pure oxygen feed gas was absorbed, a LHO sump, a single 5.3-m³ culture tank, a microscreen drum filter, a particle trap, a pump

sump, and a heat exchanger (Fig. 1). Each RAS was stocked with 1000 juvenile rainbow trout to begin the study, at which time mean rainbow trout weight was 197 ± 1 g and mean fish density was 37 kg/m^3 in the culture tank. The number of rainbow trout was reduced in each RAS after fish density reached a set maximum density of 80 kg/m^3 in the culture tank.

2.2. Ozone

Three of the six RAS were equipped with ozone generators (Model G22, Pacific Ozone Technology, Benecia, CA) for ozone addition to the inlet water of the culture tank while the other three RAS did not have ozone input (controls). A portion (typically <10%) of the oxygen contained in a nearly pure oxygen feed gas was converted to ozone as the gas passed through the generator's Corona discharge cell. After exiting the ozone generator, the ozonated-oxygen feed gas was transferred into the recirculating flow within the LHO unit and immediately before the recirculated water returns to the fish culture tank. Ozone concentration was monitored and controlled via oxidation-reduction potential (ORP) using a differential ORP digital sensor equipped with a platinum electrode (Model DRD1R5, Hach Company, Loveland, CO) and displayed by an SC100 Universal Controller (Hach Company, Loveland, CO). The ORP probe was located in front of the water inlet within each fish culture tank. Proportional-integral-derivative (PID) control of the ozone generator output was utilized during this study in order to maintain an ORP set point of approximately 250 mV within the culture tank. The resulting ozone dose, which ranged from 20 to 25 g of ozone per kilogram of feed (0.25–0.28 mg ozone/L), was not intended to disinfect the water (i.e., reduce bacterial loads) but was expected to improve general water quality.

2.3. Water exchange/flows

Makeup water (spring water) was added continuously within the pump sump of each RAS at a rate of approximately 1 L/min or

0.26% of the total recirculated flow. Thus, approximately 99.74% of the water flow was recirculated in each system, which provided a mean system hydraulic retention time of approximately 6.7 days, excluding the backwash flushed through the drum filter and settler. Retention time of the water recirculating through the fish tank was 15 min.

2.4. Feeding

During the first month of the study, fish were fed equal rations with feeding events occurring every other hour, around the clock, using automated feeders (T-drum 2000CE, Arvo-Tec, Finland). Feed rates were established during this period using standardized feeding charts and observations of feeding activity and wasted feed. After 1 month, feeding was adjusted for each RAS individually, if an obvious reduction in feeding response or significant amounts of wasted feed were observed. Thereafter, fish in each RAS were fed to satiation, but feeding was not necessarily equal among individual RAS or between treatments. Feeding was also reduced accordingly on several occasions when some rainbow trout were harvested to reduce density.

2.5. General water quality

Water samples were collected weekly from the side drains of the culture tanks. Water was tested for TSS, true color, nitrite nitrogen, total ammonia nitrogen, nitrate nitrogen, 5-day carbonaceous biochemical oxygen demand (cBOD₅), total heterotrophic bacteria plate counts, and dissolved and total organic carbon according to methods reported elsewhere (Davidson et al., 2009). In addition, dissolved oxygen, temperature, and ORP were monitored continuously using a SC100 Universal Controller (Hach Company, Loveland, CO). Over the 6-month duration of the study, the mean water temperature for RAS with and without ozone was 15.2 ± 0.0 and 15.1 ± 0.0 °C, respectively. Alkalinity was controlled via addition of sodium bicarbonate to achieve a target concentration of 200 mg/L as CaCO₃. Additionally, pH was primarily controlled by degassing of carbon dioxide and the alkalinity concentration maintained within each RAS.

2.6. Water and fillet sample collection for measurement of off-flavor compounds

Water samples were collected from each of the six RAS at the culture tank inlet (after injection of ozonated-oxygen feed gas; Fig. 1) at two different times as follows: (1) when fish were at maximum feed levels [overall mean of 5.8 kg/day/tank (1.1 kg/day/m³ tank volume) and feed loading rate of 4.5 kg/day/m³ of makeup water] and densities (overall mean of 69 kg/m³ tank volume) and (2) approximately 2 months later and just prior to harvesting of the trout with mean feed levels of 4.5 kg/day/tank (0.9 kg/day/m³ tank volume), feed loading rate of 3.2 kg/day/m³ of makeup water, and overall fish densities of 62 kg/m³. Water samples were collected from sampling ports after opening the ports and allowing water to run for 1 min. Individual water samples were placed in 20-mL glass scintillation vials (with foil-lined caps). Vials were filled completely so that no air bubbles were observed after the vial was capped and inverted. These samples were maintained at 4 °C until shipping by overnight express to the U.S. Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit (NPURU), University, MS.

Samples of trout were also obtained at the same time as water sampling. Five trout were removed from each RAS tank, euthanized by cranial percussion, and filleted. Fillets were placed in separate plastic zip-lock bags and immediately frozen until overnight shipment to the NPURU laboratory for analysis of geosmin and MIB

levels by solid-phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS) following microwave distillation (Lloyd and Grimm, 1999). For each fillet, two 20-g portions from the anterior end of the fillet were used to obtain distillates following microwave distillation, and each distillate sample was analyzed using SPME–GC–MS.

2.7. Determination of geosmin and MIB levels

Water samples and microwave distillates of trout fillet samples were processed prior to the determination of geosmin and MIB levels by micropipetting 0.6 mL aliquots into individual 2-mL glass crimp-top vials containing 0.3 g sodium chloride/vial. The method used to quantify levels of geosmin and MIB was similar to the solid-phase microextraction (SPME) procedure by Lloyd et al. (1998). The vials were heated at 40 °C for 20 min before the volatile compounds were absorbed onto a 100- μ m polydimethyl siloxane solid-phase microextraction fiber (Supelco, Bellefonte, PA). The fiber assembly was then shaken for 10 min during the absorption period and desorbed for 2 min at 250 °C in the injection port of a HP 6890 gas chromatograph–mass spectrometer (GC–MS) (Agilent, Palo Alto, CA) with a 5973 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/in² 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. The capillary column used was a DB-5 (5%-phenyl-methylsiloxane, 30 m, 0.25 mm inside diameter, 0.25 μ m film thickness; J&W Scientific, Folsom, CA). The retention time for geosmin was 6.8 min, and for MIB was 5.2 min. Standards for MIB and geosmin were prepared at 0.1, 0.5, 1.0, and 2.5 μ g/L in deionized water. These standards were obtained from Wako Chemicals USA, Inc., Richmond, VA, and were included at the beginning, middle, and end of each group of samples analyzed using a CombiPal autosampler (LEAP Technologies, Inc., Carrboro, NC). Each sample was run in triplicate (Schrader et al., 2003).

2.8. Data analysis

Means and standard errors were determined for rainbow trout fillet analysis results while means and standard deviations were determined for water sample analysis results. Analysis of variance and Tukey's test for the comparison of the means ($\alpha = 0.05$) was performed for each sampling date to determine significant differences between treatment and controls for levels of each off-flavor compound. Data analysis was generated using SAS software, Version 9.1 (SAS Institute Inc., Cary, NC).

3. Results and discussion

Mean levels of geosmin and MIB in trout fillets from RAS in which water was ozonated and in fillets from RAS without ozonation were not significantly different ($p > 0.05$; Table 1). In this study, ozone at the levels introduced into the RAS water prior to the culture tanks was not effective in reducing levels of these two off-flavor compounds in the fish flesh. Overall, mean MIB levels in fillets from the RAS ranged from 15.0 to 52.1 ng/kg. Mean geosmin levels in fillets from the different RAS were highly variable for the second sampling date (8–20–08), with mean geosmin levels ranging from 52.4 to 384.3 ng/kg. These levels of MIB and geosmin are below the estimated sensory detection thresholds in rainbow trout of 55 ng/kg (Persson, 1980) and 900 ng/kg (Robertson et al., 2005), respectively. However, there were individual fillet samples

Table 1Mean (\pm S.E.) levels of geosmin and 2-methylisoborneol (MIB) in trout fillets from the study on ozonation using six recirculating aquaculture systems (RAS).

Sampling date	RAS #/treatment	Mean geosmin ^a (ng/kg)	Mean geosmin (ng/kg) per treatment	Mean MIB ^a (ng/kg)	Mean MIB (ng/kg) per treatment
6-12-08	2 (ozone addition)	50.6 \pm 5.3	57.8 ^b \pm 4.7	20.9 \pm 8.9	21.3 ^b \pm 0.4
	3 (ozone addition)	56.3 \pm 5.2		21.0 \pm 7.8	
	6 (ozone addition)	66.5 \pm 14.1		22.1 \pm 9.7	
	1 (no ozone)	64.1 \pm 6.0		19.4 \pm 7.6	
	4 (no ozone)	34.8 \pm 6.0		23.4 \pm 16.4	
8-20-08	5 (no ozone)	22.8 \pm 3.0	173.1 ^b \pm 105.7	15.0 \pm 2.1	29.6 ^b \pm 7.5
	2 (ozone addition)	61.8 \pm 8.9		43.8 \pm 11.4	
	3 (ozone addition)	73.2 \pm 6.3		18.4 \pm 4.2	
	6 (ozone addition)	384.3 \pm 39.7		26.5 \pm 3.6	
	1 (no ozone)	104.9 \pm 13.4		29.8 \pm 7.6	
8-20-08	4 (no ozone)	82.8 \pm 8.9	80.0 ^b \pm 15.2	52.1 \pm 6.4	40.5 ^b \pm 6.5
	5 (no ozone)	52.4 \pm 4.3		39.6 \pm 7.4	

^a Each mean was obtained using five different fish (fillet samples) from each RAS; two 20-g portions were analyzed from each fillet and results were averaged to determine the mean level for the fillet.

^b No statistical difference between means of off-flavor compound level for ozone addition and no ozone on the same sampling date ($p > 0.05$, Tukey's test).

from the second sampling that had MIB levels above 55 ng/kg, and therefore, the entire crop was not "on-flavor" and not considered marketable without a depuration process. In fact, Freshwater Institute staff typically depurate rainbow trout (900–1300 g/fish) in a single-pass culture tank without feed and for at least 5 days prior to harvest to clear off-flavor. At present, we are unable to provide an explanation for the higher geosmin levels in fish from RAS 6 (Table 1) compared to the other RAS used in this study.

Comparison of geosmin and MIB levels in water from RAS receiving ozonation to levels in RAS without ozonation found no significant differences ($p > 0.05$; Table 2). Overall, mean geosmin levels in the water were at least an order of magnitude lower compared to the levels detected in the trout fillets from the respective RAS. Mean MIB levels were also generally lower in water samples compared to levels in respective fillet samples. These differences in off-flavor compound levels in water and fish flesh are similar to observations by Schrader and Summerfelt (2010) and indicate the strong potential for the bioaccumulation of geosmin and MIB in RAS-cultured fish flesh during the grow-out cycle. Previous studies by Johnsen and Lloyd (1992) with channel catfish (*Ictalurus punctatus*) found significant bioaccumulation of MIB in fish flesh within hours of exposure. Further studies are required to confirm a similar pattern of geosmin and MIB bioaccumulation from RAS water into the fish flesh while fish are raised in RAS.

Ozonation has been demonstrated to be effective in reducing geosmin and MIB concentrations in water. For example, ozone dosages of 1, 2, and 4 mg/L at a contact time of 12 min reduced an initial MIB concentration of 100 ng/L in river water by 58%, 65%,

and 75%, respectively (Koch et al., 1992). In another study, Glaze et al. (1990) determined that 0.1 mg/L of ozone with a contact time of 20 min reduced initial geosmin and MIB levels of 100 ng/L in aqueduct water by 35% and 40%, respectively, and 0.2 mg/L of ozone (20 min contact) reduced 100 ng/L of geosmin and MIB by 86–92% and 73–83%, respectively. Previous research has demonstrated that OH[•] mediated reactions play a more prevalent role than ozone reactions during geosmin or MIB oxidation (Westerhoff et al., 2006).

In the current study, ozone addition was at significantly lower levels than those used in the above mentioned studies. The concentration and flow of ozone in the feed gas supplied to one of the three RAS receiving ozonation were measured and used to quantify that approximately 20–25 g of ozone were added to the recirculating flow for every 1 kg of feed fed daily, which was equivalent to an ozone dose of approximately 0.25–0.28 mg/L. This ozone dose was used to maintain an ORP of 248 mV, which extrapolates to less than 1 μ g/L of dissolved ozone residual according to data published by Summerfelt et al. (2009); in fact, an ozone residual concentration of 1 μ g/L is not expected until ORP reaches approximately 350 mv. A dissolved ozone concentration below 1 μ g/L is safe for rainbow trout in freshwater (Bullock et al., 1997). Even though the recirculated water was subjected to the continuous addition of ozone, the ozone addition had no significant effect ($p > 0.05$) in reducing levels of geosmin and MIB in the water or trout fillets compared to RAS with no ozone addition.

The water quality results listed in Table 3 serve as a reference to the conditions encountered in this study. Ozone application at the relatively low dosage was able to significantly reduce TSS, cBOD₅,

Table 2Mean (\pm S.D.) levels of geosmin and 2-methylisoborneol (MIB) in water from the tank inlet (post-ozonation) from the study on ozonation using six recirculating aquaculture systems (RAS).

Sampling date	RAS #/treatment	Mean geosmin ^a (ng/L)	Mean geosmin (ng/L) per treatment	Mean MIB ^a (ng/L)	Mean MIB (ng/L) per treatment
6-12-08	2 (ozone addition)	0 \pm 0	3.0 ^b \pm 1.5	0 \pm 0	0 ^b \pm 0
	3 (ozone addition)	5 \pm 1.5		0 \pm 0	
	6 (ozone addition)	4 \pm 0.6		0 \pm 0	
	1 (no ozone)	3 \pm 0.6		5 \pm 0.6	
	4 (no ozone)	3 \pm 0.6		0 \pm 0	
8-20-09	5 (no ozone)	2 \pm 0.6	2.7 ^b \pm 0.3	0 \pm 0	1.7 ^b \pm 1.7
	2 (ozone addition)	1 \pm 0		6 \pm 1	
	3 (ozone addition)	1 \pm 0		2 \pm 0	
	6 (ozone addition)	4 \pm 1.2		2 \pm 0.6	
	1 (no ozone)	1 \pm 0		14 \pm 0.6	
8-20-09	4 (no ozone)	2 \pm 1.2	1.3 ^b \pm 0.3	1 \pm 0	5.3 ^b \pm 4.3
	5 (no ozone)	1 \pm 0		1 \pm 0	

^a Each mean was obtained from the analysis of a water sample analyzed in triplicate using SPME–GC–MS. Values of "0" indicate levels were below the instrument detection limit of 1 ng/L.

^b No statistical difference between means of off-flavor compound level for ozone addition and no ozone on the same sampling date ($p > 0.05$, Tukey's test).

Table 3

Mean (\pm S.E.) water quality parameters of the culture tank for RAS operated with and without ozone over the duration of the study and at maximum feeding and density when samples were taken for off-flavor compounds.

Water quality variable	RAS ^a	
	No ozone	Ozone addition
Alkalinity (mg/L)	205 \pm 1	196 \pm 1
Dissolved oxygen (mg/L)	9.9 \pm 0	9.8 \pm 0
Dissolved organic carbon (mg/L)	15.3 \pm 1.5	13.7 \pm 1.4
ORP ^b (mV)	155 \pm 1	248 \pm 1
pH	7.66 \pm 0.01	7.60 \pm 0.02
Temperature ($^{\circ}$ C)	15.1 \pm 0	15.2 \pm 0
Total organic carbon (mg/L)	15.9 \pm 1.6	13.0 \pm 1.3
Total ammonia-N (mg/L)	0.47 \pm 0.01	0.45 \pm 0.02
Nitrite-N (mg/L)	0.050 \pm 0.003	0.042 \pm 0.008
Nitrate-N (mg/L)	71 \pm 1	84 \pm 3
Total suspended solids (mg/L)	8.7 \pm 1.8	3.4 \pm 0.4
Total heterotrophic bacteria (cfu/mL)	2.0 \times 10 ⁵	9.2 \times 10 ¹
5-Day cBOD ^c (mg/L)	3.6 \pm 0.5	1.7 \pm 0.1
True color (Pt-Co)	53 \pm 2	4 \pm 0

^a Mean of three recirculating aquaculture systems per treatment for the duration of the study.

^b Oxidation–reduction potential.

^c Carbonaceous biochemical oxygen demand.

total heterotrophic bacteria counts, and true color in the recirculating water compared to the non-ozonated control systems, which achieved the desired affect. However, it is unfortunate that ozone application at these low dosages was inadequate to also reduce levels of geosmin and MIB accumulation. Variations in water quality (e.g., alkalinity and pH) between RAS at different production facilities will impact the ozone dose requirement for geosmin and MIB oxidation. For example, geosmin and MIB oxidation decreases at lower pH and temperature (Westerhoff et al., 2006). Conversely, lower alkalinity will enhance geosmin and MIB oxidation (Bruce et al., 2002). Higher ozone dosage may be more important in the efficiency of geosmin and MIB oxidation rather than the amount of contact time (Koch et al., 1992). While higher ozone doses could be applied to the RAS used in this study, UV irradiation (e.g., 50 mJ/cm² of UV irradiation for 100 μ g/L ozone; see Summerfelt et al., 2004) would be necessary to remove residual ozone prior to the culture tank to prevent lethal effects towards the fish. Future studies will help determine if higher ozone dosages can be useful in reducing geosmin and MIB off-flavor problems in RAS.

4. Conclusions

Continuous ozone addition (248 mV or <1 μ g/L) did not significantly reduce levels of geosmin and MIB in RAS water and subsequently in trout filets. These results indicate that “low-dose” ozone addition with the intended goal of improving certain water quality parameters (e.g., TSS, color, etc.) will not provide benefits in the management of off-flavor problems related to geosmin and MIB.

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