## Aquacultural Engineering
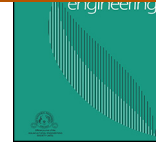
Short communication

# Evaluation of the impact of nitrate-nitrogen levels in recirculating aquaculture systems on concentrations of the off-flavor compounds geosmin and 2-methylisoborneol in water and rainbow trout (*Oncorhynchus mykiss*)

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### ARTICLE INFO

### ABSTRACT

Aquatic animals raised in recirculating aquaculture systems (RAS) can develop preharvest "off-flavors" such as "earthy" or "musty" which are caused by the bioaccumulation of the odorous compounds geosmin or 2-methylisoborneol (MIB), respectively, in their flesh. Tainted aquatic products cause large economic losses to producers due to the inability to market them. Certain species of actinomycetes, a group of fila-mentous bacteria, have been attributed as the main sources of geosmin and MIB in RAS. Previous studies have demonstrated that certain nutritional factors can stimulate or inhibit bacterial biomass and geosmin production by certain actinomycetes. In the current study, the effects of two nitrate-nitrogen ($NO_3^-$-N) levels (20–40 mg/L and 80–100 mg/L) on geosmin and MIB levels in culture water and the flesh of rainbow trout (*Oncorhynchus mykiss*) raised in RAS were monitored. Water and fish tissue samples were collected over an approximately nine-week period from six RAS, three replicates each of low and high $NO_3^-$-N, and analyzed for geosmin concentrations using solid phase microextraction–gas chromatography–mass spectrometry. Results indicated no significant difference in geosmin concentrations in water or fish flesh between the low and high $NO_3^-$-N RAS. Therefore, higher $NO_3^-$-N levels that may occur in RAS will not adversely or beneficially impact geosmin-related off-flavor problems.

## 1. Introduction

Preharvest "off-flavors" are a significant problem in aqua-culture species cultured in recirculating aquaculture systems (RAS) due to rendering the final product as unpalatable and unmarketable. The most common types of off-flavors reported in fish raised in RAS are "earthy" and "musty" which are caused by the compounds geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol (MIB) [(1-*R-exo*)-1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol], respectively. For indoor RAS, geosmin and MIB-related off-flavors in finfish have been attributed to the presence of certain species of actinomycetes (e.g., *Streptomyces* spp., *Nocardia* spp.) (Guttman and van Rijn, 2008;

Schrader and Summerfelt, 2010). These bacteria can be prolific pro-ducers of geosmin and MIB, and the associated compounds can rapidly accumulate in the flesh of the fish. Removal or purging of these off-flavor compounds from fish flesh to sufficient levels to provide an acceptable product for consumers can take days or weeks depending on various factors such as intensity of the off-flavor, adipose content of the fish flesh, and water temperature (Tucker, 2000). Depuration of these metabolites from fish flesh can be achieved by moving the fish to taint-free water. This approach is currently utilized by some United States of America (USA) pro-ducers to manage these common off-flavor problems in RAS.

The intensities and predictability of geosmin and MIB-related off-flavor episodes within RAS have not been fully elucidated. According to reports from various producers and several recent studies within the USA (Schrader and Summerfelt, 2010; Schrader et al., 2010; Burr et al., 2012), some commercial RAS systems can have chronic and high concentrations of geosmin and MIB in the cultured aquatic products while other RAS production systems have little to no incidences of these taints. These differences are likely due to a combination of the presence or absence of the

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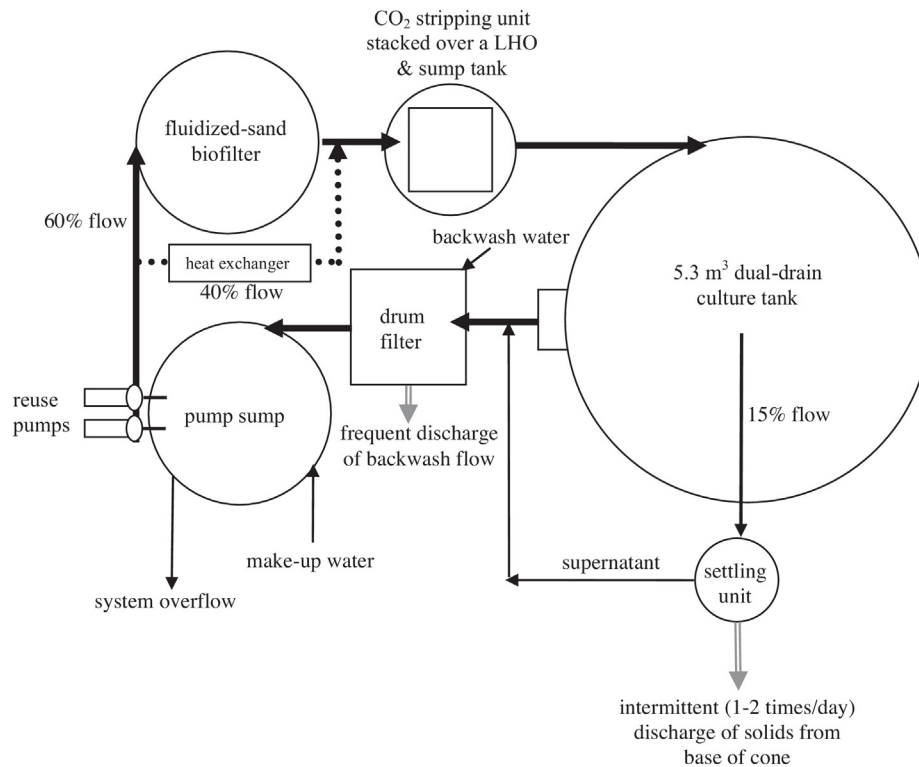E-mail address: kevin.schrader@ars.usda.gov (K.K. Schrader).

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

odor-producing bacteria and the effect of environmental conditions and/or nutritional factors on production of geosmin and/or MIB by the responsible bacteria. Previous studies have demonstrated the impact of environmental conditions and certain nutritional factors on geosmin and biomass production by *Streptomyces halstedii* (Blevins et al., 1995; Schrader and Blevins, 1999, 2001). The potential impacts of various environmental and nutritional factors on geosmin and MIB off-flavor episodes in RAS may provide producers with more information to predict and manage such episodes.

In a previous study by Blevins et al. (1995), nitrogen source (e.g., nitrate-N, ammonium-N) and concentration were determined to directly impact geosmin and biomass production by *S. halstedii*. Specifically, low concentrations of nitrate-N ($NO_3^-$-N) stimulated geosmin production while higher concentrations favored geosmin and biomass production by *S. halstedii*. Geosmin is a secondary metabolite of certain species of actinomycetes, and, therefore, reduced or limiting concentrations of $NO_3^-$-N may induce a shift to secondary metabolism and greater geosmin production.

There have not been any previous published studies on the effects of $NO_3^-$-N levels on geosmin and/or MIB off-flavor problems in RAS. Therefore, we decided to monitor the potential effects of two concentration ranges of nitrate-nitrogen ($NO_3^-$-N) on geosmin and MIB levels in culture water and finfish raised in RAS. This study was performed in conjunction with another study that was primarily being conducted to determine the potential chronic impacts of nitrate-N levels on rainbow trout (*Oncorhynchus mykiss*) welfare and performance, and, therefore, these $NO_3^-$-N levels could be encountered in commercial RAS. Water samples were monitored over an approximately two-month period and fish fillets were obtained at the end of the study to evaluate the impacts of low (20–40 mg $NO_3^-$-N/L) and high (80–100 mg $NO_3^-$-N/L) nitrate concentrations on geosmin and MIB levels in RAS culture tank water and in the rainbow trout fillets.

## 2. Materials and methods

### 2.1. Recirculating aquaculture systems

For this study, six replicated RAS ($9.5\,m^3$) units located at The Conservation Fund Freshwater Institute (TCFFI), Shepherdstown, WV, were used. These RAS have a chronic history of producing geosmin-related off-flavors in fish cultured with these systems due to the presence of geosmin-producing species of actinomycetes (Schrader and Summerfelt, 2010). Three RAS were randomly assigned to contain 20–40 mg $NO_3^-$-N/L levels in the culture tank water while the culture tank waters in the other three RAS were maintained at 80–100 mg $NO_3^-$-N/L. Each RAS unit was comprised of the following: (1) a fluidized-sand biofilter; (2) a forced-ventilation cascade aeration column; (3) a low head oxygenation (LHO) unit where pure oxygen feed gas was absorbed; (4) a LHO sump; (5) a single $5.3\,m^3$ culture tank; (6) a microscreen drum filter; (7) a particle trap; (8) a pump sump; and (9) a heat exchanger (Fig. 1). Each RAS was stocked on May 2, 2011, with 2050 juvenile rainbow trout at the beginning of the study and at which time mean rainbow trout weight was $16.4 \pm 0.3$ g and mean fish density was $6\,kg/m^3$ in the culture tank. At the conclusion of the 3-month study, maximum fish densities based on culture tank volume were $67.0 \pm 2.0\,kg/m^3$. During the study, mean water temperature was maintained at $15.5\,°C$.

### 2.2. Water exchange/flows

Makeup water flow rates were maintained equally for all six RAS throughout the study. Initially, 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 six RAS 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, respectively. These adjustments in water flow rates were made in order to maintain $NO_3^-$-N treatment concentrations and to prevent the accumulation of other water quality and ionic concentrations. The system hydraulic retention times resulting from the adjustments to makeup water rates were 2.5, 1.7, and 1.2 days, respectively.

### 2.3. Feeding

Rainbow trout were fed a standard 42/16 (protein/lipid) trout diet throughout the study (Zeigler Brothers, Inc., Gardners, PA). Fish were fed equal rations, with feeding events occurring every other hour and around the clock using automated feeders (T-drum 2000CE, Arvotec, Huutokoski, Finland).

### 2.4. General water quality

Nitrate-nitrogen concentrations for the high $NO_3^-$-N treatment were controlled by continuously dosing a sodium nitrate stock solution into the LHO sump using a peristaltic pump to complement the natural accumulation of $NO_3^-$-N resulting as an end product of nitrification. Nitrate-nitrogen concentrations within the control (low $NO_3^-$-N) systems were created only as an end product of the nitrification process and controlled by adjusting water exchange rates. All six fluidized sand biofilters were fully acclimated from a previous study and capable of providing complete nitrification when the present study was initiated. 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. Previous on-site studies have indicated that some portion of the $NO_3^-$-N that is produced within the six RAS is subsequently removed via passive denitrification (Davidson et al., 2011). Denitrifying bacteria are likely sheltered within hidden anaerobic biofilms somewhere within the unit processes of the six RAS creating conditions for low-level nitrate removal.

Water samples were collected weekly from the side drain of each tank and tested on-site for a variety of parameters. All water quality parameters measured at TCFFI were analyzed according to methods described in APHA (2005) and HACH (2003). Nitrate-nitrogen concentrations were analyzed using a cadmium reduction technique (Hach Method 8171).

### 2.5. Collection of water and fillet samples

Water samples were collected from each of the six RAS at the sidebox outflow from the culture tank to the drum filter (Fig. 1) beginning on June 6, 2011, and then again approximately three weeks later followed by additional sampling every 5–7 days until the final sampling on August 8, 2011. 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.

Rainbow trout fillet samples were obtained at the end of the study. Six trout were removed from each RAS culture tank, euthanized by cranial percussion, and filleted. Fillets were immediately placed in separate plastic freezer bags and frozen until overnight shipment to the NPURU laboratory for analysis of geosmin and MIB levels. For each fillet, one 20-g portion from the anterior end of the fillet was used to obtain distillate following microwave distillation and the procedures of Lloyd and Grimm (1999). Each distillate

sample was analyzed using solid phase microextraction–gas chromatography–mass spectrometry (SPME-GC–MS).

### 2.6. Determination of geosmin and MIB concentrations

Water samples and microwave distillates of rainbow trout fillet samples were processed prior to the determination of geosmin and MIB levels. For each sample, 0.6-mL aliquots were micropipetted into individual 2-mL, glass, crimp-top vials containing 0.3 g sodium chloride per vial. The method used to quantify levels of geosmin and MIB was similar to the SPME-GC–MS procedure used by Lloyd et al. (1998). Specifically, vials were heated at 40 °C for 20 min before the volatile compounds were absorbed onto a 100 μm polydimethyl siloxane solid-phase microextraction fiber (Supelco, Bellfonte, PA). The fiber assembly was then shaken for 10 min during the absorption period and then desorbed for 2 min at 250 °C in the injection port of a HP 6890 gas chromatograph–mass spectrometer (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) the first ramp rate was 30 °C/min to 100 °C; (3) the second ramp rate was 20 °C/min to 300 °C with an isotherm time of 2 min; and (4) the maintenance of flow pressure was at 124 kPa (18 lb/in$^2$) with helium used as a carrier gas. The molecular ion base peaks were monitored at the ratio of molecular mass to ionic charge (m/z) of 168, 95, and 135 for MIB and at m/z of 182, 112, and 126 for geosmin. A DB-5 capillary column [(5%-phenyl)-methylpolysiloxane, 30 m, 0.25 mm inside diameter, 0.25-μm film thickness; J&W Scientific, Folsom, CA] was used. 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 μg/L in deionized water. The original standards were obtained from Wako Chemicals USA, Inc., Richmond, Virginia., 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).

### 2.7. 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. Comparison of the grouped RAS mean geosmin concentrations in trout flesh between treatments (low $NO_3^-$-N versus high $NO_3^-$-N) was performed using the unpaired t-test ($\alpha$ = 0.05). Data analysis was generated using SigmaPlot software, Version 11.0 (Systat Software Inc., San Jose, CA, USA).

## 3. Results and discussion

The MIB levels measured in the culture water of the various RAS were relatively low (1–7 ng/L), and most of the water samples contained MIB levels near the instrumental detection limit of 1 ng/L. Because MIB levels fluctuated very little during the sampling period, there was no discernible impact of lower nitrate and higher nitrate levels on MIB concentrations (data not shown). Geosmin levels in the water samples were substantially higher throughout the study period (Fig. 2), and, therefore, geosmin was considered to be the more important compound contributing to off-flavor in trout from this study. Overall, geosmin levels remained <20 ng/L on most sampling dates. However, a large increase in geosmin levels occurred on day 43 (July 20, 2011) in RAS 4 (20–40 mg $NO_3^-$-N/L), 5 (80–100 mg $NO_3^-$-N/L), and 6 (80–100 mg $NO_3^-$-N/L). The increase in geosmin levels cannot be attributed to differences in $NO_3^-$-N levels because it occurred under both conditions and did not occur in the other three RAS utilized in this study. Instead, there is likely

**Table 1**
Measured water quality variables for the low nitrate-N and high nitrate-N RAS.

| Variable | RAS[a] | Sampling day | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 23 | 30 | 36 | 43 | 50 | 56 | 61 |
| BOD[b] | Low nitrate-N | 6.21 | 2.22 | 2.88 | 3.44 | 2.61 | 3.01 | 4.16 | NA[c] |
| | High nitrate-N | 8.18 | 3.93 | 4.27 | 4.30 | 3.05 | 2.93 | 3.18 | NA |
| pH | Low nitrate-N | 7.70 | 7.49 | NA | NA | 7.42 | NA | 7.33 | NA |
| | High nitrate-N | 7.66 | 7.46 | NA | NA | 7.40 | NA | 7.31 | NA |
| TSS[d] | Low nitrate-N | 7.32 | 5.62 | 2.06 | 2.77 | 2.32 | 2.76 | 6.21 | NA |
| | High nitrate-N | 16.99 | 5.10 | 6.52 | 8.44 | 2.75 | 2.54 | 2.78 | NA |

[a] Mean of respective low nitrate-N ($n = 3$) and high nitrate-N ($n = 3$).
[b] Biological oxygen demand (mg/L).
[c] Not available.
[d] Total suspended solids (mg/L).

another factor(s) involved in the dramatic increases in geosmin levels. Assessment of measured water quality variables (Table 1) did not provide any correlating factors that could be attributed to the increase in geosmin production within RAS 4, 5, and 6. Other variables not measured in the study may have induced greater geosmin production from actinomycetes and/or may have lead to a shift in the microbial communities to greater biomass (colony forming units) of geosmin-producing actinomycetes and subsequent geosmin production.

The MIB concentrations present in the trout flesh (Fig. 3A) were approximately one order of magnitude less than geosmin concentrations (Fig. 3B). Concentrations of MIB in trout flesh were not significantly different ($p > 0.05$) between low $NO_3^-$-N RAS and high $NO_3^-$-N RAS. During the study, geosmin concentrations in the trout flesh were well below the geosmin sensory threshold estimated to be 900 ng/kg (Robertson et al., 2005). Overall, geosmin concentrations in the trout flesh were also not significantly different between low and high $NO_3^-$-N RAS, except that RAS 5 (high $NO_3^-$-N) contained higher geosmin levels compared to all other RAS (Fig. 3B). Comparison of the mean geosmin concentrations between the grouped RAS (low $NO_3^-$-N versus high $NO_3^-$-N) determined no significant difference. Geosmin concentrations in the trout flesh were higher than MIB concentrations, and these results are in agreement with the levels of these compounds as measured in the RAS culture tank waters. Although the in vitro study by Blevins et al. (1995) indicated increasing geosmin/biomass production by *S. halstedii* with increasing $NO_3$-N concentrations, the highest $NO_3^-$-N concentration evaluated in their study was 0.83 mg $NO_3^-$-N/L which is well below the treatment levels of $NO_3^-$-N evaluated in the

present study and, in general, the concentrations that would be expected in low-exchange RAS. It is possible that the $NO_3^-$-N concentrations utilized in the present study were substantially high enough and not limiting, and, therefore, there was no dramatic shift to secondary metabolism and enhanced geosmin production by the actinomycetes present in the different RAS. In addition, other forms of nitrogen (e.g., ammonium-N) were available to the actinomycetes in the RAS, though nitrate-N appears to favor the growth of actinomycetes compared to ammonium-N (Shapiro, 1989).

The resulting geosmin levels in the culture tank water of the different RAS (Fig. 2) indicates that geosmin level/production within RAS is not at a constant level but can flux. Large increases in geosmin levels in the system water could further exacerbate the
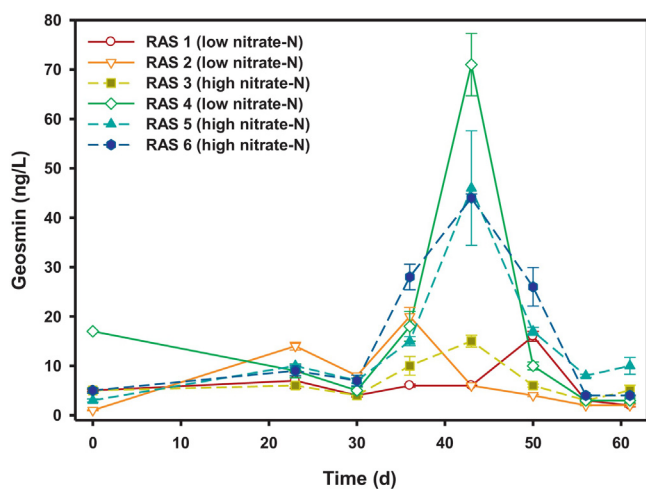


**Fig. 2.** Mean concentrations of geosmin in the culture tank water of six recirculating aquaculture systems (RAS) containing low or high levels of $NO_3^-$-N. Error bars represent the standard deviation of the mean.
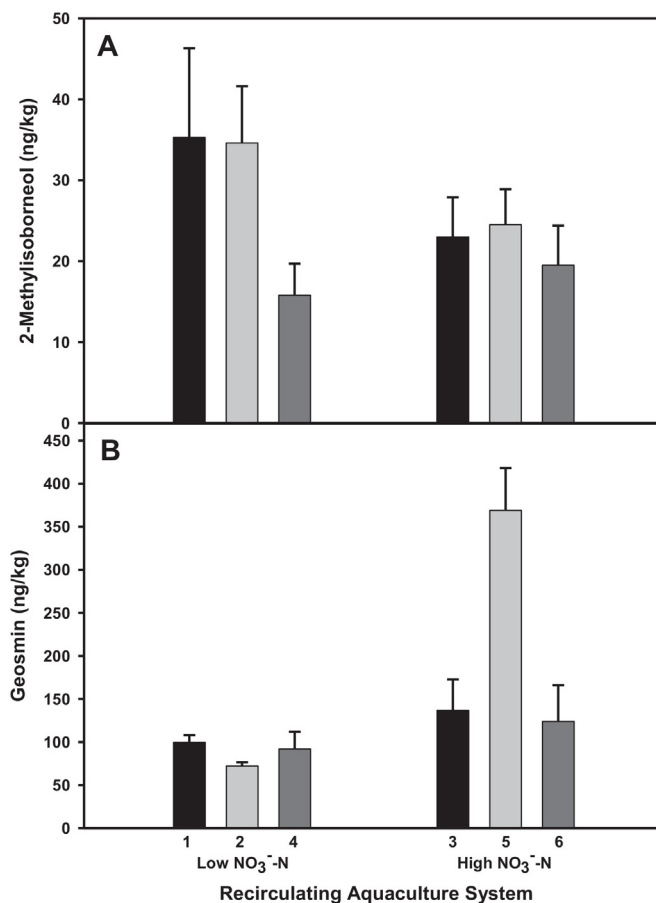


**Fig. 3.** Mean concentrations of 2-methylisoborneol (A) and geosmin (B) in the flesh of rainbow trout from recirculating systems containing low or high levels of $NO_3^-$-N. Recirculating aquaculture systems: 1, 2, and 4 = 20–40 mg $NO_3^-$-N/L; 3, 5, and 6 = 80–100 mg $NO_3^-$-N/L. Error bars represent the standard error of the mean ($n = 6$).

off-flavor problem due to the accumulation of higher concentrations of geosmin (and possibly also MIB) in the fish flesh. Additional studies to elucidate the cause(s) for such dramatic increases in off-flavor compound levels in RAS will necessitate enumeration of the responsible off-flavor compound-producing actinomycetes to determine the factors that play a role. Once these factors are identified, it may be possible to better predict the development and intensity of off-flavor episodes in RAS and develop management strategies to mitigate geosmin and MIB-related off-problems in RAS.

In conclusion, the difference between the two $NO_3^-$-N treatment levels (20–40 mg $NO_3^-$-N/L and 80–100 mg $NO_3^-$-N/L) did not result in differences in geosmin concentrations in RAS culture tank waters or in trout flesh. Therefore, it appears that adjustments of the $NO_3^-$-N levels utilized in this study are unlikely to provide a beneficial or adverse impact on geosmin-related off-flavor problems in RAS.

## Acknowledgements

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