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Published in:
Journal of Applied Microbiology

DOI (link to publication from Publisher):
10.1111/jam.15358

Publication date:
2022

Document Version
Accepted author manuscript, peer reviewed version

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Article type : Original Article

Impact of water quality parameters on geosmin levels and geosmin producers in European recirculating aquaculture systems

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Abbreviated running headline: Off-flavour-causing bacteria in RAS

Abstract

Aims: Geosmin is associated with off-flavour problems in recirculating aquaculture systems (RAS) and represents an economic problem for the aquaculture industry. This study aims at investigating factors influencing the composition of the bacterial microbiota, in particular the presence of geosmin producers and the environmental and farming factors favouring geosmin accumulation.

Methods and Results: Several water quality parameters were correlated to the composition of the microbiota with special emphasis on the presence of geosmin producers within 26 different RAS from four European countries. Three novel groups of geosmin-producing bacteria were quantified to identify potential correlations with geosmin concentration.

Conclusions: The microbiome differed significantly between systems. However, phosphate levels, calcium levels, and redox potential correlated to geosmin concentration in the water and the presence of the *Actinomycetales* geosmin-producers but not with the presence of other groups of geosmin-producing bacteria. Oxygen levels and conductivity were found to negatively correlate with geosmin concentration. A large proportion of the detected geosmin producers represented novel taxonomic groups not previously linked with this activity.

Significance and Impact of the Study: These results improve our understanding of the diversity of microbiota in RAS and the water quality parameters favouring the populations of geosmin-producing bacteria and the geosmin production.

Keywords: Fish, biofilter, off-flavour, geosmin, microbial community, *Actinomycetales*, myxobacteria, *Sorangium* sp.

Introduction

Geosmin is a secondary metabolite that gives an earthy off-flavour and has a deleterious effect on the taste of water and food items. This causes severe economic problems in miscellaneous systems such as wine and liquor production, drinking water, and in aquaculture (Ludwig et al. 2007; Du et al. 2011; Auffret et al. 2011; Behr et al. 2013). This aromatic compound can enter the fish through the gills, skin, and intestinal tract (From and Hørlyck 1984; Howgate 2004) and accumulates primarily in the adipose tissue of the fish due to its hydrophobic nature. Depuration in clean water combined with fasting is therefore frequently used to reduce the levels of geosmin. However, this is a time-consuming and laborious process and increases production costs and

reduces fish yield. The primary source of geosmin in the environment is as a secondary metabolite of several microorganisms and it is likely that the composition of the microbiota is a major defining factor for the presence of geosmin producing organisms and the potential for geosmin production. Research has therefore focused on preventing geosmin production by identifying the microorganisms involved in geosmin production and the environmental conditions that favour their activity (Howgate 2004; Robertson et al. 2006; Davidson et al. 2014; Sarker et al. 2014; Azaria and van Rijn 2018).

Cyanobacteria, myxobacteria and *Actinomycetales* (in particular *Streptomyces*) have been described as the main geosmin-producers in aquatic environments (Guttman and van Rijn 2008; Su et al. 2013; Sarker et al. 2014; Yamada et al. 2015). Culture-independent and quantitative approaches have shown that geosmin-producing bacteria constitute 0.001-1 % of the total bacterial count in modern RAS (Lukassen et al. 2017). These geosmin-producing bacteria primarily affiliate within the order of *Actinomycetales*, *Myxococcales*, and species associated with the genus *Sorangium*. These observations are based on the phylogenetic study of the gene coding for the geosmin synthase (*geoA*) which is a bi-functional enzyme within a family of related terpene synthases (Jiang et al. 2007). The functional *geoA* gene has been shown to be a suitable molecular marker to investigate the diversity of putatively geosmin-producing organisms (Cane and Watt 2003; Giglio et al. 2008; Yamada et al. 2015; Lukassen et al. 2017).

Several water quality parameters have been studied to determine their effect on the levels of geosmin and the overall population of the geosmin-producing community in laboratory, pilot scale or full-scale commercial RAS (Guttman and van Rijn 2008; Schrader et al. 2013, 2015; Azaria and van Rijn 2018). High levels of organic matter have been shown to correlate with production of geosmin in pure cultures (Saadoun et al. 2001), in drinking water (Parinet et al. 2010), and in aquaculture systems (Guttman and van Rijn, 2008; Schrader et al., 2013). Similarly, oxygen, and phosphate levels also appear to positively correlate with the production of geosmin in both cyanobacteria and *Streptomyces* isolates (Saadoun et al. 2001; Schrader and Blevins 2001; Parinet et al. 2010) and the presence of bacteria affiliated with the deltaproteobacterium *Sorangium* correlates positively with phosphate in RAS (Auffret et al. 2013). Total nitrogen content has been suggested to favour geosmin production in rivers used as drinking water reservoirs (Parinet et al. 2013). This observation was supported by *in vitro* studies on cyanobacteria and in a drinking water system where ammonium was shown to correlate positively with geosmin levels (Saadoun et al. 2001; Parinet et al. 2010). Elevated temperature has also been suggested to stimulate the production of geosmin in pure culture studies with *Streptomyces* and cyanobacteria (Zhang et al. 2009; Schrader et al. 2015). On the other hand, increased

salinity was found to negatively affect the geosmin level in fish ponds and on an *Streptomyces avermitilis* isolate (Lovell and Broce 1985; Řezanka and Votruba 1998). Finally, conflicting results have been reported in regard to the effect of nitrate concentration in relation to geosmin production. Schrader et al. have reported that nitrate concentration did not impact geosmin levels in RAS (Schrader et al. 2013). Meanwhile, other studies have reported that high nitrate concentrations can inhibit geosmin production in cyanobacteria (Saadoun et al. 2001; Robertson et al. 2006) and still other reports have shown that high levels of nitrate were associated with increased geosmin concentrations (Oh et al. 2017).

A better understanding of the environmental factors and conditions favouring the presence of geosmin-producing bacteria and their activity could allow the selection of designs or operational procedures in RAS, which would help reduce the levels of geosmin. Therefore, the present study aimed to increase knowledge on the correlation between geosmin production in RAS and water quality. To this end, the level of geosmin and the presence of bacteria harbouring the functional gene *geoA* were investigated in 26 European RAS and analysed in order to identify any statistical correlations to water quality parameters. The resulting statistical correlations illustrate water quality parameters associated with increased abundance of geosmin and geosmin-producing bacteria and provide important knowledge that can be used to reduce the presence of geosmin-induced off-flavour through maintenance and operational management.

Materials and Methods

Aquaculture plant description and sampling

Samples were taken from 26 RAS located in the Netherlands (12), Denmark (8), France (3) and Switzerland (3) (Table S1). All plants in the Netherlands were indoor facilities while all the others were outdoor plants. Samples collected in the Netherlands represented systems farming turbot (*Scophthalmus maximus*) (n=3), yellowtail kingfish (*Seriola lalandi*) (n=2), eel (*Anguilla anguilla*) (n=3), pikeperch (*Sander lucioperca*) (n=3), and Nile tilapia (*Oreochromis niloticus*) (n=1). All of the Danish systems farmed trout (*Oncorhynchus mykiss*), while all of the French and Swiss systems farmed sturgeons (*Acipenser baerii*). Sixteen plants treated the recirculated water using a moving-bed filtration and the remaining used a fixed-bed filtration approach. Additional information such as the type of feed given to the fish was also collected.

Moving-beds were sampled by collecting 10 randomly selected plastic carriers while filter material from RAS using a fixed-filter system were sampled by cutting 1 cm^2 pieces out at various locations in the filters. Water samples for DNA analysis (100 mL), analysis of water quality parameters (100

mL), and geosmin concentration (50 mL) were collected immediately after passage of the biofilter and after passage through the fish tanks (hereafter referred to as the in- and outflows, respectively). Water quality parameters were measured immediately after collection while water samples for geosmin concentration measurements were collected in completely filled glass bottles (with no head space), closed with lids with a Teflon inlay. All samples collected were transported on ice to the laboratory and water samples for DNA analysis were filtered immediately after returning to the laboratory through a 0.45 µm cellulose ester membrane filter and the filters were kept at -20 °C until further molecular analysis. Samples collected for geosmin concentration measurements were send on ice to Tzabam Laboratories (Israel) for analysis.

Water quality parameters and geosmin concentration measurements

Dr. Lange cuvette test kits were used to measure concentrations of Chemical Oxygen Demand on both filtered and unfiltered samples (COD, LCK 314), phosphate (LCK 349), calcium, magnesium, and water hardness (LCK 327) with a DR 3900 spectrophotometer (Hach Lange GmbH) in the unfiltered samples according to the recommendations provided by the manufacturer.

The rest of the water quality parameters were all measured directly in both the inflow and outflow of the sampled fish tanks. All measurements were conducted as average of five replicates. These parameters were: Conductivity, salinity, total dissolved solids (TDS) and temperature (CO 310 Conductivity meter, VWR, United Kingdom), redox potential, and pH (HQ11d pH/ORP Meter, Hach, USA) and the concentrations of dissolved oxygen (DO 210 Oxygen meter, VWR, United Kingdom), nitrate (LAQUAtwin Compact Nitrate Meter, Horiba, China) and nitrite (Quantofix nitrite strips, Macherey-Nagel, Germany).

For the geosmin concentration measurements, the water samples were analysed commercially by Tzabam Laboratories (Israel) using solid-phase microextration (SPME) as described elsewhere (Lloyd et al. 1998). All measurements were conducted in triplicates.

DNA extraction

The cellulose ester membrane filters were split into three and DNA was extracted in triplicates with the Fast-Prep soil kit (MP Biomedicals, USA), bead-beated in 2 times 40 s at 6 $m \cdot s^{-1}$ according to the manufacturer's instructions. Bead beating was performed on a FastPrep FP120 (MP Biomedicals, USA). DNA was extracted from the biofilter samples by carefully cutting the

biofilter material, with approximately 0.25 g filter material, into small pieces and transfer of these pieces to a bead-beating tube. Extractions were performed in triplicates using PowerSoil® DNA Isolation Kit (MoBio, USA) following the recommendations of the manufacturer.

Quantitative PCR and amplicon sequencing

Quantification of the *geoA* gene by qPCR was conducted using primers targeting important phylogenetic groups of *geoA*-containing *Actinomycetales* (group 1; geoA_g1F/geoA_g1R), myxobacteria (group 3; *geoA*_g3F/*geoA*_g3R), *Sorangium* sp. (group 5; geoA_g5F/geoA_g5R) and overall *geoA*-containing bacteria (Cyc primers). Primers and qPCR conditions are described elsewhere (Ludwig et al. 2007; Lukassen et al. 2017). The *geoA*-containing *Streptomyces coelicolor* (DSM 40233) was used to create a standard curve as described elsewhere (Lukassen et al. 2017). Two biofilter samples could not be amplified (sample 4 and 19) and no qPCR results are available for these samples.

Bacterial community profiling in water and biofilter samples were conducted by amplicon sequencing of the V1-3 region of the 16S rRNA gene using an Illumina MiSeq platform as described elsewhere (de Jonge et al. 2020).

Bioinformatic and statistical analysis

Raw sequencing data was processed into amplicon sequencing variants (ASV) (Edgar 2016) using the AmpProc pipeline version 5.1 (https://github.com/eyashiro/AmpProc). The taxonomic unit ASV (also termed ZOTU, Edgar 2016) allows for a detailed measurement of biological sequence variation compared to clustering into operational taxonomic units based on homology. The pipeline was executed in single read mode for the reverse read of the V1-3 amplicon, using Silva release S138 as the reference database (Quast et al. 2013).The results were analysed in R version 4.0.2 (R Development Core Team 2021) through the RStudio IDE version 1.4.1103 (http://www.rstudio.com).

Alpha and beta diversity analysis and heatmaps visualising microbial community structure were generated using the ampvis2 package (Andersen et al. 2018). Beta diversity analyses were performed using Hellinger transformed abundance counts. All other visualisations were generated using the ggplot2 (Wickham 2016) and corrplot packages (Wei and Simko 2017). Correlation analysis was performed using Spearman's correlations and the cor.mtest function from base R. Differences in alpha diversity (mean species diversity) between different types of samples were tested by Wilcoxon rank sum testing with Benjamini-Hochberg correction for multiple testing.

Non-parametric pairwise comparisons were made on quantified *geoA* data to determine significant differences between the inflow and outflow water and between the two different types of biofilter, using Wilcoxon testing using Benjamini-Hochberg correction for multiple testing. Non-parametric testing was chosen because the data did not meet the assumptions of normal distribution based on Shapiro testing.

Results

Water quality parameters

Water quality parameters measured at each of the 26 investigated full-scale RAS showed distinct profiles for each plant (Table S1). All measurements performed on site showed high reproducibility and varied less than 1 % (n=5), and only measurements of geosmin showed standard errors of the mean up to 10 % (n=3) (data not shown). In general, COD and, nitrogen oxides (NOx) were only measured at low levels while O_2 remained high throughout measurements. Geosmin levels ranged from 0-21 $ng \cdot L^{-1}$ and in many systems these values were above the human detection threshold of approximately 4 $ng \cdot L^{-1}$ in water (Watson et al. 2016). COD were very similar between filtered and unfiltered samples (data not shown), which is consistent with a low level of suspended solids. Phosphate concentrations were found to contribute significantly to geosmin levels when analysed by principal component analysis ($P < 0.001$).

Diversity and characteristics of the microbial communities

Sequencing of 16S rRNA gene amplicons revealed the presence of a diverse microbial community composition across all analysed samples. A total of 177 samples (142 water, 31 biofilter, 3 river and 1 depuration tank sample) were successfully sequenced for the 26 RAS and at least 5,000 sequences were obtained from each sample and used for further analysis. Samples that yielded less than 5,000 reads were discarded from the dataset based on a rarefaction curve (Fig. S2). A total number of 9,424 ASVs were identified at an average estimated richness of 1,432 ± 836 and 1,359 ± 711 ASVs for the in- and outflow water samples, respectively (Fig. 1), and 957 ± 293 ASVs in the biofilter samples. The aquaculture systems could be divided into two groups on the basis of their alpha diversity with patterns corresponding to the farmed fish species and type of farming: the farms farming eel, pikeperch and tilapia displayed a lower estimated richness (Chao1, as described by Chao 1984). Meanwhile, farms producing sturgeon, trout, yellowtail

kingfish, and turbot had a lower alpha diversity (Fig. 1).

Statistical testing using the Wilcoxon rank sum test revealed significant differences ($p \leq 0.05$) in estimated richness between the biofilter samples and the water samples ($p = 0.027$) but not between the other sample types ($p > 0.05$). Shannon index values were significantly different between the biofilter and the inflow and outflow ($p = 0.015$) and the biofilter and river water ($p = 0.045$) samples, as well as the river water and inflow ($p = 0.032$) and outflow water ($p = 0.015$) samples.

The general microbial community structure present in the water samples from all 26 RAS was characterized by five prokaryotic phyla, which together accounted for 90 to 99 % of total read abundance per sample. The phylum *Proteobacteria* was the most abundant in almost all samples, averaging at around 50 % of total reads in all RAS, while other major contributors included the phyla *Bacteroidota*, *Actinobacteriota*, *Firmicutes*, and *Fusobacteriota*.

The genus *Flavobacterium* was the most abundant population across all samples (Fig. S3), especially in the farms producing pikeperch and sturgeon (1.1-35.9 % of total reads). Meanwhile, *Rhizobacter* was uniquely and abundantly associated with trout farming (18.8 % of total reads). The RAS using marine water were characterised by the abundant presence of the genera *Granulosicoccus*, *Francisella*, and *Roseovarius*, while *Polynucleobacter*, *Aurantimicrobium*, and *Novosphingobium* were exclusively observed in freshwater farms. *Aestuariivirga* was uniquely observed in the indoor freshwater farms.

Principal component analysis (PCA) and PERMANOVA were performed to explore the beta diversity and the results showed that the microbial community in the water from all plants formed clusters according to the farmed fish species and the farming systems (Fig. 2, $p < 0.001$, R^2=0.963 and 0.8601 for the effect of the fish species and farming systems, respectively). The freshwater systems formed two distinct clusters, the first cluster containing the low diversity indoor RAS farming eel, tilapia, and pikeperch while a separate cluster with a high diversity corresponded to the outdoor RAS (farming trout and sturgeon). The marine RAS farming turbot and yellowtail kingfish, on the other hand, formed a third separate and distant cluster. Furthermore, the systems farming trout employing both moving-bed and fixed bed-filters formed a joint cluster regardless of the type of biofilter, suggesting that the type of biofilter is less influential compared to the type of fish raised (data not shown).

Distribution of *geoA*-harbouring bacteria and presence of uncharacterised geosmin producers

The number of bacteria harbouring a copy of *geoA* corresponded to 0.8 %, 0.9 % and 0.3 % in the inflow water, outflow water and biofilters, respectively, as assessed using the total 16S rRNA gene copies and assuming an average of 10 rRNA operons and a single *geoA* gene per bacterial genome. Interestingly, the vast majority of the copies of the *geoA* gene identified using the universal Cyc primers could not be detected using any of the three specific primer sets. This suggests the presence of a group of so far uncharacterized geosmin producers that accounted for 93 % of the *geoA* in the water and 73 % in the biofilter samples. This number was affected by the species of the fish being farmed and the percentage of the *geoA* copies represented by the three known geosmin producer clades constituted 70 % of the total *geoA* copy numbers (*Cyc*) in the systems farming sturgeon, while they represented only 7 % of the total Cyc copies in RAS farming pikeperch.

Regarding the different phylogenetic groups of *geoA*, the variant of the gene associated with the myxobacteria (group 3) was the least abundant in both the inflow and outflow water but were the most abundant in the samples from the biofilters (1200 copies·mg^{-1}). Conversely, the group 5 (*Sorangium* sp.) was found to be the most abundant overall: it was the most abundant in the water samples and second most abundant in the biofilters (4153 copies·mgL^{-1} in the water and 894 copies·mg^{-1} in the biofilters). Moreover, the distribution of the different groups of *geoA* gene appeared more variable in the water samples compared to the biofilter samples (Fig. 3a and 3b, respectively). Indeed, less than half of the water samples harboured representatives from all three groups of the *geoA* gene. On the contrary, all but one of the biofilter samples contained representatives from all three variants.

Factors influencing geosmin concentrations in the water

A multivariate statistical analysis of the water quality parameters of the water was performed to find correlations between these water quality parameters and geosmin concentration and presence of the *geoA*-containing microbial groups (as assessed by qPCR). The results showed that two water quality parameters were positively correlated with the geosmin levels: the redox potential and the calcium levels (Fig. 4). On the other hand, two factors were negatively correlated to the geosmin concentration: the oxygen concentration and water conductivity (albeit to a lesser extent). Other factors (for example, nitrate concentrations) had no correlation to the geosmin levels.

Redundancy analysis suggested that the quantity of geosmin in the water was variable relative to the microbiota composition, even within the same species of farming system (Fig. S4). It is likely that geosmin concentration is determined by the individual plants and operational conditions. The concentration of geosmin in the water was positively correlated with the bacterial load in the water, as assessed based on the number of copies of the 16S rRNA gene (Fig. 4). Moreover, there was a non-statistically significant positive association with the presence of the *geoA* gene characteristic of the *Sorangium* sp. but not with the other groups of bacteria and not with the total *geoA* copies as measured using the Cyc primers.

Factors influencing the microbiota

The total numbers of bacteria measured based on the 16S RNA copy number were not statistically different between the inflow and outflow water ($p = 0.462$). Similarly, the fish species farmed did not affect the total number of bacteria ($p = 0.215$). Interestingly, the feed type was associated with significant changes in the microbial community profile, although a direct correlation is difficult to predict as each feed type was adapted to the species farmed and since the exact composition of the used feed is not systematically recorded (Fig. S5).

While the microbial community in the water of RAS located within the same plants presented a generally similar profile (Fig. S6), each RAS showed a clear difference between biofilter and water sample (Fig. S7). Similarly, samples originating from marine RAS were distinct from that originating in freshwater RAS (Fig. S3). Furthermore, the type of fish species being farmed had a significant impact on the composition of the microbiomes ($p < 0.001$).

Factors influencing the presence of geosmin producers.

Like the composition of the microbiota, the total numbers of *geoA* copies assessed using the universal Cyc primers were similar between inflow and outflow water (3129 and 4973 copies.mL^{-1}, respectively; $p = 0.433$). Similarly, no statistical difference was recorded in the copy numbers for the *geoA* gene between moving-bed and fixed-bed biofilters ($p = 0.660$) or between marine and freshwater water systems ($p = 0.6572$).

Contrary to the general microbiota, the fish species being farmed had a significant effect on the copy numbers of *geoA* gene ($p < 0.001$). For example, the numbers of *geoA* copies were particularly low in the outflow water of the RAS farming sturgeon and only two copies·mL^{-1} were detected in one sample. On the contrary, the *geoA* copy numbers obtained with both specific and

unspecific primers were highest in the systems farming pikeperch (26,357 *geoA* copies·mL^{-1}). Moreover, the presence of geosmin-producing organisms was favoured by high levels of COD and elevated temperatures but negatively associated with salinity.

The presence of *geoA*-harbouring *Actinomycetales* (group 1) was positively associated with the total number of bacteria, redox potential, and calcium levels (Fig. 4). However, this group was strongly negatively correlated to the COD and temperature. In addition, other water quality parameters such as nitrate concentration, conductivity, as well as salinity were associated with reduced levels of the group 1 *geoA* gene.

The myxobacteria-associated variant of *geoA* (group 3) was also positively correlated to calcium levels but negatively correlated to magnesium concentrations, conductivity, and salinity (Fig. 4). There was also a small but statistically significant correlation between the numbers of these group 3 bacteria and the number of the geosmin producers belonging to the *Sorangium* sp. group.

This group of bacteria harbouring a variant of *geoA*, characteristic of *Sorangium* sp. was characterised as the group 5. Presence of bacteria of this group was strongly positively associated with high bacterial loads, in particular with bacteria belonging to group 3 as mentioned above. On the other hand, group 5 was negatively correlated with temperature and nitrate concentrations, alongside oxygen and calcium concentrations (Fig. 4), even if these later two showed a weaker correlation.

Discussion

Investigations of the bacterial community compositions and quantification of the *geoA* genes in aquaculture systems can facilitate a better understanding of the water quality parameters favouring the presence of geosmin-producing microorganisms. In this study, 26 different European RAS were investigated by comparing the microbial community compositions in the water and biofilm in the biofilters. Species diversity calculated based on these ASVs showed an alpha diversity ranging from approximately 400 to 3,000 ASVs in the most diverse systems (Fig. 1). Previously studies in aquaculture and aquatic systems have also reported diverse microbiomes with varying species diversities between individual plants (Martins et al. 2013; Qin et al. 2016; Payne et al. 2017; Rud et al. 2017). A common observation in almost all of these ecosystems is that the water phase has more diverse microbial communities than the corresponding biofilter samples. This is likely explained by the high degree of turnover in the water microbiome compared to the established and more specialised microbial communities present on the biofilters. This is

supported by the clear difference in microbial community composition between samples from the water and biofilters from the same plant in the present study (Fig. S7).

Interestingly, there was a shared general profile between water samples from different RAS originating from the same farm. This could be explained by the fact that each individual farm shares generally comparable water quality parameters (for example temperature, salinity) and management practices (same fish species farmed), as well as water source, and it is therefore plausible that these parameters influence the microbiota in similar ways. The marine RAS did not reveal higher alpha diversity compared to the freshwater RAS, which is in accordance with previously reported findings (Rud et al. 2017), and thus the diversity does not appear to be influenced by increased salinity despite large differences in the composition of the microbial communities. The influence of both salinity and the conditions prevailing and the fish species farmed on the microbial community has also been reported elsewhere (Schreier et al. 2010; Martins et al. 2013; Bakke et al. 2017). The differences in microbial communities between biofilters and water within individual RAS were less significant than the effect caused by the salinity and the fish species, suggesting that the various water quality parameters associated with the farming of each species had an important effect on the microbiome. These observations are consistent with previous reports on insignificant differences between different RAS compartments (Bakke et al. 2017) and that the sequencing depth used in this study was sufficient for the overall interpretation (Qin et al. 2016). Thus, we can assume that the analysed water and biofilm samples are representative for the 26 RAS investigated in this study. The effect of the farming system was similar to that of the species farmed. This was anticipated, because the farming systems were adapted to the species farmed and therefore, they were similar between systems farming the same fish species.

The most abundant phyla in the water phases and biofilters in both marine and freshwater RAS were *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Planctomycetes*, which is consistent with previous reports (Sugita et al. 2005; Rud et al. 2017). At the order level, the most abundant orders were *Flavobacteriales*, *Burkholderiales*, *Rhizobiales*, *Rhodobacterales*, and *Sphingomonadales*, which are commonly found within RAS and other aquatic freshwater environments (Sugita et al. 2005; Martins et al. 2013; Tang et al. 2014; Llirós et al. 2014; Rud et al. 2017). However, at present very little is known about the role and functions associated with these taxonomic groups in comparable environments.

Geosmin has been described in numerous RAS farming various fish species (Guttman and van Rijn 2008; Davidson et al. 2014; Sarker et al. 2014), but only a few studies have focused on the identification of geosmin-producing bacteria in the RAS and how they relate to the different fish

species farmed in the plant. The quantification of *geoA* gene copies using the unspecific Cyc primers showed the presence of other *geoA*-containing cells aside from the ones targeted by the three specific qPCR primer sets. The qPCR assays for the specific taxonomic groups were conducted with a TaqMan approach whereas the qPCR that unspecifically targeted the *geoA* gene was conducted using an SYBR green assay. This discrepancy could result in a methodological bias. When quantifying the *geoA* gene and 16S rRNA gene, the differences in gene copy numbers between the two genes will also influence the absolute quantification. However, the relative proportions should be uniform in all samples and thereby not anticipated to affect the conclusions of the correlational analysis forwarded in this study.

The relative content of *geoA*-containing microorganisms was estimated to account for approximately 0.9 % of the community in the water. This is similar to previously reported numbers (Lukassen et al. 2017; Azaria et al. 2020). As it is difficult to estimate the total amount of biofilm in fixed-bed biofilters, and from other surfaces in the RAS, we have not attempted to estimate the major source of geosmin-producing bacteria in RAS. However, it appears that both the water phase and the attached growth constitute significant sources of geosmin-producers confirming previous results (Guttman and van Rijn 2008).

The most abundantly identified group of *geoA* gene variants in both water phases and biofilters was the group affiliating with *Sorangium* spp. (group 5) and these microbes were potentially the most abundant geosmin-producing bacteria in RAS which is consistent with previous observations (Lukassen et al. 2017). The *geoA*-containing myxobacteria (group 3) were the least abundant group in the water while the *Actinomycetales* (group 1) were the least abundant group in the biofilters. The highest quantities of the *geoA* gene copies were found in RAS farming pikeperch (26,357 copies·mL^{-1}). These numbers of putative geosmin-producing bacteria are significantly lower than shown by another study analysing absolute *geoA* quantities in RAS farming trout (Auffret et al. 2011). However, relative to the total 16S rRNA gene content and assuming 10 rRNA operons per genome and only one gene copy for *geoA*, the numbers were comparable. When comparing numbers of geosmin producers relative to the fish species raised, it was found that representatives of *Actinomycetales* containing the *geoA* gene were most abundantly detected in the biofilters in RAS farming pikeperch and trout, and least abundantly detected in RAS farming sturgeon, eel, and in the marine water systems. Myxobacterial *geoA* was revealed in high copy numbers in the biofilters of systems farming pikeperch, eel and marine water systems, while sorangial affiliated *geoA* was found in high quantities in the biofilters of RAS farming pikeperch, in marine water RAS, and in the water of RAS farming trout, confirming previous reports (Lukassen et al. 2017). The populations of geosmin-producing bacteria

appeared more homogenous in the biofilters compared to the water samples, with the various groups of *geoA* producers more evenly represented and with smaller differences between units. This could be explained by the fact that the biofilter likely represents a more stable environment for bacteria, allowing more stable populations to settle. Notably, a very large proportion of the *geoA* detected using the universal Cyc primers could not be allocated to one of the three groups. This was in contradiction to previous studies (Lukassen et al., 2017). The reason for this difference is unknown but might have to do with the species farmed, as this was found to have an impact on the proportion of the *geoA* gene corresponding to the unknown group.

No direct correlations was found between the copy numbers of *geoA* and the levels of geosmin in the water. While intriguing, this result is consistent with what was reported in a previous study, where an opposite response of geosmin concentration and *geoA* numbers was observed following addition of peracetic acid, with the acid being associated with lower concentrations of geosmin but higher copy numbers of the *geoA* gene (Lindholm-Lehto et al. 2019). This discrepancy could be explained by the difference in gene expression under the investigated environmental circumstances. Moreover, removal of the geosmin from the water, for example by air stripping or accumulation into the fish tissues, suspended solids or other compartments or even degradation through microbial activity could also explain this lack of correlation (Azaria et al. 2017). Absorption by the fish is known to be complex and influenced by multiple factors such as the geosmin concentration in the water, the fish intestine and the amount of geosmin already present in the tissue as well as the lipid content of the fish (Lukassen et al. 2019; Schram 2020). Moreover, a large amount of geosmin producers are likely sequestered inside biofilms and the condition within these biofilms is likely to differ from that in the water. This could suggest some difference between the water quality parameters measured and those experienced by the microorganisms. The dynamics of geosmin in RAS are complex and still poorly characterized (Schram 2020).

The general low levels of COD, NO_X, high O_2, etc. indicated that all plants are well maintained and operated. A correlation analysis was performed to address how various environmental water quality parameters influenced geosmin concentrations and the growth or presence of *geoA*-containing bacteria (Fig. 4). A weak positive correlation was found between the concentration of geosmin and the redox potential and calcium levels in the water (Fig. 4). Previous reports have similarly found weak positive correlations between the concentrations of geosmin in the water and the waters' redox potential, as well as calcium levels (Parinet et al. 2010, 2013). On the other hand, geosmin levels were found to be negatively correlated to oxygen levels and, to a lesser extent, conductivity. This was in contrast with the findings from a previous study, which reported

that geosmin was present at higher concentrations in aerobic environments (Guttman and van Rijn, 2008). Similarly, no significant correlation was observed between geosmin concentrations and COD, which contrasts with previous work that reported that geosmin production was associated with elevated COD and levels of organic matter (Guttman and van Rijn 2008).

Interestingly, the number of *geoA* copies measured using the Cyc primers were positively correlated with the COD and water temperature. However, these factors were negatively correlated with the presence of the *geoA* variants belonging to the groups 1 and 5 and not correlated with *geoA* variants belonging to the group 3, supporting the suspicion that a large number of geosmin-producing organisms were not targeted by the specific primers used in the present study. Elevated temperatures have previously been shown to stimulate geosmin production in both *Streptomyces* (Zhang et al. 2009; Schrader et al. 2015) and cyanobacterial strains (Zhang et al. 2009; Schrader et al. 2015) as well as in *Anabaena* sp. (Saadoun et al., 2001). However, in the present study, temperature was not linked to the concentration of geosmin in the water.

Actinomycetales representatives containing the *geoA* gene correlated strongly with the presence of *Sorangium* sp., suggesting that both groups of bacteria might have had similar growth requirements. On the other hand, the *Actinomycetales* group had no correlation with the presence of myxobacteria (group 3) while members of *Sorangium* sp. only had a weak, non-statistically significant positive correlation to the myxobacterial group.

No correlation was found between the nitrate levels and the concentration of geosmin in the water, and negative correlations were observed with the various variants of the *geoA* gene measured, albeit not at a significant level in the case of the Cyc or the myxobacterial variant. The correlation of nitrate concentrations with geosmin production remains controversial with some studies showing no correlation (Schrader et al., 2013), while others have reported a strong negative (Saadoun et al., 2001) or positive correlation (Oh et al. 2017). In most RAS, removal of nitrate by denitrification can occur in an uncontrolled and spontaneous fashion, which takes place mostly in the sedimentation tanks. In addition, denitrification reactors are designed with the intent to remove nitrate from the systems. Anoxic zones in the water have also been shown to harbour zones with high removal of geosmin (Azaria et al. 2021) and could explain why no significant correlation were observed. Removal of nitrate by dilution would similarly result in correlations between nitrate and geosmin, although we were not able to show such a link. The feed distributed to the fish was also found to have an impact on both the composition of the microbiota and the production of geosmin. However, our data was incomplete on that subject and the matter is further complicated by the fact that the feed was adapted to both the fish farmed and the farming

system, both of which are factors known to influence both microbiota composition and geosmin production. It is however worth commenting that one of the feeds (Biomar Orbit) was specifically designed to protect biofilters and it is therefore plausible that it would have a distinct effect on the microbiome of the receiving RAS.

Conclusions

A number of water quality parameters were correlated to the level of geosmin and the geosmin-producing bacterial communities in a large number of full-scale RAS. Several of the water quality parameters were shown to influence the geosmin production (including the calcium and phosphate levels, redox potential or oxygen levels). Conversely, oxygen levels and conductivity were found to be negatively correlated with geosmin concentration. These might be relevant as operational tools for management and design of off-flavour-free RAS in the future. Reducing the amount of uneaten feed or changing the diet in order to reduce the levels of available phosphate could deselect for otherwise dominating geosmin-producing bacteria in the system and thereby decrease the geosmin levels, as it has been suggested elsewhere (Sarker et al. 2014). A complementary research would be to use a reductionist approach and assess the effect of some of these parameters on *geoA* expression in some model geosmin-producing organisms.

Moreover, the composition of the microbiota observed in the water and biofilters was variable between farms, but comparable for RAS within the same farm. Several factors were found to be particularly influential, notably the salinity and the fish species being farmed. The presence and distribution of the detected geosmin producers were variable between farms and found to be more abundant at higher temperatures and when high concentrations of COD were present in the water but correlated negatively with salinity. Finally, a large population of geosmin producers beyond those detected by our previously established primers were also detected, highlighting that not all geosmin producing populations are currently known. Together, the results of the present study add to our knowledge of what drives and suppresses the production of geosmin and related microbes in RAS systems.

Acknowledgments

This study was supported by the European Union's Seventh Framework Programme within the project:"Towards off flavour free finfish aquaculture - Spacetaste", managed by REA-Research Executive Agency (FP7/2007-2013) under grant agreement N° 604956.

Conflict of interest

No conflict of interest declared.

Data Availability Statement

The DNA sequences generated during this project have been deposited to the European Nucleotide Archive under the accession number **PRJEB46235**. The other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors Contribution Statement

MBL gathered the data and performed the initial analysis, and wrote of the first draft of the manuscript. SML and NdJ continued the analysis and carried out the statistical work and finished the writing while ES and JLN designed and supervised the project and oversaw the writing process. JLN, NdJ, and SML handled the revision process.

Figure legends

Figure 1 *Estimated richness (Chao1) of the obtained ASVs, sorted by fish species. The boxplot is coloured by farming type. Data is displayed as boxplots that bound the interquartile range (IQR) divided by the median and whiskers that extend 1.5 x IQR past the box. Outliers are shown as black points.*

Figure 2 *Principal component analysis on Hellinger transformed abundance counts displaying the impact of the fish species and farming types on geosmin concentrations. Samples are coloured by fish species, and shaped by farming type.*

Figure 3a *Quantification of geoA in water [copies·mL^{-1}] Group 1: Actinomycetales. Group 3: myxobacteria. Group 5: Sorangium. Unknown: bacteria harbouring the geoA gene measured with*

Cyc primers.

Figure 3b *Quantification of geoA in biofilters [copies·g^{-1}] Group 1: Actinomycetales. Group 3:* myxobacteria*. Group 5: Sorangium. Unknown: bacteria harbouring the geoA gene measured with Cyc primers.*

Figure 4 *Correlogram of the water quality parameters. Correlation was generated using Spearman's correlations. The colour and size of the square indicate the Spearman's rho, and correlations that were proven insignificant (p >0.05) by statistical testing are marked with a cross.*

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 *Description of the farms sampled and water quality parameters for the recirculated aquaculture systems. Parameters for the water going into (inflow). Parameters for the water going into (inflow) and out of (outflow) the fish production tank are shown as inflow/outflow. NA: Sample not measured.*

Figure S2 *Rarefaction curve of the sequenced samples. Curves are coloured by fish species.*

Figure S3 *Heatmap of the 25 most abundant bacterial genera observed in the water samples. Samples are sorted by fish species and farming type.*

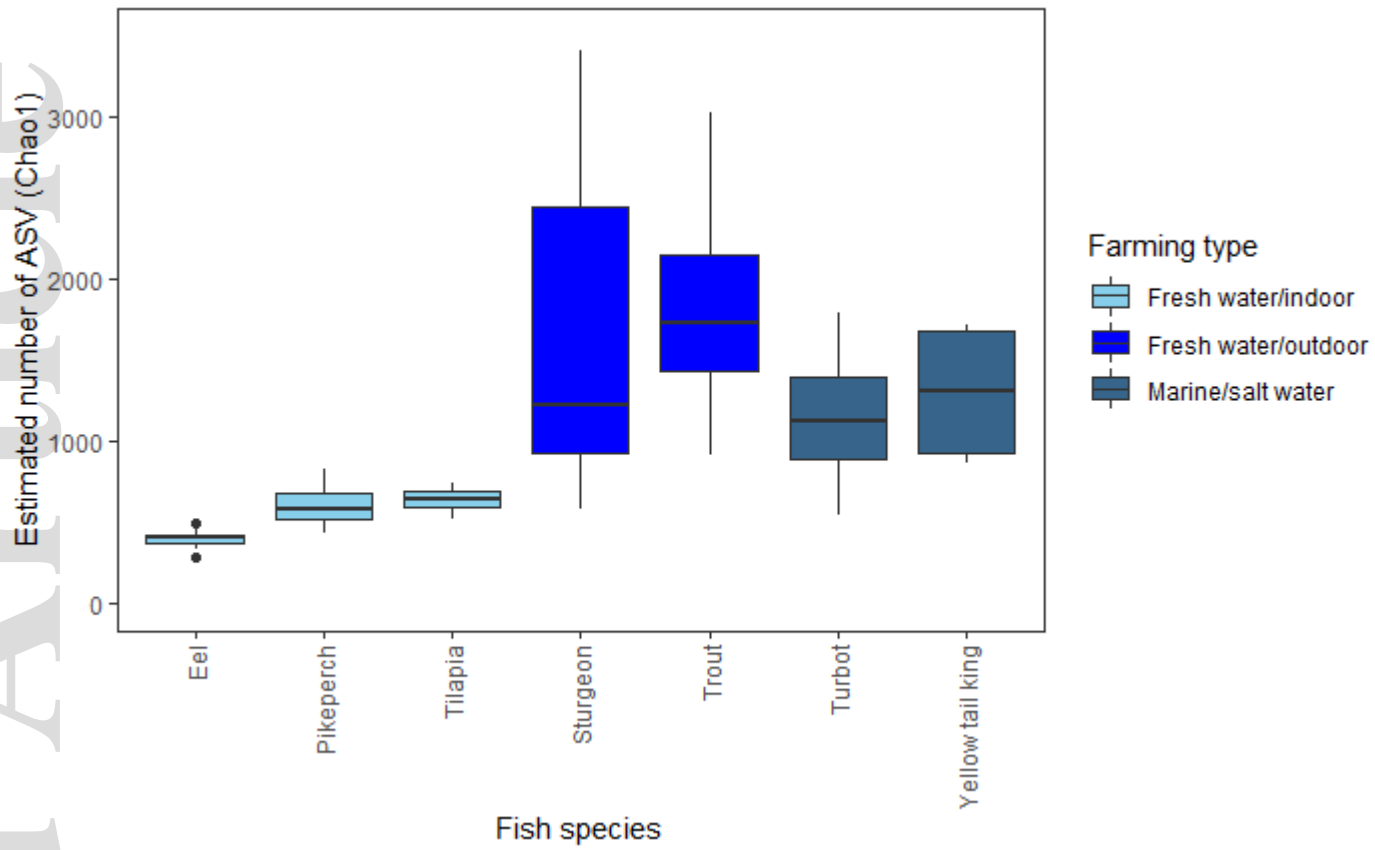
Figure S4 *Redundancy analysis of Hellinger transformed abundance counts constrained by showing the impact of several farming parameters on the composition of the microbiota, constrained by geosmin concentration. Samples are coloured by fish species, and shaped by farming type. Size of the points is scaled to the measured geosmin concentration in the water.*

Figure S5 *Principal Component analysis of Hellinger transformed abundance counts of samples where the feed source was known. Samples are coloured by fish species and shaped by feed source.*

Figure S6 *Heatmap of the 50 most abundant bacterial genera observed in the water samples.*

Samples are sorted by RAS systems and farms.

Figure S7 *Heatmap of the 25 most abundant bacterial genera observed in the biofilm and water samples. Samples are sorted by sampling location in each farm.*



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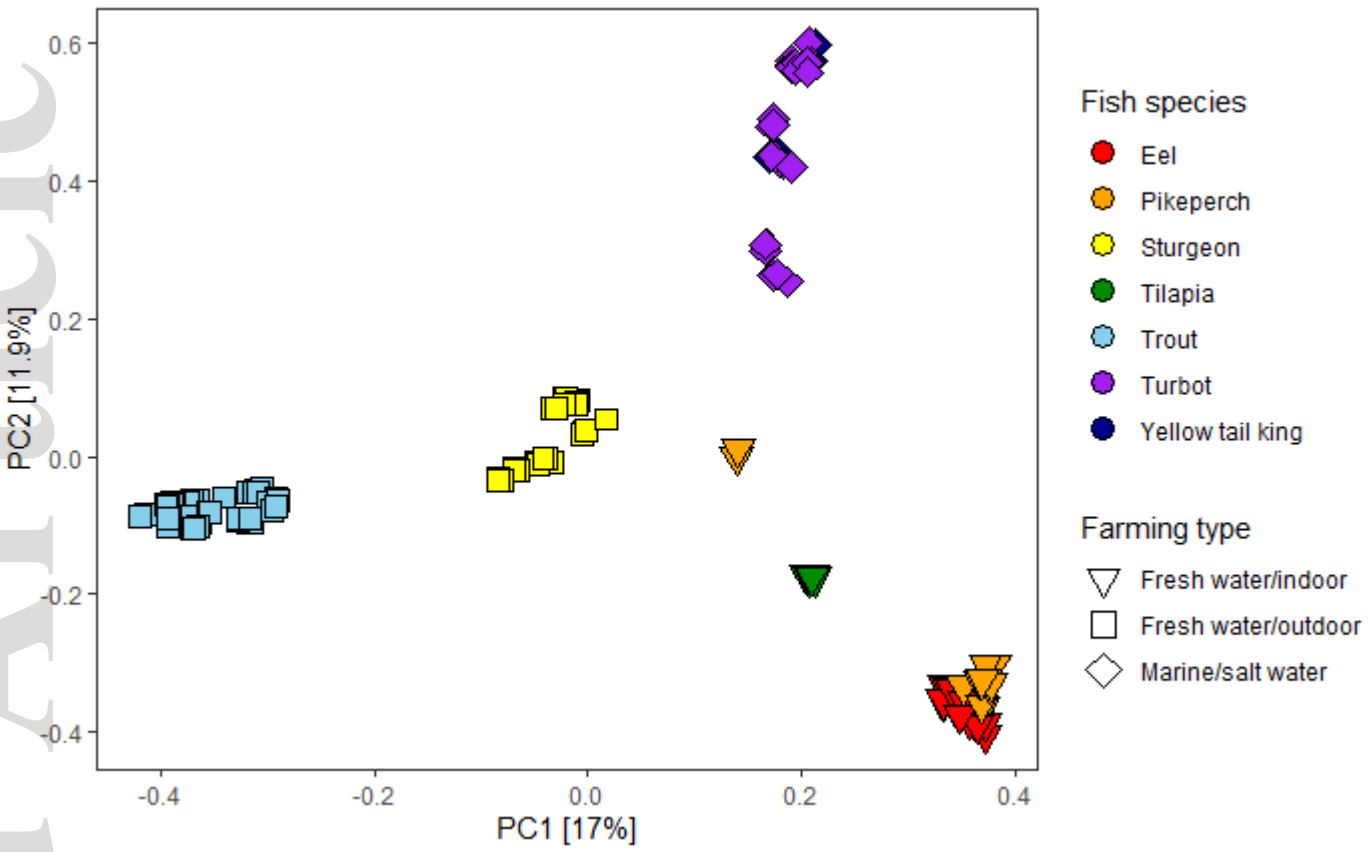

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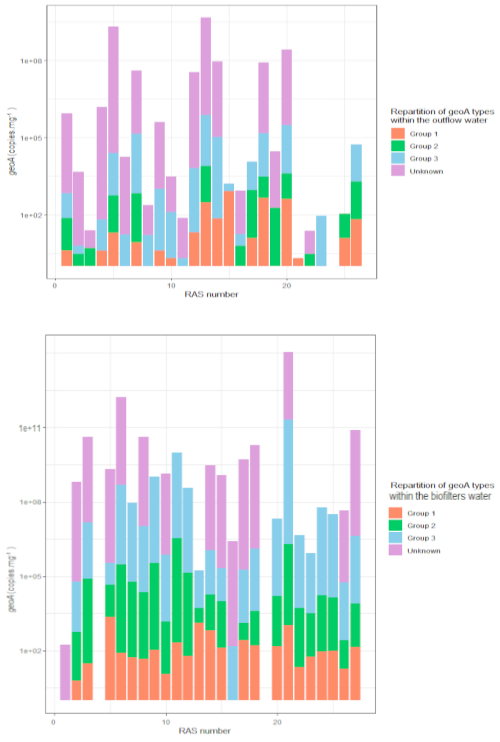

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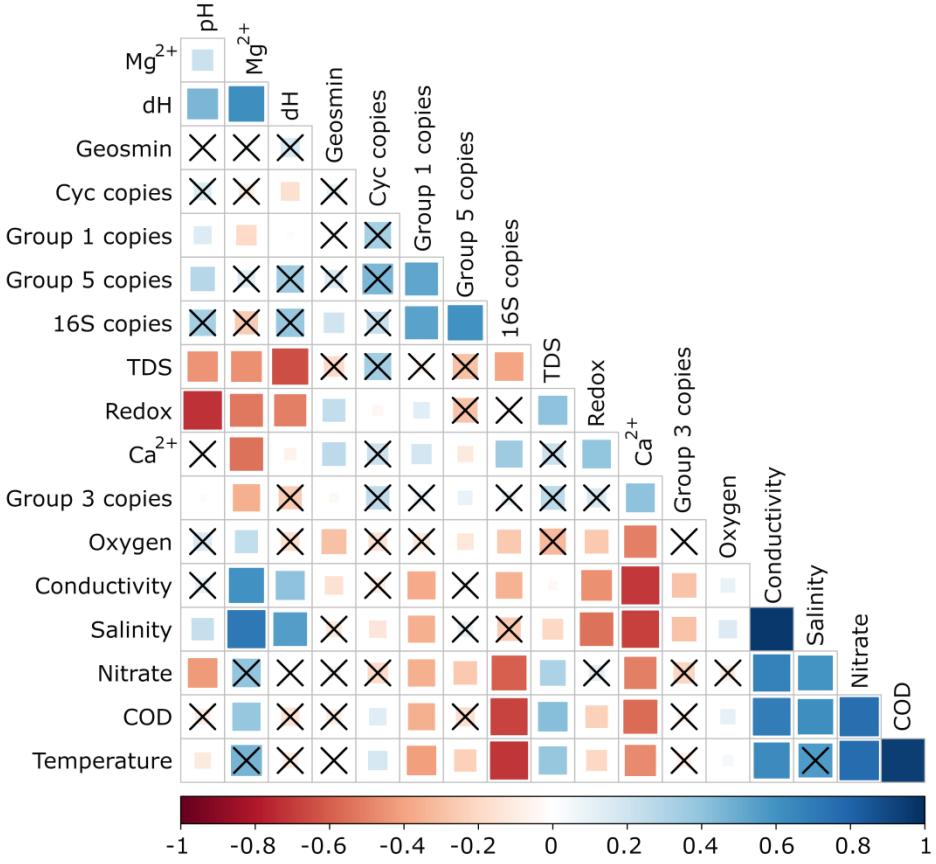

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