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Accumulation and depuration of geosmin and 2-methylisoborneol in Japanese seabass (*Lateolabrax japonicus*) fed diets containing different dietary protein and lipid levels in a recirculating aquaculture system

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Abstract

Muddy flavour caused by microbially synthesized geosmin and 2-methylisoborneol (2-MIB) represents a quality challenge for production of fish in recirculating aquaculture systems (RAS). The current study was conducted to find out if the accumulation and depuration of geosmin and 2-MIB in Japanese seabass (*Lateolabrax japonicus*) raised in RAS were affected by dietary protein and lipid levels, and to find out if these components were equally distributed in lean and fatty fish muscle tissues. A group of 540 fish with an initial weight of 0.11 kg were fed six extruded diets in triplicate tanks for 15 weeks for accumulation. A 3*2 factorial design with three dietary protein levels (420, 450, and 490 g kg^{-1}) and two dietary lipid levels (150 and 180 g kg^{-1}) was adopted. Six fish per tank were randomly selected after accumulation. Two of these were slaughtered as initial samples in a depuration trial. The other 4 fish per tank had passive integrated transponder tags implanted. The tagged fish were pooled into a 22 m^3 depuration tank and fasted for 10 days. Water from RAS were also sampled after the accumulation trial and the microbial structures were identified by high throughput sequencing. The results showed that actinobacteria accounted for 0.98% of total bacteria, which was sufficient to induce a high degree of muddy flavour in the fish. Fish fed the diets with lower protein level accumulated more lipid and had more muddy flavour components in dorsal tissues (the upper part of the fillet) than those fed the high protein diets. The contents of lipid, geosmin and 2-MIB in the ventral tissues (belly) were significantly higher than that in dorsal tissues. The concentration of geosmin was higher than that of 2-MIB in all tissues and showed a positive correlation between the concentrations of geosmin and 2-MIB. The depuration rate of both geosmin and 2-MIB was higher in the ventral tissue than that in the leaner dorsal tissue during the 10-day fasting. Overall, high lipid diet resulted in fatty fish which caused rapid accumulation of geosmin and 2-MIB in fish. The duration of depuration was not sufficient to produce acceptable quality of the fish. Thus, a depuration period exceeding 10 days proved necessary for removing muddy off-flavour from a fatty fish like Japanese seabass.

Keywords

Off-flavours; Geosmin; 2-methylisoborneol (2-MIB); Depuration; Japanese seabass; RAS

Norsk sammendrag

Smak og lukt av mudder skyldes mikrobielt syntetisert geosmin og 2-metylisoborneol (2-MIB) og utgjør en utfordring for kvaliteten ved produksjon av fisk i resirkulerende akvakultursystemer (RAS). Dette forsøket ble utført for å finne ut om akkumulering og utskillelse av geosmin og 2-MIB i Japanese seabass, *Lateolabrax japonicus*, produsert i RAS var påvirket av innholdet av protein og fett i fôret, og å finne ut om disse stoffene var distribuert likt i magre og feite muskelvev. Totalt 540 fisk med startvekt på 0,11 kg ble tildelt seks ekstruderte fôr for utvikling av muddersmak. Hvert fôr ble gitt til fisk i 3 kar, i 15 uker. Et 3*2 faktorielt design med 3 nivåer av protein (420, 450, og 490 g kg^{-1}) og to nivåer av lipid i fôret (150 and 180 g kg^{-1}) ble benyttet. Seks fisk fra hvert kar ble tilfeldig utvalgt etter 15 ukers perioden. To av disse ble slaktet og benyttet som startprøver. Resterende 4 fisk per kar fikk «transponder-chip» implantert slik at individuell fisk kunne bli identifisert. Merket fisk ble samfengt i et 22 m^3 kar for reduksjon av muddersmak og fastet i 10 dager. Prøver ble tatt av vann fra RAS etter akkumuleringsforsøket, og mikrobielle strukturer ble identifisert ved «High Througoutput Sequencing». Resultatet viste at actinobakter utgjorde 0,98% av bakteriene. Dette var tilstrekkelig for å indusere kraftig muddersmak i fisken. Fisk som fikk fôr med lavest innhold av protein hadde mer komponenter som forårsaker muddersmak i dorsale vev (øvre del av fileten) enn de som fikk fôrene med høyt proteininnhold. Innholdet av lipid, geosmin og 2-MIB i ventrale vev (buk) var signifikant høyere enn vev i dorsale prøver. Konsentrasjonen av geosmin var høyere enn den av 2-MIB i alle vev og var positivt korrelert til konsentrasjonene av geosmin og 2-MIB. Utvaskingsraten for både geosmin og 2-MIB var høyere i ventrale vev enn i magrere dorsale vev ved 10 dagers faste. I hovedsak resulterte fôr med høyt innhold av lipid i feit fisk, som forårsaket rask akkumulering av geosmin og 2-MIB fisken. Varigheten av utvaskingen var ikke tilstrekkelig for å produsere fisk av akseptabel kvalitet. Derfor er lengre utvasking enn 10 dager nødvendig for å fjerne muddersmak fra feit fisk som Japanese seabass.

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Abbreviations

- 2-MIB 2-methylisoborneol
- ANOVA Analysis of variance
- DHN Decahydro-1-naphthol
- FPP Farnesyl diphosphate
- GC-MS Gas chromatography – mass spectrometry
- GPP Geranyl diphosphate
- H_2O_2 Hydrogen peroxide
- HS-SPME Headspace solid-phase microextraction
- PCR Polymerase chain reaction
- PIT Passive integrated transponders
- qPCR Quantitative real-time polymerase chain reaction
- RAS Recirculating aquaculture system
- SAM S-adenosylmethionine
- UV Ultraviolet

1 Introduction

Geosmin and 2-methylisoborneol (2-MIB) are the most widespread sources of muddy flavour in freshwater. Both are secondary metabolites produced by microorganisms (Jiang et al., 2007). The employment of quantitative real-time polymerase chain reaction (qPCR) analysis allows the identification and quantification of the *geoA* and 2-MIB synthase genes, which encode for geosmin and 2-methylisoborneol (2-MIB) synthesis in bacteria (Lukassen, 2017; Suurnäkki et al., 2015; Wang et al., 2011). Previous studies have shown that cyanobacteria (Schrader & Dennis, 2005; Smith et al., 2008; Wang et al., 2011) and actinobacteria (Lukassen et al., 2017; Lylloff et al., 2012) are primary sources of geosmin and 2-MIB in the water. Myxobacteria from the phylum proteobacteria also produce geosmin and 2-MIB and release them into the water (Dickschat et al., 2005; Dickschat et al., 2007; Schulz et al., 2004).

Geosmin and 2-MIB are lipid-soluble compounds that are mainly absorbed via the gills or skin (Tucker, 2000) and accumulate in lipid-rich tissues (Howgate, 2004). High dietary lipid levels or low digestive protein to energy ratios in feed stimulates fat accumulation in fish tissues (Ding et al., 2010; Luo et al., 2010; Santinha, 1999), which causes off-flavours in fish. Typically, the 2-MIB levels in channel catfish (*Ictalurus punctatus*) with tissue fat content > 2.5% were three times higher than in leaner fish (< 2%) (Johnsen & Lloyd, 1992). Recirculating aquaculture systems (RAS) have low water exchange rates, resulting in high abundance of microorganisms, both in the biofilter and in the rearing water. This may produce off-flavours and cause the accumulation of these in fish. Fish with intense muddy flavours have low sales value and will not be well received in most markets. Thus, the removal of muddy flavour before harvest is necessary. The most efficient method is purging in clean fresh water; however, this is resource demanding and time consuming (Burr et al., 2012). The depuration efficiency is mainly influenced by the fat content in fish, and fatty fish need more time to be purged in freshwater than lean fish (Johnsen & Lloyd, 1992; Johnsen et al., 1996). In addition to purging, oxidants such as ozone, H_2O_2, and ClO_2 have also been extensively used to improve water quality. These strong oxidants can disinfect water by reducing the concentration of microbes (Westerhoff et al., 2006), and directly oxidize geosmin or 2-MIB in fish meat (Zhang et al., 2016). However, the low level (0.25-0.28 mg L^{-1}) of ozone used in the RAS was not effective at reducing levels of off-flavours, either in the fish fillets or water (Schrader et al., 2010). Lindholm-Lehto and Vielma (2019) recently reviewed the challenges of controlling off-flavours in RAS. They also concluded that purging with fresh water is the most effective and

economical method to reduce off-flavours in fish, although many methods have been studied and tested for the removal of off-flavours, including biological degradation or physical absorption.

The aim of this study was to investigate how the accumulation, distribution, and depuration of geosmin and 2-MIB were affected by dietary protein and lipid levels in Japanese seabass (*Lateolabrax japonicus*) raised in a freshwater RAS.

2 Literature background

2.1 Introduction of geosmin and 2-MIB

The off-flavours in drinking water and fish meat cause muddy taste and odour which results in high complaints of customers although these off-flavours are not toxic (Watson, 2003). At the present, geosmin and 2-methylisoborneol (2-MIB) are considered the major components of off-flavours and both are secondary metabolites produced by microorganisms (Dickschat et al., 2007; Jiang et al., 2007). A C^{14} radio labelling experiment was conducted and revealed that geosmin and 2-MIB are most likely derived from sesquiterpenoid and monoterpene precursors, respectively (Bentley & Meganathan, 1981). Similarly, the whole biosynthetic pathway of geosmin and 2-MIB were revealed by another radio labelled feeding experiments which used [$^{2}H^{10}$] leucine and [4,4,4,5,5,5-$^{2}H_6$] dimethylacrylate feeds for investigating biosynthesis of geosmin as well as [methyl-^{13}C] methionine, [4,4,6,6,6-$^{2}H_5$]- and [5,5,6,6,6-$^{2}H_5$] mevalolactone feeds for investigating biosynthesis of 2-MIB (Dickschat et al., 2005; Dickschat et al., 2007). In general, geosmin is the product of the conversion of farnesyl diphosphate (FPP) achieved by an approximate 725-amino acids protein encoded by *geoA* (Giglio et al., 2008; Jiang & Cane, 2008). And the biosynthesis of 2-MIB is mainly divided into two reactions: 1) from geranyl diphosphate (GPP) to 2-methyl-GPP achieved by geranyl diphosphate 2-methyltransferase and 2) from 2-methyl-GPP to 2-MIB achieved by MIB synthase (Giglio et al., 2011).

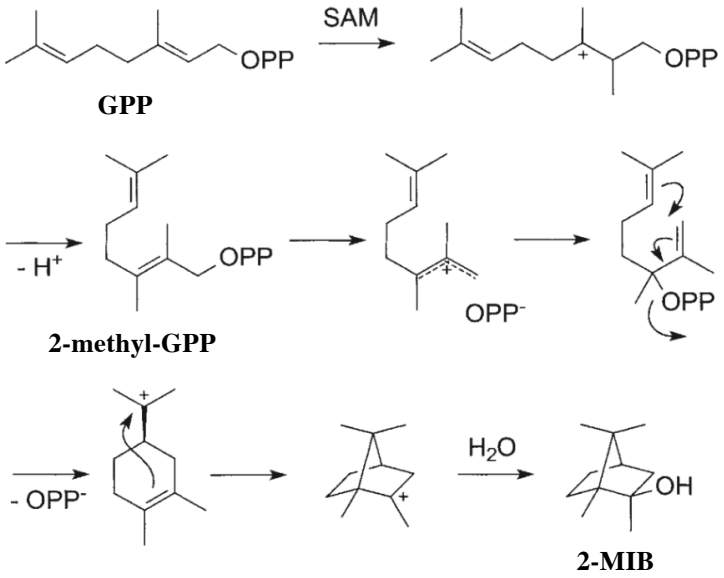

Figure 2-1 The biosynthetic pathway of 2-MIB (Giglio et al., 2011).

2.2 Sources of geosmin and 2-MIB

Several studies have been conducted to identify geosmin and 2-MIB producers where actinobacteria, cyanobacteria, and proteobacteria are designated to be the major producers in the water (Lukassen, 2017). The putative bacteria were isolated and cultured from the environments and produced geosmin and 2-MIB in the media were analyzed. Then PCR amplification of bacterial 16s rRNA was then conducted for identifying the specific species in the media. It could detect most of the off-flavour-producing bacteria in the water when combined isolation, culture, and PCR methods. For example, myxobacteria of proteobacteria like the species *Chondromyces crocatus* (Schulz et al., 2004) and cyanobacteria like the genera *Nostoc*, *Calothrix* (Suurnäkki et al., 2015) can produce geosmin while genus *Pseudanabaena* of cyanobacteria (Izaguirre & Taylor, 1998; Izaguirre et al., 1999) can produce 2-MIB. Some other species, such as genus *Streptomyces* of actinobacteria (Guttman & van Rijn, 2008; Klausen et al., 2005; Zaitlin & Watson, 2006) and genus *Phormidium* of cyanobacteria (Jüttner & Watson, 2007) can produce both geosmin and 2-MIB. Furthermore, the employment of qPCR could quantify the geosmin and 2-MIB producing genes (*geoA* and 2-MIB synthase gene), which could efficiently assess the abundance of relevant bacteria in the environment (Auffret et al., 2011). For example, Lukassen et al. (2017) identified and quantified several geosmin producing bacteria from six aquaculture systems in two European countries (Scotland and Denmark).

2.3 Factors affecting geosmin and 2-MIB production in water

Generally, the abundance of off-flavour-producing bacteria in the water is the major factor affecting the concentrations of geosmin and 2-MIB in the water. Other nutritional and environmental factors, such as phosphorus concentration in water or water temperature, mainly influence the bacterial abundance or structure which then changes water geosmin and 2-MIB levels.

2.3.1 Phosphorus

Phosphate is a major nutrient for bacteria, and it can regulate the metabolism of several bacteria (Lindholm-Lehto & Vielma, 2019). A previous study conducted by Schrader and Blevins (2001) showed that the maximal growth and highest geosmin production of *Streptomyces halstedii* occurred at the highest tested phosphorus concentration (36.2 μmol L^{-1}). This study demonstrated that phosphate could promote the geosmin production in several bacteria. Auffret et al. (2013) also revealed the significant correlations between phosphate and major bacterial population in the RAS. In another study, a high positive correlation was found between geosmin and 2-MIB levels in farming water with the phosphate levels in the inlet water of the farm (Robertson et al., 2006). Furthermore, Sarker et al. (2014) have conducted a study which evaluated the effect of low and high phosphorus levels diets on geosmin accumulation in rainbow trout (*Oncorhynchus mykiss*). The study showed that fish fed high phosphorus feeds caused higher water phosphorus levels, water geosmin levels and tissue geosmin levels than these in fish fed low phosphorus feeds. It was assumed that phosphorus excreted by fish fed high phosphorus feeds caused this geosmin problem.

2.3.2 Nitrogen (nitrate, nitrite)

High correlations (both positive and negative) were also found between nitrate or nitrite and major bacterial population in the RAS biofilter (Auffret et al., 2013). This indicats that nitrate and nitrite can stimulate or inhibit the growth of certain bacteria. However, the geosmin production may not be impacted by nitrate levels in the RAS, where no significant difference was found between high (80-100 mg L^{-1}) and low (20-40 mg L^{-1}) nitrate levels on geosmin production in the previous study (Schrader et al., 2013). Saadoun et al. (2001) also studied the effects of nitrate-nitrogen and ammonium-nitrogen on the growth and the geosmin synthesis of *Anabaena* sp.. This study found a high correlation between increasing ammonium-nitrogen and geosmin production while no strong correlation was found between nitrate-nitrogen levels

and geosmin production. Klausen et al. (2005) collected freshwater samples which were supplied to aquaculture systems. The results showed that the geosmin and 2-MIB levels in oligotrophic stream (Funder) with 50-70 μmol L^{-1} total dissolved nitrogen (TDN) was lower than these in other two eutrophic streams with 230-480 μmol nitrogen L^{-1}. In general, nitrate and nitrite may not adversely or beneficially impact the off-flavours production in the water, however, ammonium nitrogen will stimulate the off-flavours production by bacteria.

2.3.3 Temperature

Normally, high water temperature will promote metabolism and growth of bacteria, which causes high bacterial abundance in warm water. Robertson et al. (2006) have found a significant correlation between water temperature and either geosmin or 2-MIB levels in rainbow trout. Water with high temperature caused higher geosmin and 2-MIB levels in fish than these in low-temperature water. Johnsen et al. (1996) have estimated a model for 2-MIB uptake in channel catfish:

$$\text{MIB in fillet tissue } (\mu g\ kg^{-1}) = -0.61 + 4.2 * \log(h+1) + 0.0076 * T * h + 0.089 * T,$$

where T indicated water temperature and h indicated duration time in hours in the equation. This model revealed a positive correlation between 2-MIB accumulation rate and water temperature.

2.4 Accumulation of geosmin and 2-MIB in farmed fish

The presence of off-flavours in fish farmed in freshwater is one of the important problems of fillet quality in aquaculture. The degradation rates of both geosmin and 2-MIB in natural conditions are rather slow. This indicates that the concentrations of geosmin and 2-MIB in the water will increase continuously. Fish farmed in off-flavour contaminated water will uptake them through gills and skin. These off-flavours will then accumulate in lipid-rich tissues, such as ventral (belly) tissues (Howgate, 2004). Geosmin and 2-MIB are highly potent off-flavours with strong odor and taste which can be readily detected by the human senses. Persson (1980) has concluded the sensory threshold concentrations of geosmin and 2-MIB in fish fillet are 0.59 and 0.075 μg kg^{-1} while these in water are 15 and 42 ng L^{-1}, respectively. However, the sensory thresholds were rather different in previous studies because of individual differences, the threshold concentration of geosmin in rainbow trout was 0.9 μg kg^{-1} (Robertson et al., 2005) while that was 1.5 μg kg^{-1} reported by (Robertson & Lawton, 2003). Furthermore, Grimm et al. (2004) compared sensory detection of flavour checkers and instrumental method to geosmin and 2-MIB in channel catfish, and this study concluded the sensory threshold concentrations of geosmin and 2-MIB are 0.25-0.5 and 0.1-0.2 μg kg^{-1}, respectively.

The recirculating aquaculture system is an intensive farming mode with rather low water exchange rate, such as only 0.26% in a rainbow trout farm (Schrader et al., 2010), 0.2-3.1% in an Atlantic salmon (*Salmo salar*) farm (Davidson et al., 2014) and 5% in an arctic charr (*Salvelinus alpinus*) farm (Houle et al., 2011). The low water exchange rate resulted in geosmin and 2-MIB accumulation in the systems and could not be removed with wastewater. Table 2-1 summarizes reports from previous studies about the accumulation of geosmin and 2-MIB in water and fish from RAS or pond farms. They revealed that the concentrations of geosmin and 2-MIB in the RAS water were higher than these in pond water. Table 2-1 also shows that fish farmed in RAS accumulated more geosmin and 2-MIB in flesh than these off-flavours in fish farmed in semi-intensive ponds. The concentrations of geosmin and 2-MIB in the fish farmed RAS, meanwhile, almost excessed the sensory threshold according to the conclusion from Grimm et al. (2004). The major reason affecting the accumulation of geosmin and 2-MIB is believed the concentrations of these off-flavours in the farming water. In Table 2-1, a strong correlation between fillet off-flavour contents and water off-flavour levels was evident. High concentrations of geosmin and 2-MIB in the water resulted in high accumulation of these in fish flesh. Varga et al. (2015) farmed same species with same fish size in different ponds, which

resulted in different accumulation of geosmin and 2-MIB in common carp (*Cyprinus carpio*). The major reason for these differences was believed the different environments caused different water off-flavour levels which affected the accumulation of geosmin and 2-MIB in fish. In another study, Robertson et al. (2006) also found that the concentrations of geosmin and 2-MIB in rainbow trout fluctuated with the water geosmin and 2-MIB levels. The feeding habit may also be the reason which affects the accumulation of geosmin and 2-MIB in fish flesh. For example, Petersen et al. (2014) farmed two fish species, tilapia and pangas, in the same pond. Their results showed that pangas accumulated almost five times more geosmin than tilapia. In this study, the panga is a benthic species and live on the bottom of pond while tilapia prefer to live in the middle or surface water. And the sediment of pond might accumulate more geosmin and 2-MIB than water in the pond (Nielsen et al., 2006). Thus, benthic species accumulate geosmin and 2-MIB more readily than fish swimming in the free water bodies.

Table 2-1 Reported accumulation of geosmin and 2-MIB in fish from different farms.

Farming mode	Species	Fish size	Concentration of geosmin		Concentration of 2-MIB		Sources
			Fish flesh	Environment	Fish flesh	Environment	
RAS	Arctic charr (*Salvelinus alpinus*)	NR	0.703 μg kg^{-1}	37 ng L^{-1}, water 381 ng L^{-1}, biofilm	0.008 μg kg^{-1}	1 ng L^{-1}, water 4 ng L^{-1}, biofilm	(Houle et al., 2011)
RAS	Atlantic salmon (*Salmo salar*)	99 g	0.2 μg kg^{-1}	7 ng L^{-1}, water	0.9 μg kg^{-1}	132 ng L^{-1}, water	(Burr et al., 2012)
RAS	Atlantic salmon (*Salmo salar*)	3-5 kg	0.265-0.516 μg kg^{-1}	NR[2]	0.555-0.993 μg kg^{-1}	NR	(Davidson et al., 2014)
RAS	Largemouth bass (*Micropterus salmoides*)	NR	0.005-0.041 μg kg^{-1}	NR	0.017-0.067 μg kg^{-1}	NR	(Schrader et al., 2005)
RAS	White sturgeon (*Acipenser transmontanus*)	NR	0.675-1.177 μg kg^{-1}	NR	0.026-0.035 μg kg^{-1}	NR	(Schrader et al., 2005)
RAS	Rainbow trout (*Onchorhynchus mykiss*)	308 g	1.25-1.5 μg kg^{-1}	36.1 ng L^{-1}, water	0.1-0.2 μg kg^{-1}	28.5 ng L^{-1}, water	(Petersen et al., 2011)
RAS	Rainbow trout (*Onchorhynchus mykiss*)	197 g	0.08 μg kg^{-1}	1.3 ng L^{-1}, water	0.041 μg kg^{-1}	5.3 ng L^{-1}, water	(Schrader et al., 2010)
Pond (clayey soil)	Common carps (*Cyprinus carpio*)	Market size	0.12 μg kg^{-1}	0.07 ng g^{-1}, soil	0.18 μg kg^{-1}	0.018 ng g^{-1}, soil	(Varga et al., 2015)
Pond (alkaline soil)	Common carps (*Cyprinus carpio*)	Market size	0.02 μg kg^{-1}	0.045 ng g^{-1}, soil	0.11 μg kg^{-1}	0.015 ng g^{-1}, soil	(Varga et al., 2015)
Pond (marshy soil)	Common carps (*Cyprinus carpio*)	Market size	0.13 μg kg^{-1}	0.17 ng g^{-1}, soil	0.19 μg kg^{-1}	0.035 ng g^{-1}, soil	(Varga et al., 2015)
Pond (earthen)	Rainbow trout (*Onchorhynchus mykiss*)	300-400 g	1.54 μg kg^{-1}	5 ng L^{-1}, water	0.2 μg kg^{-1}	NR	(Robertson et al., 2006)
Pond (earthen)	Tilapia (*Oreochromis niloticus*)	182-199 g	0.011 μg kg^{-1}	4.1 ng L^{-1}, water	NA[1]	NA	(Petersen et al., 2014)
Pond (earthen)	Pangas (*Pangasianodon hypophthalmus*)	689-870 g	0.054 μg kg^{-1}	4.1 ng L^{-1}, water	NA	NA	(Petersen et al., 2014)

[1] NA: not analyzed.
[2] NR: not reported.

2.5 Depuration and elimination of geosmin and 2-MIB

Purging with clean water is the most economical and the simplest method to remove off-flavours in farmed fish although it is time consuming and water wasting. Prior to harvest, the fish are transferred to depuration ponds which are disinfected in advance and are not connected to other production units. Although a previous study which kept the feed regime during the depuration period could decrease tissue geosmin to less than sensory threshold (0.9 μg kg^{-1}) (Robertson et al., 2005), fish still should be fasted to enhance the depuration effect in industries (Petersen et al., 2011). The purging time is commonly one week, or else fish will significantly lose weight and tissue lipid when the purging time exceeded one week with starvation (Burr et al., 2012). In most studies, the rate of depuration was rather high in the first 24 hours and became lower after that (Johnsen & Lloyd, 1992; Robertson et al., 2005). During the depuration period, the purging water should be replaced regularly by clean water without off-flavour. This can reduce the purging time. Schram et al. (2017) found that the water renewal rate in the depuration ponds did not affect the geosmin depuration process. However, Davidson et al. (2020) in a depuration study with semi-RAS found that high water exchange rate system significantly reduced the required purging time.

Some oxidants are commonly used in the depuration period to shorten purging time. These oxidants disinfect water and thereby reduce the abundance of off-flavours producing bacteria and oxidize the off-flavours directly. Ozone and hydrogen peroxide (H_2O_2) are the widest used oxidants because of convenience and safety. Glaze et al. (1990) have evaluated the effect of several oxidants and their combinations to remove off-flavours in water. They concluded that the combination of ozone and H_2O_2 (around 1:1 ratio) was more efficient to eliminate water geosmin and 2-MIB than the effect of ozone and H_2O_2 used individually. Other studies conducted by Koch et al. (1992) and Westerhoff et al. (2006) also reached same conclusions, also when the ratio of ozone and H_2O_2 was slightly different. In general, both studied ratios successfully removed near 90% of water geosmin and 2-MIB in 15 min. The effect was also evident when these oxidants were applied in the depuration period. Davidson et al. (2014) evaluated the effect of H_2O_2 to depuration ponds with Atlantic salmon from RAS. The results showed that H_2O_2 might enhance the depuration process both for geosmin and 2-MIB. In another study, Zhang et al. (2006) treated fish meat from bighead carp (*Hypophthalmichthys nobilis*) with ozone, and the study showed that around 50% of muscle geosmin was removed after 20 min. However, ozone was not found effective in reducing water either geosmin or 2-

MIB levels in farmed rainbow trout when using ozone in the RAS (Schrader et al., 2010). This adversely result may have been caused by organic particles from feces and uneaten feed which may destruct and consume the OH radicals from ozone and H_2O_2, and thereby reduce the effect to elimination of geosmin and 2-MIB in the water (Ho et al., 2004).

Besides from oxidants, sludge derived from RAS has also used to eliminate geosmin and 2-MIB in the water. Guttman and van Riji (2009) found that both sterilized and non-sterilized sludge could remove 2-MIB and geosmin, and non-sterilized sludge was more efficient than sterilized sludge on 2-MIB and geosmin elimination. This means that sludge from RAS could reduce 2-MIB and geosmin levels by the combination of chemical and/or physical sorption and biological degradation. Other studies have also found that non-sterilized sludge has higher capacity to remove 2-MIB and geosmin than sterilized sludge. Sterilized sludge was, however, still able to reduce water 2-MIB compared to untreated control groups (Azaria et al., 2017; Ma et al., 2016). However, these studies were both experimented in small reactors in laboratories and the results cannot be applied in full-scale industrial RAS before their effects have been verified.

2.6 Development of recirculating aquaculture systems

With the growing of world population and the decrease in fish capture from nature, aquaculture is a preferred way to satisfy the fish-protein requirements in the world. Compared to traditional farming modes, RAS farming has several advantages. Critical factors can be monitored to control precise production. According to the water exchange rate of RAS, FAO have divided the RAS into low level RAS, intensive RAS and super intensive RAS and the degrees of water recirculation in system are 95.9%, 98.6% and 99.6%, respectively. A recirculating aquaculture system can also be divided into production units and water treatment units.

2.6.1 Production

The production unit mainly includes culture tank and feeding system. The size of tanks is usually based on the production and stocking density. Stocking density is the major factor which affects the fish welfare, water quality and the production. High stocking density may cause serious stress to farmed fish and exceed the capacity of the system. However, some species, such as Atlantic salmon and rainbow trout, fish may be dominant and aggressive when the tank stocking density is too low (North et al., 2006). Thus, an optimal stocking density will maximum the yield and satisfy the fish welfare together. The limits of stocking density in different life stages, farming methods and certain farming environment are rather different. The regulation in Norway have limited the stocking density of Atlantic salmon on growing stage that 25 kg m^{-3} for sea cages and 80 for land-based systems (RAS). Liu et al. (2017) have evaluated the effect of different stocking density on growth performance and welfare related indicators of Atlantic salmon, and the study concluded that 50 kg m^{-3} might be the limit for Atlantic salmon farmed in RAS. In a review article, Malone (2013) have also suggested that as much as 60 kg m^{-3} may be an acceptable stocking density in RAS. Based on stocking density and estimated production, the size of tanks can be calculated. There are also many tank shapes can be used in RAS, such as circular and rectangle. At the present, circular tank is the dominant shape in industries because of the structure and self-cleaning ability (Malone, 2013).

Feeding system is also a major part of production units. Low feeding rate cannot satisfy the nutrition requirement of farmed fish as well as high feeding rate causes low feed conversion rate (FCR) and feed wasting. Feed cost is a large part of the total cost in aquaculture regardless farming methods. Mowi ASA has reported that feed cost was accounted for 40% of total cost in 2019 (MOWI annual report 2019). Thus, an optimal feeding rate with standardized feeding

route is rather necessary in industries. For standardizing and simplifying the management, automatic feeders are widely used in industrial RAS at the present. Most of the automatic feeders can be controlled by designed programs and they are able to feed fish according to the pre-determined schedules without human operators (Tanveer et al., 2018; Yeoh et al., 2010). At the present, for monitoring the precise feeding conditions, vision monitors with artificial intelligence is studied to control the feeding strategy (Antonucci & Costa, 2020).

2.6.2 Water treatment

Water treatments mainly includes particle removal, disinfection, and water quality controlling (Losordo et al., 1998). Uneaten pellets and feces are the major sources of organic particles in RAS, and they are both collected by a drain on the bottom of the tank. These organic particles must be removed firstly before other water treatments or else they can be a shelter for microorganisms and reduce the effect of disinfection. Recently, Xiao et al. (2019) have reviewed major physical filters used for particle removal in industrial RAS, and these filters can be used individually or multiply to satisfy the different recirculating systems.

High density in RAS may increase the probability of infection with several diseases. Although, vaccination is widely used in farmed fish, disinfection is still necessary to control diseases with killing pathogens. Ultraviolet (UV) light is a common way used in RAS. It is harmless to fish farmed in tanks. However, the effect of UV light on disinfection will be significantly affected by the water transmittance and the velocity of water flow (Xiao et al., 2019). Ozonization is another way which is said to facilitate particle removal, clarify water, stable the water quality and control disease (Gonçalves & Gagnon, 2011). The largest problem on ozone using is the toxicity of ozone residual to fish, exceed dosage of ozone used in aquaculture may damage skin and gills of fish, reduce growth, and even cause death (Powell & Scolding, 2018). To minimum the toxicity of ozone, Spiliotopoulou et al. (2018) have evaluated the effect of different dosage of ozone on water quality in a laboratory scale recirculating system. The result showed that the ozone dosage at 87 mg O_3 L^{-1} h^{-1} could significantly improve water quality and no mortality was found during the experiment. Furthermore, combing ozonization and UV light may be more efficient than separate application of ozone or UV light. An experiment conducted by Summerfelt et al. (2009) showed that UV light could enhance the disinfection effect of ozone on controlling heterotrophs and coliform in the water.

The main water quality parameters in RAS mainly includes pH, temperature, dissolved oxygen, nitrogen (ammonia, nitrite, and nitrate), and salinity (just for marine species). Ammonia is excreted nitrogen from fish and is toxic in its un-ionized form, NH_3 (Tomasso, 1994). To minimize the toxicity of ammonia, a biofilter is necessary in the system. In the biofilter, nitrification converts ammonia to nitrite and nitrate by nitrobacteria. Xiao et al. (2019) also reviewed the major biofilters used in RAS and listed the disadvantages and advantages. pH in the system is usually monitored on real time, and lime water is a common reagent for pH adjustment. Low pH may promote the growth of pathogens and is harmful to fish gills as well as high pH will increase the toxicity of ammonia where high pH water will result in increasing of un-ionized ammonia (Colt, 2006). Oxygen is also related to the nitrification process, which slows down when the dissolved oxygen is too low. Besides of nitrification, low dissolved oxygen also impacts the growth rate and health of fish, and even may increase mortality. Colt (2006) suggested that the dissolved oxygen should be maintained above 5-6 mg L^{-1} in the system for most species. Some species can tolerate low oxygen levels, such as catfish, to which above 3 mg O_2 L^{-1} is acceptable. Oxygenation is a common way used in industrial aquaculture systems to satisfy the oxygen requirements. Another water quality index is temperature which is mainly affect the growth rate and feed intake of farmed fish. Water temperature should be adjusted according to the habit of farmed fish. For example, Atlantic salmon is a cold-water species with the bast growth rate on post-smolt stage at 12-14 °C (Handeland et al., 2008), and the thermal limits for growth and survival are around 22.5 °C and 28 °C, respectively (Corey et al., 2020; Elliott & Elliott, 2010). However, the optimal temperature for growth of Nile tilapia (*Oreochromis niloticus*) is around 26 °C and the growth performance may be impacted when the water temperature is below 22 °C (da Silva et al., 2021). Another study also proved that Nile tilapia ceased feeding when the water temperature was below 13-18 °C and that mortality occurred when the temperature was below 11 °C (Atwood et al., 2003). Thus, a cooling system is necessary to Atlantic salmon during the summertime as well as a heating system for Nile tilapia during the wintertime.

3 Materials and methods

Healthy Japanese seabass with initial weight of 0.11 kg were placed into 18 tanks at the beginning of the experiment in RAS (30 fish per tank). Six extruded diets with three crude protein levels (420, 450, and 490 g kg^{-1}) and 2 crude lipid levels (150 and 180 g kg^{-1}) were produced. Table 3-1 shows the main chemical compositions of these diets. Each diet was randomly assigned to three tanks. Fish were hand-fed 3 times per day in a 15-week accumulation trial. Feed intake was assessed daily, based on uneaten pellets that were counted and removed by siphoning. In the 16th week, a ten-day depuration period with starving was initiated. Water and fish tissues were sampled for analysis at the end of the accumulation and depuration period.

Table 3-1 Main chemical compositions of the experimental diets (g kg^{-1} dry matter).

Proximate composition	Diet no. (Protein/Lipid)					
	D1 420/150	D2 420/180	D3 450/150	D4 450/180	D5 490/150	D6 490/180
Dry matter	924.5	932.5	934.1	931.2	926.5	927.5
Crude protein	422.7	410.7	458.2	451.6	494.0	495.6
Crude lipid	151.6	185.5	154.3	187.0	140.8	174.3
Gross energy	223.9	231.3	225.1	229.5	224.6	231.9
Ash	72.7	70.7	71.9	72.2	74.4	72.0
CP/GE[1], g MJ^{-1}	18.9	17.8	20.4	19.7	22.0	21.4

[1] CP/GE, the ratio of crude protein to gross energy.

3.1 Recirculating aquaculture system, water quality and depuration period

The recirculating aquaculture system comprises 24 culture tanks (Volume: 1 m^3) and several water treatment units, which supplied by Goldbill (Ningde, Fujian, China). The rearing water from tanks was collected in drum filter for removal of particles and water was disinfected by UV light after that. Recycled water then was pumped into biofilter where was filled with filter media and the nitrification process was carried out by nitrobacteria. A heat exchanger (ZWH-KFX-BT2011, Zhengxu Technology Co. Ltd. Dongguan, Guangdong, China) was installed and kept the constant water temperature after biofilter. The treated water was then pumped back to culture tanks and the water flow was 8 to 9 L min^{-1} in each of 24 tanks. The total

volume of water in the system was about 24 m^3 and 12.5% of water was replaced by freshwater every day. When calculating the degree of reuse of RAS, this equation was used:

$$R = \left(1 - \frac{Q_B}{Q_T}\right) * 100\%$$

Where R indicated degree of reuse, Q_B indicated the size of new incoming water, and Q_T indicated total water flow in the system. Thus, the degree of reuse in experimental RAS is around 98.93%.

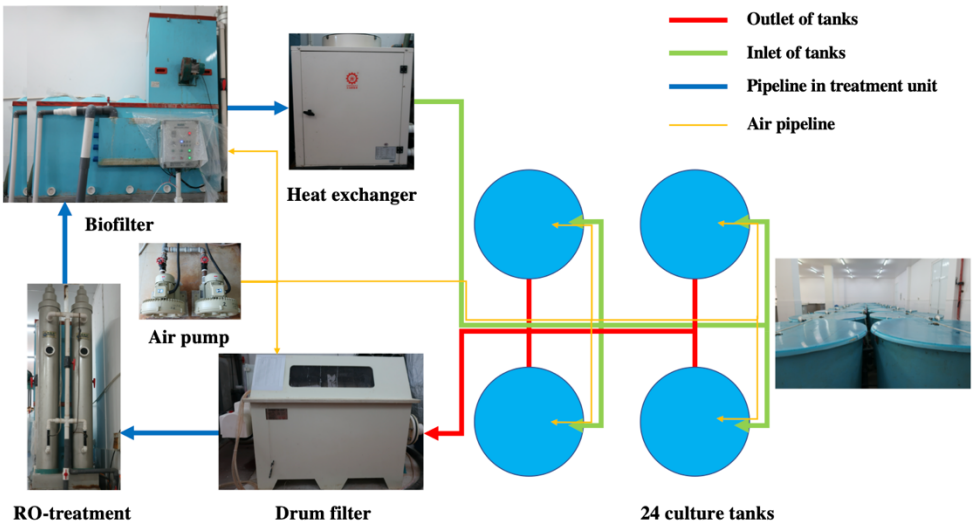

Figure 3-1 The experimental recirculating aquaculture system.

Water quality was measured in the tanks every day after feeding. Oxygen was assessed using a Dissolved oxygen meter (AZ8401, Az Instrument Corp., Taiwan), and ranged from 5.5 to 6.5 mg L^{-1} in the tanks. Water temperature decreased gradually from 27.5 °C to 22.5 °C, and the pH was maintained above 6.5 by adding lime slurry (pH=10) every day. A "Water quality regulator" (Miracle Animal, FFC research institute, Okayama, Japan) was added into the system weekly for controlling concentrations of total ammonia and nitrite, which were below 5 mg $L^{-1}$and 0.25 mg L^{-1}, respectively. Total ammonia and nitrite were assessed using commercial testing kits (Yi'er Biology Engineering Co., Ltd., Guangzhou, Guangdong, China). Seawater, disinfected with sodium hypochlorite, with salinity at 25 ppt, was added twice (on

the 7th and 9th weeks, 2 m^3 new water each time) into the system. This resulted in salinity in the RAS at 2 ppt. The depuration tank was filled with 22 m^3 of freshwater, and this water was partially replaced by 11 m^3 tap water on the 5th day of depuration. The water was sufficiently aerated to reduce residual chloride before utilization.

3.2 Water sampling and fish tissues preparation

At the end of the accumulation trial, 6 fish with average individual weights of about 0.4 kg, were randomly selected from each tank. Two fish were slaughtered, and the dorsal and ventral muscle tissues without skin and bone were sampled, sealed in cups, and stored at -80 °C. The remaining fish had passive integrated transponder (PIT) tags (Smartrac N.V., Amsterdam, Netherland) implanted after being anesthetized by 0.9 g L^{-1} of MS-222. All tagged fish were pooled into a 22 m^3 holding tank with clean fresh water and starved for 10 days during depuration. At completed depuration, two fish from the same feeding tank in the accumulation trial were identified using PIT tags and sampled for analysis. Water samples for muddy flavour analysis were collected from RAS, sealed into bottles, and stored at -80 °C.

3.3 Analysis of geosmin and 2-MIB in tissues and water samples

Geosmin and 2-MIB were extracted by headspace solid-phase microextraction (HS-SPME) using a 65 μm DVB/PDMS fibre (57310-U) in a manual holder (57330-U) (Sigma-Aldrich, St. Louis, MO, USA). Approximately 5 g of fish tissue was weighed and homogenized with ultrapure water. The mixture was transferred to a 10 ml glass bottle with 5 μl of internal standard solution (DHN) (10 ng μl^{-1} Decahydro-1-naphthol in methanol) and heated in a microwave reactor for 3 min. Pure nitrogen (99.999%, 75 ml min^{-1}) was used to carry the stream (steam and off-flavours) to a condenser that held a temperature of 4 °C. Water for the analysis of muddy flavour components was collected in 15 ml extraction bottles with Teflon-faced silicone septa caps (Agilent Technologies, Palo Alto, CA, USA). The solution volume was replenished to 10 ml with ultrapure water, and 3 g of NaCl was added. The extraction bottle was heated in a water bath using a heating magnetic stirrer (IKA RET basic, Staufen, Germany), and the SPME fibre was injected through the septa. The rotation speed was 1100 rpm, temperature was set to 60 °C, and the extraction time was 40 min. After extraction, the SPME fibre was transferred and injected into the operated injector of a gas chromatograph-mass spectrometer (GC-MS, Agilent 7890B-7000C, Agilent Technologies, Palo Alto, CA, USA) for desorption. Desorption was carried out by heating the fibre to 250 °C with a flow of

carrier gas (high-pressure He, 0.45 MPa) for 2 min. The temperature program of gas chromatography was 50°C (2 min), raised at 10 °C min^{-1} to 200 °C (1 min), and at 50°C min^{-1} to 250 °C (2 min). The temperatures of the transfer line and ion source were maintained at 280 °C and 230 °C, respectively. The electron energy was 70 eV, and quantification of geosmin and 2-MIB was performed using the selected ion monitoring mode of the m z^{-1} 112 and 95 fragments, respectively. After 21 min, the relative response ratios of geosmin and 2-MIB to DHN in the tissues were measured.

To convert the relative response ratios to actual values, the standard curves of geosmin and 2-MIB in tissues were plotted. Mixed standard samples were prepared by blending equal weights of the dorsal tissues and ventral tissues. A standard solution (100 ng μl^{-1} (+/-)-geosmin and 2-methylisoborneol, Sigma-Aldrich, St. Louis, MO, USA) was diluted to 10 ng μl^{-1} using methanol in advance, and eight pieces of mixed standard tissues were injected with 0, 0.5, 1, 2.5, 12.5, 25, 50, and 75 µl of the diluted standard solution, respectively. The relationships between the response ratio and concentration were linear, and the actual concentrations of geosmin and 2-MIB in tissues were calculated from these curves.

Fifty millilitres of water sample was added to a 100 ml extraction bottle with 15 g of NaCl, 5 µl of DHN, and a small magnetic rotor. For the standard curve, each of six 150 ml extraction bottles were filled with 120 ml of ultrapure water, 36 g of NaCl and 5 µl of DHN. Aliquots of 0, 0.3, 0.6, 1.2, 3.0, and 4.8 µl of the diluted standard solution were then added to the bottles, which were subjected to the same protocol as the tissues.

3.4 Analysis of fat content in fish tissues

The tissues remaining after off-flavour extraction were dried (temperature: -50 °C, pressure: <0.1Pa, duration: 24h) in a vacuum freeze dryer (SJIA-10N-50A, Shuangjia Instrument Co. Ltd., Ningbo, Zhejiang, China). Dried tissues were ground mildly and analysed for fat content by SoxROC Extractor (SX-360, OPSIS AB, Furulund, Sweden).

3.5 Analysis of bacterial composition in the water

One liter of water was sampled for analysis of bacterial compositions from drum filter and culture tanks in RAS, respectively. Water samples were filtered and concentrated in Millipore filter membranes (0.45 µm, aqua system, Sangon Biotech, Shanghai, China) by vacuum

filtration. Millipore filter membranes with microorganisms were stored in -80 °C and were delivered with dry ice to a commercial company. The bacterial community structure in RAS water was assessed by Shanghai OE Biotech Co. Ltd. (Shanghai, China), using High Throughput Sequencing of 16S rRNA by Illumina MiSeq (Illumina Inc. San Diego, CA, USA). The raw data after sequencing were analysed step by step using Trimmomatic, Flash, split_libraries (QIIME) and UCHIME and were presented as valid tags which generated operational taxonomic units (OTUs) using Vsearch software with 97% similarity cutoff. The observed species, Shannon-Wiener Index and Simpson's diversity Index were calculated based on OTUs.

3.6 Ethics statement

This study did not involve any endangered species. Japanese seabass (*Lateolabrax japonicus*) is not the protected species by Chinese law. It is a commercially harvested and farmed species in China. During the feeding period and sampling procedures, the experimental fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (Decree No. 2 of Ministry of Science and Technology, issued in 1988).

3.7 Statistical analysis

Statistical analysis, plots, correlation (Spearmen's test), regression analysis and curve fitting were carried out in R-studio (Boston, MA, USA). The Shapiro-Wilk normality test and homogeneity test of variances were carried out in advance. Significant differences between dietary treatments and their interactions were tested by one-way and two-way analysis of variance (ANOVA), using a significance (α) of 0.05. Bonferroni's test and Duncan's test were used for multiple comparisons (post hoc tests) when variances were equal, or the Kruskal-Wallis test and Nemenyi test were used.

4 Results and discussion

4.1 Microbes synthesizing components causing muddy flavour and oxidizing nitrogen in the RAS water

The species diversity indices from the RAS water were obtained from the results of high-throughput sequencing. The observed species were 698 and 729 in tanks and drum filter, respectively. The Shannon-Wiener Index were 6.26 and 6.61, while the Simpson's diversity Index were 0.94 and 0.95 in tanks and drum filter, respectively. The microbial structures showed that proteobacteria was the predominant phylum in the RAS water, accounting for 54% of the total bacteria (Figure 4-1, A). Actinobacteria and cyanobacteria, which are most likely responsible for the synthesis of muddy flavours, accounted for 0.98% and 0.03% of the total bacteria, respectively. Although Myxobacteria were below the detection limit, these levels are consistent with previous observations that actinobacteria and cyanobacteria can cause intense off-flavours, even when these bacteria represent only low (0.007-0.9%) proportions of the total bacteria (Lukassen et al., 2017). In Figure 4-1, B, nitrobacteria were also assessed. *Nitrosomonas*, which oxidizes ammonia to nitrite, represented 0.72%, and *Nitrospira*, which oxidizes nitrite, accounted for 0.99%. Although the nitrate nitrogen does not directly affect the geosmin and 2-MIB levels in the water (Schrader et al., 2013), low nitrogen level in the water might be an efficient way to control the growth of nitrogen-dependent actinobacteria and cyanobacteria (Cottingham et al., 2015; Dai et al., 2018; Saadoun et al., 2001).

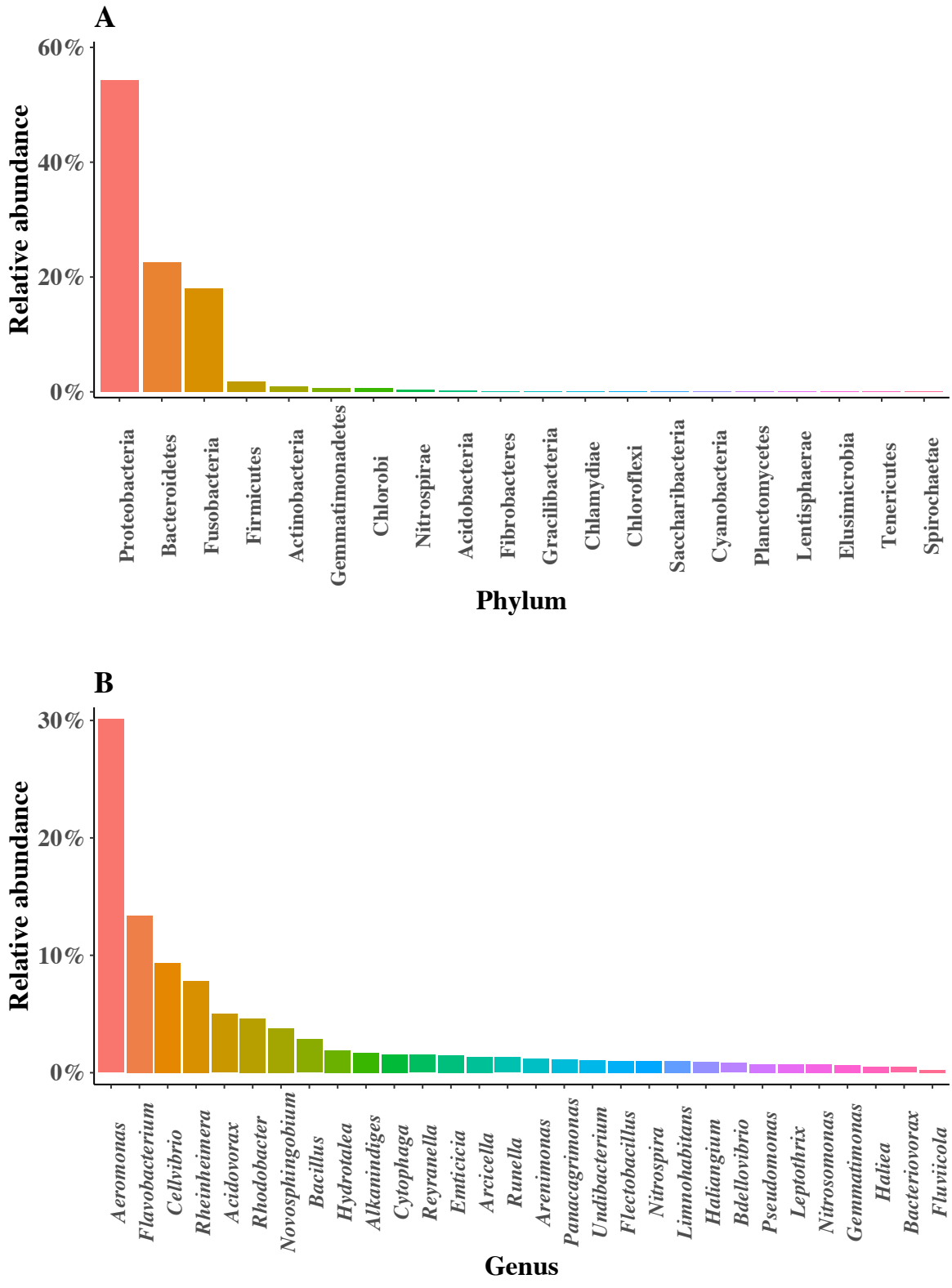

Figure 4-1 The microbial structure in the RAS water.

4.2 Distribution of geosmin and 2-MIB in rearing water as well as dorsal and ventral tissues

The concentrations of geosmin and 2-MIB were 45.9 and 21.4 ng L^{-1} in the depuration tank, respectively (Figure 4-2). The water in RAS tank and drum filter contained as much as 169.0-183.6 ng geosmin L^{-1} and 41.3-45.3 ng 2-MIB L^{-1}, respectively.

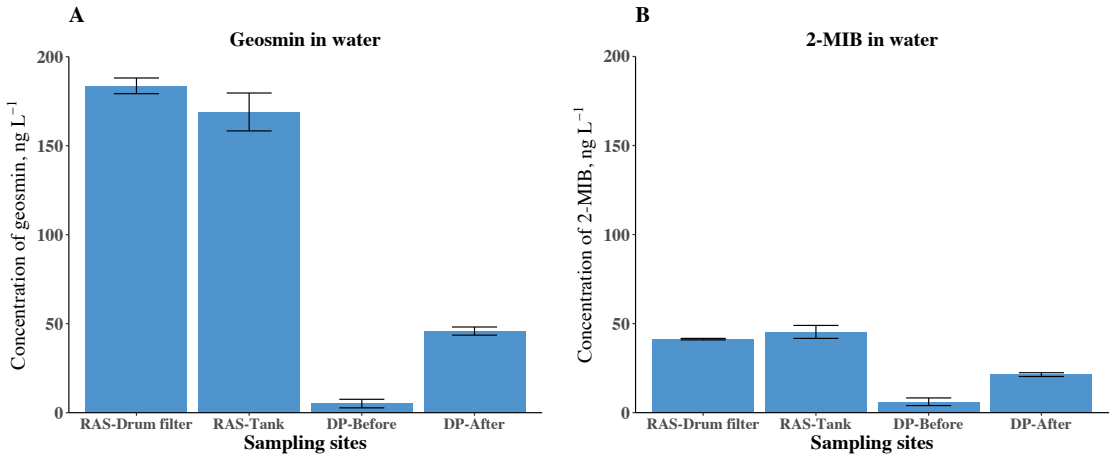

Figure 4-2 Concentrations of geosmin and 2-MIB in the water. DP-Before and DP-After indicated the water from depuration tank before onset of the depuration period and after the depuration period, respectively. Data presented as mean ± SE.

No significant interaction between dietary protein and lipid were seen, with respect to lipid, 2-MIB nor geosmin in dorsal or ventral tissues (Table 4-1). However, the main effects were significant ($P < 0.05$). The lipid concentration in the dorsal tissues was inversely related to the dietary protein concentration, and the lipid concentration in this tissue was increased by raising dietary lipids in dietary dry matter from 150 to 180 g kg^{-1}. Increasing dietary protein from 420 to 450 g kg^{-1} resulted in reductions in both 2-MIB and geosmin, while no additional reduction was obtained by a further increase in dietary protein to 490 g kg^{-1}. The one-way ANOVA between different diets also revealed that the dorsal tissue lipid in Diet 2, the lowest protein level and highest lipid level treatment, was significantly higher ($P < 0.05$) than those in other diets, while the lowest dorsal tissue lipid was observed in Diet 5, which had the highest dietary protein to lipid ratio. This observation was consistent with previous findings of a positive correlation between lipids in feed and tissues, and a negative correlation between dietary protein and tissue lipids (Santinha, 1999). The lipid content affected the muddy flavours in tissues and caused significantly higher concentrations of geosmin and 2-MIB in the dorsal tissues from Diet 2 when compared to other diets ($P < 0.05$). The ANOVA did not reveal a significant difference in ventral tissue geosmin or 2-MIB, although the concentrations were higher than those in the dorsal tissues. The only significant difference observed in the ventral tissue was an increase in tissue lipid concentration when

dietary lipid was increased from 150 to 180 g kg^{-1}. It is believed that fish accumulate geosmin and 2-MIB gradually, and that a long time is needed to reach saturation. The lipid content in ventral tissues was higher ($P < 0.001$) than that in the dorsal tissues. Thus, more time may be required to sufficiently accumulate high levels of geosmin and 2-MIB in ventral tissues to obtain significant differences.

As can be seen from the clusters in Figure 4-3, the tissue lipid content affected the accumulation of both geosmin and 2-MIB. Prior to depuration, the leaner dorsal tissues (circles) concentrated the muddy flavours to less than 7.6 µg kg^{-1} for geosmin and 0.9 µg kg^{-1} for 2-MIB. However, the concentration of geosmin in the fattier ventral tissues (triangles) ranged from 11.1 to 35.3 µg kg^{-1} and the concentration of 2-MIB in these tissues ranged from 0.8 to 2.7 µg kg^{-1}. Thus, the results in Table 4-1 and Figure 4-3 (A and C) confirmed that the fatty ventral tissue more readily accumulated off-flavour components than leaner dorsal tissue. The ANOVA also revealed significant ($P < 0.001$) difference between dorsal and ventral tissues both on lipid, geosmin and 2-MIB. This imbalanced distribution of lipid and muddy flavours in fish is in consistent with previous findings on barramundi (*Lates calcarifer*) (Percival et al., 2008). Furthermore, Johnsen and Lloyd (1992) found that channel catfish with more than 2.5% accumulated body fat contained 3 times as much 2-MIB as leaner fish with less than 2.5% body fat when exposed to water with 0.5 µg 2-MIB L^{-1} for eight hours. Rohani et al. (2009) also found that fish fed the higher crude fat feeds could have more intensive off-flavours than fish fed the lower crude fat feeds. However, body fat content and dietary composition may not be the only factors controlling the uptake and deposition of components causing off-flavours. Experiments with rainbow trout have shown that there was no significant correlation between tissue lipid (1.9-10.6 %) and geosmin or 2-MIB in rainbow trout (Petersen et al., 2011). The concentrations of geosmin and 2-MIB in the rearing water seemed to be the main driving force for accumulation in trout tissues, but Petersen also found a significant positive correlation between fish size and accumulation of 2-MIB and geosmin. Simultaneously, no significant correlation was found between sensory traits and tissue lipid in yellow perch (*Perca flavescens*) (González et al., 2006) or in barramundi (Frank et al., 2009).

Table 4-1 Concentrations (wet weight) of lipid, geosmin and 2-MIB in dorsal and ventral tissues of Japanese seabass fed diets with different levels of protein and lipid.[1]

	Dorsal tissue			Ventral tissue		
	lipid, g kg^{-1}	2-MIB, μg kg^{-1}	geosmin, μg kg^{-1}	lipid, g kg^{-1}	2-MIB, μg kg^{-1}	geosmin, μg kg^{-1}
One-way ANOVA						
D1 (420/150)	13.5^{bc}	0.67^{ab}	4.76^{b}	70.3	1.71	19.1
D2 (420/180)	20.8^{a}	0.73^{a}	6.58^{a}	72.6	1.70	20.3
D3 (450/150)	12.4^{bc}	0.55^{bc}	3.03^{c}	55.6	2.45	29.8
D4 (450/180)	14.3^{b}	0.42^{c}	3.47^{c}	80.6	1.76	21.1
D5 (490/150)	10.1^{c}	0.47^{c}	3.08^{c}	46.9	1.84	17.4
D6 (490/180)	11.7^{bc}	0.41^{c}	2.85^{c}	78.6	1.21	18.7
Pooled s.e.m.[2]	3.42	0.12	1.33	12.2	0.36	4.08
Two-way ANOVA *P*-value						
Protein	**0.007**	**0.006**	**<0.001**	0.621	0.493	0.155
Lipid	**0.048**	0.255	0.304	**0.037**	0.301	0.647
Protein * Lipid	0.226	0.464	0.158	0.368	0.740	0.349
Main effect of protein[3]						
420	16.6^{a}	0.69^{a}	5.54^{a}	71.4	1.70	19.7
450	13.2^{ab}	0.50^{b}	3.20^{b}	68.1	2.10	25.4
490	10.9^{b}	0.44^{b}	2.96^{b}	59.6	1.58	17.9
Main effect of lipid[4]						
150	11.8^{b}	0.55	3.48	56.1^{b}	1.98	21.4
180	14.6^{a}	0.48	3.90	77.2^{a}	1.55	20.0

[1] means of three replicate tanks. Means in each column with different superscripts are significantly different ($P < 0.05$).

[2]s.e.m., standard error of means.

[3]420, 450, and 490 g kg^{-1} of dietary crude protein.

[4]150 and 180 g kg^{-1} of dietary lipid.

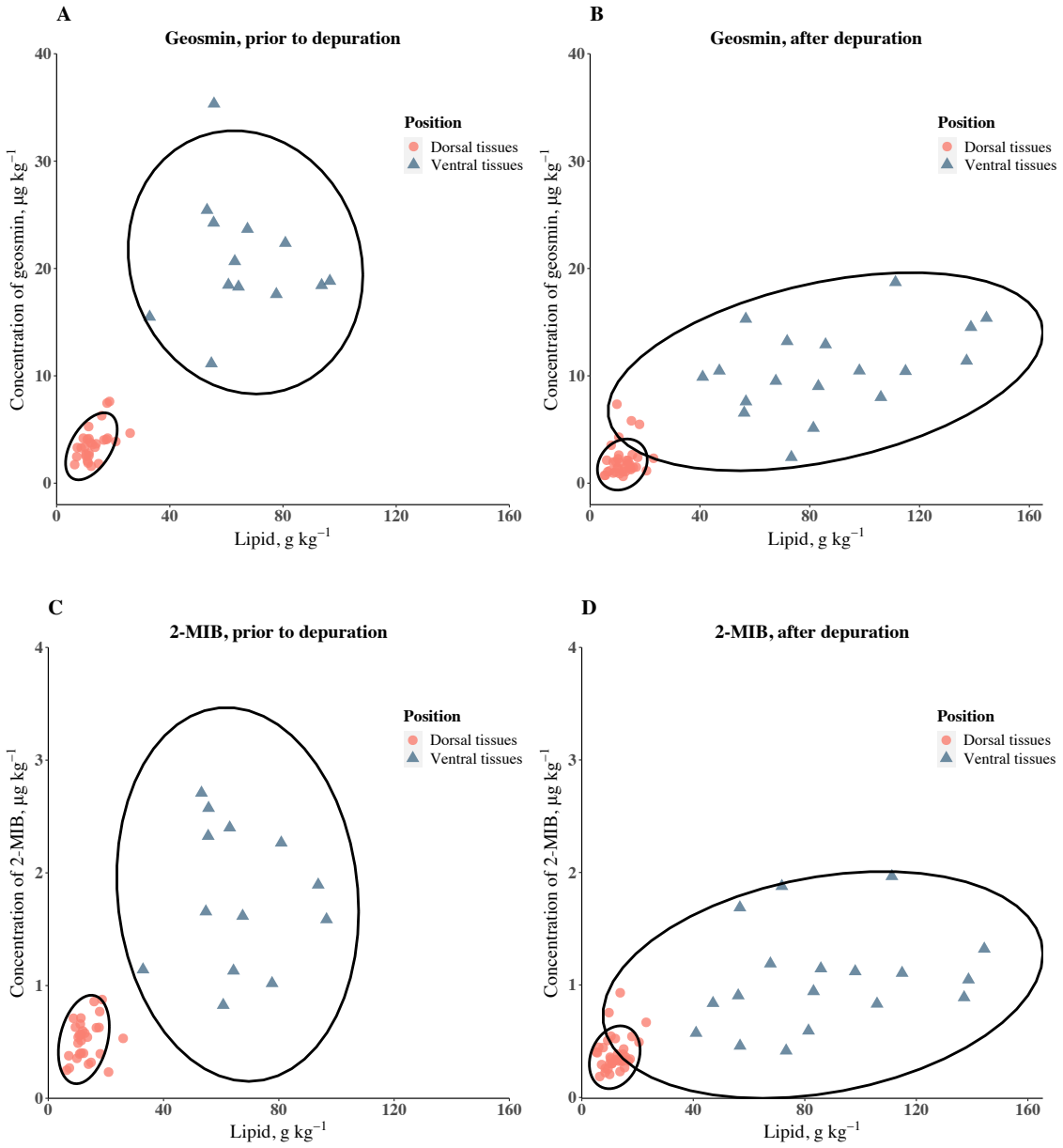

Figure 4-3 Scatter plots of geosmin and 2-MIB related to lipids in fish with 95% confidence intervals.

As shown in Figure 4-4, there was a significant correlation between tissue geosmin and 2-MIB levels. According to the linear regression analysis, the slope of tissue geosmin to 2-MIB is 11.4, which is higher than the ratio of geosmin to 2-MIB in the RSA water (4.07) (value can be calculated in Figure 4-2). This indicates that the absorption rate of geosmin was higher than that of 2-MIB in Japanese seabass, which is consistent with the hypothesis of a previous review (Rurangwa & Verdegem, 2015).

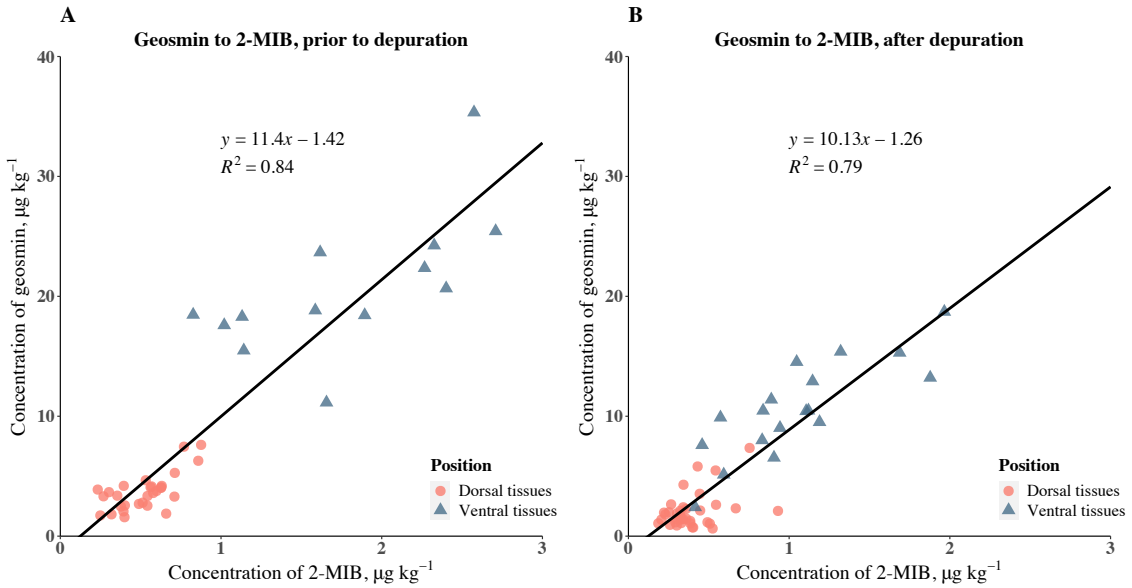

Figure 4-4 Scatter plots of geosmin related to 2-MIB in fish.

4.3 Depuration of geosmin and 2-MIB from Japanese seabass

Depuration with freshwater is the most common procedure used to remove muddy flavours, and notable changes can be seen during the first 24 h of treatment (Johnsen & Lloyd, 1992; Robertson et al., 2005). For example, the concentrations of muddy flavours can decrease to below the detection threshold after 7 days (Petersen et al., 2011; Robertson et al., 2005). In this experiment, the effect of depuration was evident. After 10 days of depuration, the concentration of geosmin in the ventral tissue was reduced to less than 18.71 µg kg^{-1}, while that of the dorsal tissue still had values between 0.6 and 7.4 µg kg^{-1} (Figure 4-3 B & D). Simultaneously, the concentration of 2-MIB in ventral tissue was reduced to less than 2.0 µg kg^{-1}, while the concentration of 2-MIB in the leaner dorsal tissue remained between 0.2 and 0.9 µg kg^{-1}. It was also found that the clusters were far away before depuration, and they became closer and overlapped after depuration. There were significant differences in geosmin and 2-MIB levels

between the ventral and dorsal tissues. After depuration, the dorsal clusters dropped slightly and were closer than before. However, the ventral clusters dropped significantly, indicating that the concentration of geosmin and 2-MIB in ventral tissues decreased dramatically (nearly 50%) when the tissue lipid content increased. The results indicate that clearance of both geosmin and 2-MIB during depuration was slower in dorsal than in ventral tissue. It is believed that the correlation between depuration rate and concentrations of geosmin and 2-MIB in fish may be positive, and high geosmin and 2-MIB contents resulted in high depuration rates in fish tissues. An accumulation and depuration experiment in crucian carp (*Carassius carassius*) was conducted and it showed the same conditions that both the accumulation rate and depuration rate in the ventral tissues were higher than these in dorsal tissues (Yang et al., 2019).

Similar ratios of geosmin to 2-MIB obtained from prior to depuration and after depuration are also shown in Figure 4-4 A & B. This demonstrated that the depuration rate of geosmin was similar to that of 2-MIB in both the dorsal and ventral tissues. This result was consistent with a previous study that showed the similar depuration rates (approximately 75% removal of geosmin and 2-MIB in 10 days) in Atlantic salmon (Davidson et al., 2014), although Rurangwa and Verdegem (2015) suggested that the depuration rate of geosmin should be slower than that of 2-MIB.

Both geosmin and 2-MIB were initially found at only low levels in the depuration pond water (Figure 4-2), and the concentrations of both were significantly increased when the depuration period was completed. The combination of fasting fish and adding clean freshwater into the depuration tank resulted in the removal of geosmin and 2-MIB from fish tissues. However, the current purging procedure was not sufficient to render the fish tissues without muddy flavour when the threshold concentrations for detection are 0.25-0.5 μg kg^{-1} for geosmin and 0.1-0.2 μg kg^{-1} for 2-MIB (Grimm et al., 2004). Previous experiments on fatty fish, such as Arctic charr (Houle et al., 2011), also indicated that high lipid content in the fish complicates purging if only fresh water is employed. Davidson (2014) subsequently tried disinfecting the depuration tank with H_2O_2 prior to purging fish, and a rapid reduction of geosmin and 2-MIB levels in Atlantic salmon was observed, which indicates that the pre-treatment of depuration tanks or water with environmentally friendly oxidants might be useful to help purge fatty fish when only fresh water is employed.

5 Conclusion

In conclusion, diets with high fat content or low protein content resulted in increased concentration of geosmin and 2-MIB in tissues when fed to Japanese seabass in RAS. The concentration of geosmin was higher than that of 2-MIB in all tissues and a positive correlation between tissue geosmin and 2-MIB was also found. The rate of clearance during 10-day depuration of both geosmin and 2-MIB was faster in lipid-rich ventral tissues than that in the leaner dorsal tissues. In the current RAS, purging was facilitated by daily replacement of a portion of the rearing water. In general, the main factors affect the concentrations of geosmin and 2-MIB in fish are the production of geosmin and 2-MIB both in the depuration tank and in RAS, and the fat content in farmed fish.

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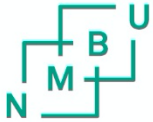

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