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[Article]

Distribution of Off-Flavor Compounds and Isolation of Geosmin-Producing Bacteria in a Series of Water Recirculating Systems for Rainbow Trout Culture

KEVIN K. SCHRADER*
U.S. Department of Agriculture, Agricultural Research Service,
Natural Products Utilization Research Unit, National Center for Natural Products Research,
Post Office Box 8048, University, Mississippi 38677-8048, USA

STEVEN T. SUMMERFELT
The Conservation Fund Freshwater Institute, 1098 Turner Road, Shepherdstown, West Virginia 25443, USA

Abstract.—Preharvest off-flavor in aquaculture products results in large economic losses to producers due to delayed harvest. The common off flavors "earthy" and "musty" are due to the presence of geosmin and 2-methylisoborneol (MIB), respectively. Although certain species of cyanobacteria are responsible for these problems in pond-cultured fish, the microbial sources of geosmin and MIB in recirculating aquaculture systems (RASs) are still being explored. In this study, we investigated (1) the distribution of geosmin and MIB within six replicated RASs producing rainbow trout *Oncorhynchus mykiss* and (2) the microorganisms responsible for earthy off-flavor in the flesh of RAS-cultured trout. Water, biosolids, and fish samples were collected when fish were at maximum feed levels and before harvest. Each RAS contained a fluidized-sand biofilter, a cascade aeration column, a low-head oxygenation (LHO) unit, an LHO sump, a 5.3-m^3 culture tank, a drum filter, a pump sump, and a heat exchanger. Solid phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS) was used to determine the levels of geosmin and MIB in the collected samples. Water and biosolid samples were used to inoculate media for culturing microorganisms capable of producing geosmin, MIB, or both (e.g., actinomycetes, cyanobacteria, fungi, and myxobacteria). Microbial isolates producing an earthy–musty odor were subjected to SPME–GC–MS analysis to verify geosmin and MIB production. Geosmin levels were higher and more prevalent than MIB in water, biosolids, and trout fillets. In addition, geosmin levels were higher in biosolids than in water. The earthy-odor-producing actinomycetes *Nocardia cummidelens*, *N. fluminea*, *Streptomyces luridiscabiei*, and *Streptomyces* cf. *albidoflavus*, were isolated from biosolids contained within the RAS drum filters and heat exchangers, and these isolates were subsequently confirmed to be geosmin producers. No other geosmin-producing microorganisms were isolated. These isolates were the likely source of the geosmin in the RAS water and contributors to the earthy off-flavor in the trout.

Microbial sources of preharvest off-flavor in aquaculture products create large economic losses to the industry due to delays in harvest and reduction of market demand from inconsistent product quality (Tucker 2000). The most common off flavors acquired before harvest are "earthy" and "musty" and are attributed to the presence of the compounds geosmin and 2-methylisoborneol (MIB), respectively, in the flesh of the cultured aquatic animal. The production of geosmin and MIB can occur in certain species of actinomycetes, cyanobacteria, and fungi (Jüttner and Watson 2007). In addition, geosmin production has been confirmed in several species of myxobacteria such as *Myxococcus fulvus* (Yamamoto et al. 1994), *Stigmatella aurantiaca* (Dickschat et al. 2005), and *Chondromyces crocatus* (Schulz et al. 2004). Although species in the order Myxobacteriales are commonly associated with terrestrial environments, myxobacteria have been isolated from freshwater habitats (Carlson and Pacha 1968; Yamamoto et al. 1994). So far, the only geosmin-producing fungal species that have been discovered are commonly found in soil.

Research on identifying the microbial sources of earthy and musty off flavors in aquaculture, especially in the pond-raised channel catfish *Ictalurus punctatus* industry over the past two decades, has focused on cyanobacteria (blue-green algae). There are few published cases confirming the microbial sources of geosmin production in other systems used in aquaculture, e.g., recirculating aquaculture system (RAS). One of the earliest reports discussing the potential microbial sources of off-flavor compounds such as geosmin and MIB in RAS was by Flick et al. as reported by Avault (1994). In that report, the off-flavor in hybrid striped

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* Corresponding author: kevin.schrader@ars.usda.gov

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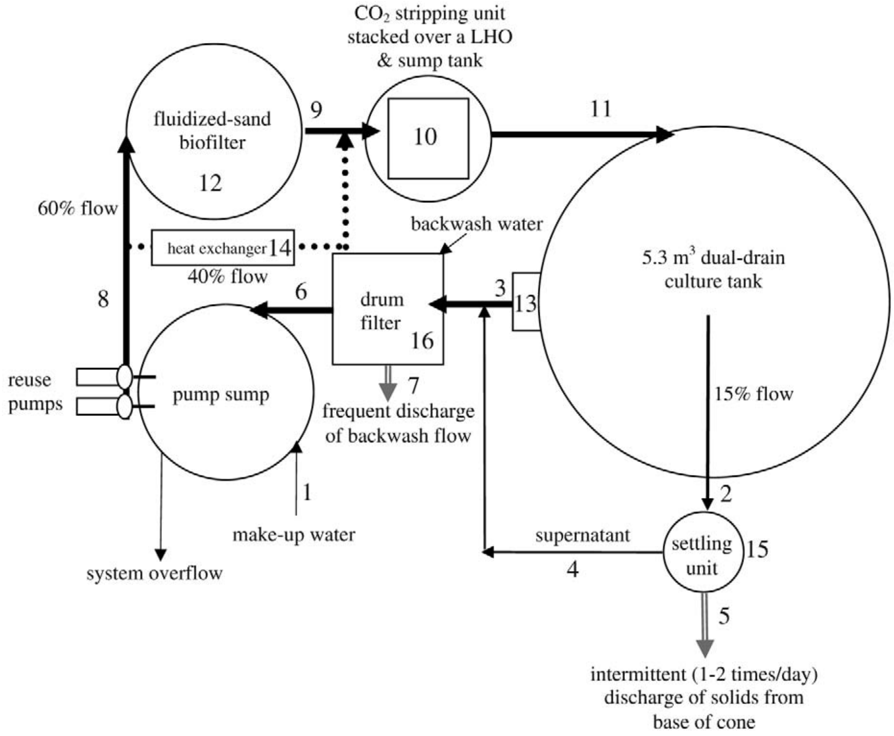

FIGURE 1.—Process flow diagram of one of the six replicated recycling systems (after S. T. Summerfelt et al. 2009). Each system recirculates 380 L/min (100 gal/min). A purified oxygen feed gas is added at the low-head oxygenation unit and air is ventilated through the cascade aeration (CO_2 stripping column). Sampling locations are numbered as in Table 1.

bass *Morone saxatilis* × *M. chrysops* was attributed to the detection of MIB, but not geosmin, in the RAS, and the scientists detected the presence of "streptomycetes spp." in the rotating biological filter and biological tower of the RAS. However, no mention is made as to the isolation of odor-producing microorganisms and subsequent confirmation of MIB production by "streptomycetes spp."

In a recent study, Guttman and van Rijn (2008) isolated two streptomycete species closely related to *Streptomyces roseoflavus* and *S. thermocarboxydus* from a zero-discharge RAS used to culture hybrid tilapia (Nile tilapia *Oreochromis niloticus* × blue tilapia *O. aureus*), and they confirmed the production of geosmin and MIB by both streptomycete species. The relatively high concentrations of geosmin and MIB in the organic-rich aerobic components of the RAS correlated with the presence of the streptomycete isolates.

In this study, we investigated the potential microbial sources of geosmin and MIB in the water within a series of RAS (six individual systems) and in the flesh of RAS-cultured rainbow trout *Oncorhynchus mykiss*. This series of six RAS has previously been identified as producing trout with earthy off-flavor problems. Our focus was to isolate microorganisms capable of producing geosmin, MIB, or both (e.g., actinomycetes, cyanobacteria, fungi, and myxobacteria) and subsequently confirm geosmin or MIB production from isolates obtained from various sampling sites containing high amounts of organic matter within the six RAS.

Methods

Recirculating aquaculture systems.—Six individual recirculating systems located at The Conservation Fund Freshwater Institute, Shepherdstown, West Virginia, were included in this study. Each recirculating system contained 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-m^3 culture tank, a microscreen drum filter, a particle trap, a pump sump, and a heat exchanger (Figure 1). Approximately 99.74% of the water flow was recirculated in each system, which provided a mean system hydraulic retention time of approximately 6.7 d. The water temperature during the study was 15 ± 1°C. A constant 24-h photoperiod was provided and fish in all systems were fed equal portions once every 2 h.

Sample collection.—Samples were collected from each of the six RAS at different locations (Table 1; Figure 1) and at two different times as follows: (1) when fish were at maximum feed levels (overall mean of 5.8 kg/tank per day or 1.1 kg/m^3 per day) and densities (overall mean of 69 kg/m^3; June 12, 2008), and (2) approximately 2 months later and just before harvesting the trout with mean feed levels of 4.5 kg/tank per day (0.9 kg/m^3 per day) and overall fish densities of 62 kg/m^3 (August 20, 2008). Water and biosolids samples were collected to determine geosmin and MIB levels at various sites within the RAS. In addition, biosolids samples and water samples containing high total suspended solids (TSS) were used as sample material for the isolation and culture of potential geosmin- and MIB-producing microorganisms. Water samples from sampling ports were collected 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 shipped by overnight express to the U.S. Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit (NPURU), University, Mississippi. Biosolids samples were collected using a clean spatula, placed in individual 20-mL glass scintillation vials, and stored at 4°C until overnight shipping.

Samples of rainbow trout were also obtained at the same time as water and biosolids 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 spectrometery (SPME–GC–MS) following microwave distillation (Lloyd and Grimm 1999).

Analysis for geosmin and MIB.—Water samples and microwave distillates of rainbow trout fillet samples were processed before we determined geosmin and MIB levels by placing 0.6-mL aliquots into 2-mL, glass, crimp-top vials containing 0.3 g sodium chloride per vial. For those biosolids samples that were too thick to micropipette, a slurry of the sample was made by adding 3–6 mL (depending upon the thickness of the sample) of carbon-filtered and double-deionized water before transferring 0.6-mL aliquots into 2-mL glass crimp-top vials. The procedures of Lloyd et al. (1998) and as modified by Schrader et al. (2003) were used to quantify geosmin and MIB using SPME–GS–MS. In addition, 1-mL aliquots of each biosolids sample were transferred to individual weigh pans for drying at 82°C to determine the dry weight.

TABLE 1.—Sampling locations and types within each of the six recirculating aquaculture systems at the Freshwater Institute. Sample numbers correspond to those in Figure 1.

Sample number	Sampling location	Sample type
1	Makeup water inlet at the pump sump	Water
2	Drain outlet at bottom of culture tank	Water
3	Side drain outlet on culture tank	Water
4	Settler overflow outlet	Water
5	Settler daily discharge	Water[a]
6	Drum filter outlet	Water
7	Drum filter backwash	Water[a]
8	Biofilter inlet	Water
9	Biofilter outlet–stripper inlet	Water
10	Stripping column outlet	Water
11	Culture tank inlet	Water
12	Top of fluidized biofilter bed	Biosolids
13	Inside standpipe on side drain of culture tank	Biosolids
14	Inside heat exchanger	Biosolids
15	Wall of settler	Biosolids
16	Wall of drum filter	Biosolids

[a] Sample contained a large amount of total suspended solids.

Media and culture conditions used for cyanobacteria, fungi, and myxobacteria.—The following media were used to culture cyanobacteria from the biosolids and high TSS water samples: (1) BG-11 (Rippka et al. 1979), (2) BG-11 (modified; van der Ploeg et al. 1995), and (3) ASM-1 (Gorham et al. 1964). Several types of cyanobacterial culture media were used to help increase the chances of culturing odor-producing cyanobacteria. Approximately 2 mL of each media type were pipetted into separate wells of 48-well microplates (Costar, Corning, Inc., Corning, New York), and 100 μL of each biosolids slurry sample and high TSS water sample were micropipetted into separate wells. Plates were placed at 15°C under continuous light from a warm white, fluorescent, 8-W bulb and at two different ranges of photon flux densities of 2.05–2.38 μE/m^2 per second and 9.33–10.72 μE/m^2 per second. Plates were incubated for 8 weeks and inspected visually after 1 week of incubation and weekly afterwards for the growth of algae and cyanobacteria. Periodically, plate lids were slightly raised and checked by olfaction using individuals familiar with the odors of geosmin and MIB to detect the presence of earthy or musty odors. Samples were removed from microplate wells observed to contain algal and cyanobacterial growth and observed microscopically (400×) for identification to the genus level using the taxonomic schemes and classification keys of Wehr and Sheath (2003) and Komárek and Anagnostidis (2005).

Corn meal agar (CMA) (BBL; Becton, Dickinson and Co., Sparks, Maryland) plates were used for the culture of fungi. Aliquots of 0.1-mL serial dilutions of the biosolids slurry samples and high TSS water samples were spread-plated onto CMA plates. Plates were incubated for 1 week at 15°C. The lids of plates containing fungal colonies were raised slightly to detect the presence of an earthy or musty odor.

Agar plates of casitone-yeast (CY) medium were prepared for the isolation of myxobacteria (Yamamoto et al. 2000). Aliquots of 0.1-mL serial dilutions of biosolids samples and high TSS water samples were spread-plated onto CY agar plates. Plates were incubated at 15°C for 1 week and then inspected visually for the presence of myxobacteria colonies and plate lids were slightly raised to detect the presence of an earthy or musty odor.

Isolation and identification of odor-producing actinomycetes.—Based upon the study by Guttman and van Rijn (2008), samples containing higher amounts of organic matter (biosolids samples and high TSS samples) were considered more likely to provide odor-producing actinomycetes. Biosolids samples and high TSS water samples were serially diluted, and these diluted samples were used to inoculate 1% yeast extract–1% dextrose (YD) agar (pH 7.5) plates, actinomycete isolation agar (AI) (Bacto; Becton, Dickinson and Co.) plates, and starch–casein (SC) agar (Kuster and Williams 1964) plates by the spread-plate technique. Serially diluted (1:10) biosolids and high TSS water samples were routinely shaken 25 times in a back-and-forth motion within a distance of approximately 0.5 m and over a 10-s period before spread-plating. This shaking routine was used to help release actinomycetous propagules (AP) from organic matter aggregates while preventing excessive fragmentation of actinomycetous filaments that could cause an overestimation of AP numbers (Schrader and Blevins 1993). Total heterotrophic bacteria (enumerated as colony-forming units [CFU]) were also determined for biosolids and high TSS water samples. Duplicate sets of plates were prepared, with one set incubated at 15°C and the other set at 25°C. Two different incubation temperatures were used to aid in the isolation of actinomycetes.

At 7 d after incubation, colonies bearing resemblance to actinomycete colony morphology (e.g., chalky appearance, "biting" into agar surface) were enumerated and then streaked for isolation onto the same type of agar from which they were obtained and incubated at the temperature in which they had originally grown. After 7 d of incubation, plates were evaluated for odor production by olfaction. Isolates identified as producing typical odors associated with geosmin and MIB were chosen to perform analyses to detect production of those compounds. An isolated colony (2–5 mm in diameter) was aseptically removed from an agar plate containing a suspected geosmin-producing actinomycete and aseptically transferred to a 2-mL glass crimp-top vial containing 0.6 mL of ultrapure water (geosmin and MIB free) and 0.3 g sodium chloride. These vials were then immediately analyzed by SPME–GC–MS to detect and confirm production of geosmin, MIB, or both by the isolate.

Colony morphologies of the various actinomycete isolates were documented to help with presumptive identification and designation of actinomycetes into suprageneric groups and subsequently to arrange isolates into four representative subgroups based upon similar colony morphoplogy and pigment production. Genotypic identification of a representative of each subgroup of geosmin-producing isolates was performed by phylogenetic analysis (Accugenix, Inc., Newark, Delaware) using comparative 16S rRNA gene sequencing (500 base pairs [bp]). For each representative isolate, genomic DNA was extracted and purified, and target DNA (portion of 16S ribosomal gene) amplified using polymerase chain reaction (PCR) with the bacterial primers 531R (5′- TAC CGC GGC TGC TGG CAC –3′) and 005F (5′- TGG AGA GTT TGA TCC TGG CTC AG –3′). Each PCR product was purified and sequenced using dye-terminator sequencing chemistry to fluorescently label each nucleotide of the PCR product. Fluorescently labeled product was analyzed on an automated fluorescent DNA sequencer to provide an electropherogram and sequence the sample. Each generated sequence was compared with the Accugenix, Inc., database. For each isolate sample, a phylogenetic tree was generated by neighbor-joining and closest-match methods, and final identification was made based upon genetic percent difference (distance measurement) and the phylogenetic tree. A similar polyphasic approach has been described for the identification of certain species of *Streptomyces* and *Nocardia* by Maldonado et al. (2000) and Anderson and Wellington (2001), respectively.

Results and Discussion

Dense phytoplankton growth was observed in many of the inoculated microplates wells after 8 weeks of incubation. The only types of phytoplankton observed to grow in the three types of cyanobacterial culture media used in this study were several species of green algae identified as *Chlorococcum* sp., *Desmococcus* sp., and *Stichococcus* sp. and one species of cyanobacteria tentatively identified as *Pseudanabaena* sp. The growth of the *Pseudanabaena* sp. was observed in microplate wells containing each type of culture media

TABLE 2.—Abundance of actinomycetous propagules (AP) in biosolids samples on two dates. Abundance is based on the dry weight of the slurry mixture made of each biosolids sample and represents a compilation of colony counts obtained from dextrose and actinomycete isolation agar plates; CFU = colony-forming units of total heterotrophic bacteria based on the dry weight of the slurry mixture.

	Jun 12, 2008			Aug 20, 2008		
Sampling location[a]	AP/mg	Total CFUs/mg	AP/CFU (%)	AP/mg	Total CFUs/mg	AP/CFU (%)
Biofilter (1)	4.9×10^2	4.7×10^5	0.1	ni[b]		
Standpipe (1)	ni[b]			1.1×10^2	1.2×10^4	0.9
Heat exchanger (1)	1.2×10^2	4.5×10^4	0.3	6.6×10^2	2.9×10^4	2.3
Wall of settler (1)	ni[b]			5.6×10^2	1.9×10^5	0.3
Drum filter (1)	1.2×10^3	8.6×10^4	1.4	2.5×10^3	7.5×10^4	3.4
Heat exchanger (2)	5.0×10^2	4.2×10^4	1.2	4.7×10^2	1.8×10^4	2.6
Drum filter (2)	3.4×10^2	6.9×10^4	0.5	7.6×10^1	2.8×10^4	0.3
Biofilter (3)	6.7×10^1	2.8×10^4	0.2	ni[b]		
Heat exchanger (3)	6.5×10^1	5.5×10^5	0.01	3.5×10^2	1.4×10^5	0.3
Drum filter (3)	3.6×10^2	5.1×10^4	0.7	4.9×10^2	1.4×10^5	0.4
Biofilter (4)	ni[b]			1.7×10^3	2.5×10^4	6.6
Heat exchanger (4)	1.2×10^2	1.8×10^6	0.01	3.3×10^2	6.3×10^4	0.5
Drum filter (4)	7.0×10^1	8.4×10^4	0.1	6.9×10^3	1.1×10^5	6.5
Standpipe (5)	ni[b]			7.1×10^1	3.6×10^4	0.2
Heat exchanger (5)	8.2×10^1	1.8×10^6	0.01	3.4×10^2	1.3×10^4	2.6
Drum filter (5)	5.9×10^2	1.3×10^6	0.1	1.3×10^3	2.6×10^4	4.9
Heat exchanger (6)	2.6×10^1	1.1×10^5	0.02	8.0×10^2	3.8×10^4	2.1
Drum filter (6)	ni[b]			3.0×10^3	6.2×10^4	4.8

[a] Numbers in parentheses indicate the recirculating aquaculture system units in the series. Actinomycete colonies were not observed on the spread plates of biosolids samples from other sampling locations for either sampling period.

[b] None isolated.

used (ASM-1, BG-11 [Rippka et al. 1979], or BG-11 [modified] [van der Ploeg et al. 1995]), maintained at either the low or high light intensities, and inoculated with biosolids sample material obtained from the following locations of the six RASs: (1) wall of the settler, (2) wall of the drum filter, and (3) top of the biofilter bed. Microplate wells containing this cyanobacterial isolate did not produce any earthy or musty odors. In contrast to southeastern U.S. catfish production ponds typified by bloom-forming planktonic cyanobacteria, the RAS used in our study were not conducive for the growth of similar planktonic types of cyanobacteria and cyanobacterial bloom formation due to the rapid circulation and filtration of the water and low ambient light levels, respectively.

Although the growth of several different types of fungi was observed on the inoculated CMA agar plates, no earthy or musty odors were detected from these plates. Myxobacteria were not observed to be present on any of the inoculated CY agar plates.

Very few actinomycete colonies were observed on YD, AI, and SC agar plates incubated at 15°C, while plates incubated at 25°C revealed substantially higher numbers of actinomycete colonies. One possible reason for the vast difference in actinomycete colonies observed at 15°C compared with 25°C may be due to the over-growth of the agar surfaces by other types of bacteria that grew faster at 15°C than at 25°C. Based upon observations of actinomycete colonies on plates incubated at 25°C, AP were determined to be present in biosolids samples obtained from the various locations in the RAS (e.g., biofilter, standpipe on tank side drain, wall of settler, heat exchanger, and drum filter), though not at each sampling location at each sampling time (Table 2). Higher AP counts were obtained on YD and AI agar plates than on SC agar plates, and, therefore, counts from these plates were used to compile the data presented in Table 2. Actinomycetous propagules were present in each biosolids sample collected from the heat exchangers and drum filters of all six RAS, and AP were more abundant in biosolids samples obtained from the heat exchangers and drum filters than at other sampling locations within the RAS (Table 2). The lack of isolation of AP at each sampling location in our study does not necessarily indicate their lack of presence at such sites. Guttman and van Rijn (2008) concluded that aerobic organic-rich conditions present at certain sites (e.g., drum filter) within the RAS used in their study stimulated the growth of actinomycetes. While it is possible that AP may be present throughout the RAS in water and suspended organic matter, our results suggest that aerobic organic-rich conditions present at certain sampling sites in our study (e.g., heat exchanger, drum filter) were more conducive to provide a sufficient abundance of biomass containing propagules to permit isolation and detection via

TABLE 3.—Mean abundance and mean percentage of actinomycetous propagules (AP) in biosolids samples from the heat exchangers and drum filters in the six recirculating aquaculture systems on two dates. *P*-values are from paired *t*-tests ($\alpha = 0.05$; df = 5). See Table 2 for other details.

	Mean AP/mg			Mean AP/CFU (%)		
Sampling location	Jun 12	Aug 20	*P*	Jun 12	Aug 20	*P*
Heat exchangers	1.5×10^2	4.9×10^2	0.031	0.26	1.73	0.011
Drum filters	4.3×10^2	2.4×10^3	0.131	0.47	3.38	0.053

conventional spread-plate techniques. During in vitro studies, Guttman and van Rijn (2008) also found that geosmin production increased in organic matter from the RAS. In our study, the mean abundance of AP and the mean percentage of AP/CFU of total heterotrophic bacteria significantly ($P < 0.05$) increased in biosolids samples from the heat exchangers from the first sampling to the second sampling based upon plate count results (Table 3). There was also an increase in the mean abundance of AP and mean percentage of AP/CFU of total heterotrophic bacteria in biosolids samples from the drum filters, though these increases were not significant ($P > 0.05$) (Table 3). Although AP can be filaments, metabolically inactive arthrospores, or both, our preliminary results along with those of Guttman and van Rijn (2008) suggest that actinomycetes, including odor producers, are metabolically active in the submerged organic matter at aerobic sites within the RAS. Future in vivo studies will attempt to verify that there is a concomitant increase in the levels of geosmin and abundance of AP in biosolids samples within these RAS during a rainbow trout grow-out cycle to harvest time.

Among the spread plates (YD, AI, and SC) of the biosolids samples from the two samplings, over 70 actinomycete colonies exhibiting a chalky appearance were identified and streaked for isolation on separate YD agar plates Approximately 50 of these isolates produced an earthy odor based upon olfactory detection. By distinguishing the uniqueness of colony morphologies and color, these earthy-odor isolates were further designated into four distinct subgroups, with each subgroup essentially representative of a single species. Geosmin production was confirmed by GC–MS for each subgroup species while MIB was not detected. Subsequently, a representative isolate from each subgroup was chosen for identification via phylogenetic analysis and representative isolates were identified as *Nocardia cummidelens* (similarity: 100%), *N. fluminea* (similarity: 99.40%), *Streptomyces luridiscabiei* (similarity: 100%), and *Streptomyces* cf. *albidoflavus* (similarity: 100% at genus level). The taxonomic identification of *N. fluminea* also used the phylogenetic tree and relative close matches of related species (Accugenix Inc.). The later isolate was initially matched by phylogenetic analysis to a group of five closely related species (*S. albidoflavus*, *S. coelicolor*, *S. limosus*, *S. odorifer*, and *S. sampsonii*). However, this *Streptomyces* isolate was later designated as *S.* cf. *albidoflavus* based upon phenotypic characteristics including spore chain morphology of flexuous and spore mass characteristics (aerial is white while color of substrate hyphae is yellow-brown with no diffusible pigments). These characteristics matched those described for the type species *S. albidoflavus*, with the subjective synonyms for this type species including *S. coelicolor*, *S. limosus*, *S. odorifer*, and *S. sampsonii* (Locci 1989).

TABLE 4.—Geosmin-producing isolates of actinomycetes obtained from various sites within the six recirculating aquaculture systems (RAS) from two sampling events.

		RAS	
Species	Location	Jun	Aug
Nocardia cummidelens	Top of biofilter bed		4
	Standpipe on tank side drain		1, 5
	Heat exchanger		1, 5, 6
	Drum filter	1, 3	1, 4, 5, 6
Nocardia fluminea	Standpipe on tank side drain		5
	Heat exchanger		5
	Drum filter	1, 2, 4	1, 4, 5
Streptomyces luridiscabiei	Heat exchanger	2, 3, 6	
Streptomyces cf. *albidoflavus*	Drum filter	3	
	Heat exchanger	4	

Biosolids samples obtained from the heat exchangers and the drum filters during the two samplings provided more isolates of geosmin-producing actinomycetes (Table 4). In addition, *N. cummidelens* and *N. fluminea* were isolated at more sampling locations within the six RAS compared with the two geosmin-producing species of *Streptomyces*. No geosmin-producing *Streptomyces* species were isolated from biosolids samples obtained during the second sampling (Table 4).

Among the various types of actinomycetes, geosmin-producing species of *Streptomyces* have been isolated more often than other species over the past several decades since the initial discovery of geosmin production by several *Streptomyces* spp. (*S. antibioti-*

TABLE 5.—Geosmin levels in water and biosolids samples collected during two sampling events from six recirculating aquaculture systems (RAS). Geosmin values are expressed as ng/L for water samples collected at locations 1–11 and as μg/kg dry weight for biosolids samples collected at locations 12–16; nd = not detected (below the instrumental detection threshold level of 1 ng/L).

Sampling location	RAS 1		RAS 2		RAS 3		RAS 4		RAS 5		RAS 6	
	Jun 12	Aug 20	Jun 12	Aug 20	Jun 12	Aug 20	Jun 12	Aug 20	Jun 12	Aug 20	Jun 12	Aug 20
1	1	nd	1	1	nd	nd	nd	nd	1	nd	1	nd
2	3	2	3	1	nd	1	nd	2	5	1	3	3
3	3	1	4	1	6	1	nd	2	3	1	3	3
4	2	2	4	1	4	2	nd	2	4	1	3	3
5	9	4	34	5	15	3	16	1	3	3	4	5
6	4	1	3	1	1	1	4	2	4	1	4	3
7	7	15	14	4	53	5	11	9	4	7	41	22
8	3	2	3	1	1	1	nd	1	3	1	3	4
9	3	2	3	1	4	1	4	1	2	1	5	3
10	4	1	1	1	5	2	4	1	2	1	4	3
11	3	1	nd	1	5	1	3	2	2	1	4	4
12	23.9	31.6	6.7	12.7	18.3	26.3	15.1	84.0	32.7	15.0	1.7	73.1
13	13.5	4.1	115.0	21.7	48.0	42.8	1.5	12.8	1.0	1.2	39.5	0.3
14	40.7	38.5	93.5	4.3	82.7	93.2	44.6	64.8	43.4	31.2	119.2	260.7
15	13.3	1.1	2.3	2.0	11.1	0.9	2.9	1.2	18.8	0.2	6.9	0.6
16	28.0	125.0	105.0	46.9	46.5	23.5	30.8	85.0	46.4	98.7	113.5	15.0

cus, *S. fradiae*, *S. griseus*, and *S. odorifer*) made by Gerber and Lechevalier (1965). Gerber (1979) was the first to discover geosmin production in the laboratory by three isolates of *Nocardia*, though designations of species were not made. Additional published reports of geosmin-producing *Nocardia* are lacking. This research is the first to report geosmin production by *S. luridiscabiei*, *N. cummidelens*, and *N. fluminea*. All four of the geosmin-producing isolates in our study were found to be relatively strong producers of geosmin based upon olfactory analysis. In addition, these four isolates are capable of growth at 15°C because colonies of each isolate on agar plates maintained at 15°C were observed to almost double in diameter over the course of 3–4 d. Physiology studies are underway to determine the optimal temperature for growth and geosmin production for each isolate.

For both samplings, geosmin was present at detectable levels at most of the sampling locations throughout the six RAS (Table 5). However, geosmin levels were considerably lower in water samples, including high TSS samples, compared with biosolids samples. Geosmin levels in water samples ranged from below the instrumental detection limit of 1 ng/L to as high as 6 ng/L, except for high TSS water samples in which geosmin levels ranged from 1 to 53 ng/L. In the biosolids samples, geosmin levels ranged from 200 to 260,700 ng/kg on a dry weight basis. Generally, geosmin levels were highest in biosolids samples obtained from the heat exchanger (4,300 to 260,700 ng/kg) and the drum filter (15,000 to 125,000 ng/kg). These locations also provided biosolids samples with a greater abundance of AP (Table 2). Although actinomycetes were not isolated from the settling unit and the standpipe of the tank side drain during the first sampling, it is likely that organic matter at these locations also contained geosmin-producing actinomycetes due to significantly higher levels of geosmin in the biosolids (mean of six RAS = 9,200 and 36,400 ng/kg for settling unit and standpipe, respectively) compared with the water samples of the settler inflow and outflow (mean of six RAS = 2.3 and 2.8 ng/L, respectively) and side-drain inflow and outlet (mean of six RAS = 2.8 and 3.2 ng/L, respectively). Actinomycetes were isolated from the settling unit of RAS 1 and the standpipe of RAS 1 and 5 from biosolids samples obtained during the second sampling. It is possible that distribution of AP throughout the RAS becomes greater after suitable conditions are established for actinomycete growth at aerobic organic-rich sites within the RAS.

Based upon results from the first sampling, MIB was found to be sporadically present at detectable levels (1 ng/L and higher) in only 17 of the 96 samples collectively for the six RAS (data not shown). In addition, MIB levels were quite low in water and biosolids samples combined (mean of 96 samples = 2.2 ng/L) compared with geosmin levels (Table 5). However, the second sampling found MIB present at each sampling location within each RAS, though levels were still low (mean of 96 samples = 13.4 ng/L). Geosmin and MIB were confirmed to be present in the flesh of all of the rainbow trout fillets from each sampling. The microbial source of MIB within these RAS was not identified during this study.

Based upon the results of our study, the geosmin-producing actinomycetes *N. cummidelens*, *N. fluminea*, *S. luridiscabiei*, and *S.* cf. *albidoflavus* are likely sources of geosmin present in the RAS water and subsequently in the rainbow trout cultured in the RAS. There may be additional geosmin-producing actinomycetes that were not isolated during this study, and, therefore, it is difficult to assess the exact contribution of the geosmin-producing actinomycetes that were isolated to the geosmin produced within the RAS. Conversely, the main contributors to geosmin (and MIB) off flavors in freshwater ecosystems, such as aquaculture ponds, are certain species of cyanobacteria (Jüttner 1995; Tucker 2000), and the application of algicides to production ponds is the management approach typically used by catfish producers to help selectively reduce the abundance of common odor-producing cyanobacteria (Tucker 2000). Due to the nature of RAS and the microbial sources of earthy off-flavor, the use of biocides to control the actinomycetes responsible for earthy and musty off-flavor problems would be difficult. Therefore, novel management techniques will need to be developed to help manage earthy and musty off-flavor problems in fish cultured in RAS. Although frequent cleaning of the heat exchanger and drum filter to remove organic matter would be labor intensive, future studies need to determine whether this potential solution is worthwhile before implementation on a commercial scale.

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