The ratio between digestible protein and digestible energy affects accumulation and depuration of geosmin and 2-methylisoborneol (2-MIB) in Japanese seabass (*Lateolabrax japonicus*) raised in a recirculated aquaculture system

Qiang Lu[1,2], Yuan Zou[1,2], Yuexing Zhang[1*], Liying Huang[3], Haokun Liu[4], Zhiyong Dong[1,2], Xiaolong Yin[5], Trond Storebakken[2]

[1] Zhejiang Ocean University, Marine Science and Technology College, National Engineering Research Center for Marine Aquaculture, Zhoushan, Zhejiang, 316022, China.

[2] Norwegian University of Life Sciences, Faculty of Biosciences, N-1432 Ås, Norway.

[3] Marine Fisheries Research Institute of Zhejiang, Zhoushan, Zhejiang, 316021, China.

[4] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, 430072, China.

[5] Zhoushan Fisheries Research Institute of Zhejiang, Zhoushan, Zhejiang, 316000, China.

Qiang Lu and Yuan Zou contributed equally to this work.

*Corresponding author. Phone +8615088890110; email yuexing.zhang@zjou.edu.cn

Abstract

Actinobacteria and cyanobacteria accounted for less than 1% of total of bacteria in water in a recirculated aquaculture system (RAS) during a 15-week feeding trial with 0.11-g Japanese seabass. Resulting concentration of geosmin and 2-methylisoborneol (2-MIB) in RAS water were 169 and 45 ng L^{-1}, sufficient to produce strong off-flavor. The seabass were fed diets with 42, 45 and 49% protein, and each protein level was supplemented with 15 or 18% lipid. Accumulation of off-flavors was independent of diet in fatty ventral tissue. Dietary protein significantly reduced off-flavors in lean, dorsal tissue. This was mainly rationalized by linear reduction in 2-MIB in response to increasing DP/DE, and a strong, 2nd degree polynomial response in geosmin. The ratio between geosmin and 2-MIB was slightly higher at the beginning of a 10-day period with clean water and fasting, than what was observed throughout depuration. 2-MIB remained between 0.2 and 1 µg kg^{-1} in dorsal tissue throughout depuration. Geosmin in ventral tissue ranged from 10 to more than 30 µg kg^{-1}at the termination of the feeding period and was reduced to a range from 6 to 20 µg kg^{-1} by depuration.

KEYWORDS

geosmin, 2-methylisoborneol (2-MIB), depuration, *Lateolabrax japonicus*, RAS, DP:DE ratio

1 INTRODUCTION

Geosmin and 2-methylisoborneol (2-MIB) are the most widespread sources of muddy flavours in freshwater. Both are secondary metabolites produced by microorganisms (Jiang, He, & Cane, 2007). The employment of quantitative real-time PCR (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, Xu, Shao, Wang, & Li, 2011). Previous studies have shown that cyanobacteria (Schrader & Dennis, 2005; Wang et al., 2011) and actinobacteria (Lukassen, Saunders, Sindilariu, & Nielsen, 2017; Lylloff, Mogensen, Burford, Schlüter, & Jørgensen, 2012) are primary sources of geosmin and 2-MIB. Myxobacteria from the phylum proteobacteria also produce geosmin and 2-MIB and release them into the water (Jeroen S. Dickschat, Bode, Mahmud, Müller, & Schulz, 2005; Jeroen S Dickschat et al., 2007; Schulz, Fuhlendorff, & Reichenbach, 2004).

There are some disagreements in the literature about the solubility of Geosmin and 2-MIB, as Ikai et al. (2003) state that they are water soluble, while the general understanding is that they are lipid soluble (Tucker, 2000). They 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 stimulate fat accumulation in fish tissues (Ding et al., 2010; Luo, Xu, Teng, Ding, & Yan, 2010; Santinha, 1999), and are supposed to cause off-flavours in fish. Typically, the 2-MIB levels in channel catfish (*Ictalurus punctatus*) with high tissue fat content (> 2.5%) were three times higher than in lean fish (< 2%) (Peter B. Johnsen & Lloyd, 1992). Recirculated 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 sale value and are not well received in most markets. Thus, 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, Wolters, Schrader, & Summerfelt, 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 (Peter B. Johnsen & Lloyd, 1992; Peter B Johnsen, Lloyd, Vinyard, & Dionigi, 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, Nalinakumari, & Pei, 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, neither in rearing water, nor in the fish fillets (Schrader, Davidson, Rimando, & Summerfelt, 2010). Lindholm-Lehto and Vielma (2019) recently reviewed the challenges of controlling off-flavours in RAS. They 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 MATERIALS AND METHODS

Healthy Japanese seabass (initial weight, 0.11 kg) were placed into 18 tanks at the beginning of the experiment in RAS (30 fish per tank). Six extruded diets were formulated based on a 3×2 factorial design with three crude protein levels (420, 450, and 490 g kg^{-1}) and two crude lipid levels (150 and 180 g kg^{-1}); A constant level of Peruvian fish meal (225 g kg^{-1}) and Soybean meal (203 g kg^{-1}) were included in each diet with varied inclusions of soy protein concentrate (110-170 g kg^{-1}), wheat gluten (26-130 g kg^{-1}) and canola meal (79-132 g kg^{-1}) as sources of protein. A varied inclusion of marine fish oil (70-75 g kg^{-1}) and soy oil (31-67 g kg^{-1}) were used to provide the lipid. All the diets were extruded at the Feed Technology Laboratory of the Feed Research Institute, Chinese Academy of Agricultural Sciences in Beijing. All the dry ingredients were ground with a hammer mill through a 0.18-mm screen, mixed, preconditioned and extruded in a twin-screw extruder (MY56X2A, Muyang, Jiangsu, China) with 4.0-mm die plate. The extrusion process was optimized to obtain a bulk density > 420 g L^{-1} in the pellets before drying, in order to facilitate floating of the feed after drying and coating with lipid. The obtained extruded pellets were forced-

air dried to 950 g dry matter kg^{-1} at ambient temperature and then coated with oils with a ZJB-40 vacuum coater.

Table 1 shows the main chemical composition of these diets. Each diet was randomly assigned to three of eighteen tanks. Fish were hand-fed three times per day for a 15-week accumulation trial, and feed intake and uneaten pellets were recorded daily for the first 12 weeks. Feed intake was not assessed during the last 3 weeks, due to disturbing the fish by repeated stripping of the fish to obtain feces for digestibility assessment. At the beginning of the 16th week, a ten-day depuration period with starvation was initiated. Water and fish tissues were sampled for analysis at the end of the accumulation and depuration periods.

2.1 Recirculated aquaculture system, water quality and depuration period

The recirculated aquaculture system comprises 24 culture tanks (1 m^3 in volume) and water treatment units, supplied by Goldbill (Ningde, Fujian, China). The rearing water from the tanks was collected in a drum filter for the removal of particles, and water was disinfected by UV light. Water was then pumped from the drum filter into the biofilter. The biofilter provided substrate for nitrobacteria that nitrified ammonia. After bio-filtration, the water was pumped back to the culture tanks. The water flow was 8 to 9 L min^{-1} in each of the 24 tanks. The total volume of water in the system was approximately 24 m^3, and 12.5% of the water was replaced by freshwater every day.

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 tank. Water temperature was 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 clean water was aerated to minimize residual chloride before it was distributed to the fish.

2.2 Water sampling and fish tissue 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 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 were injected with PIT tags (Smartrac N.V., Amsterdam, Netherland) 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 for depuration. After 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 different sections of RAS, sealed into bottles, and stored at -80 °C.

2.3 Analysis of geosmin and 2-MIB in fish tissues and water

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 μLof 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 fiber 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 fiber 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 fiber

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 mL of water sample were 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 with120 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.

2.4 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 analyzed for fat content by SoxROC Extractor (SX-360, OPSIS AB, Furulund, Sweden).

2.5 Analysis of microbes in water

For bacterial analysis, water samples (300 mL) were filtered through 0.2 μm polycarbonate membranes. DNA extraction and quality verification, PCR amplification, high-throughput sequencing and bioinformatic analysis were performed refer to the previous study (Li et al., 2020).

2.5.1 DNA extraction, amplification, and library construction

Total genomic DNA was extracted using DNA PowerSoil Kit following the manufacturer's instructions. Quality and quantity of DNA was verified with NanoDrop 2000 and agarose gel. Extracted DNA was diluted to a concentration of 1 ng/μL and diluted DNA used as template for PCR amplification of bacterial 16S rRNA genes with the barcoded primers and Takara Ex Taq (Takara, Japan). The 16S rRNA genes were amplified using bacterial primer set 343F (5′ - TACGGRAGGCAGCAG)/798R (5 - AGGGTATCTAATCCT). An Illumina Sequencer MiSeq (Illumina Inc. San Diego, CA, USA) was used for high-throughput sequencing by OE Biotechnology Company (Shanghai, China).

2.5.2 Bioinformatic analysis

Raw paired-end reads were subjected to quality control procedures using Trimmomatic software (Bolger et al, 2014). After trimming, paired-end reads were assembled using FLASH software (Reyon et al, 2012). Clean reads were subjected to primer sequences removal and clustering to generate operational taxonomic units (OTUs) using Vsearch software with 97% similarity cutoff (Edgar, 2011), and chimeric sequences identified and removed using UCHIME (Edgar, 2016). The representative read of each OTU was selected using QIIME package. For the observed species, Shannon-Wiener index and Simpson's diversity index were calculated based on the OTUs.

2.6 Analysis of the ratio of digestible protein on digestible energy (DP:DE)

Feces was collected at the end of the study by careful stripping from the pectoral fin towards anus pooled by tank and frozen at - 20 °C until freeze dried to constant weight and homogenized by pestle and mortar. Gross energy in feed and feces was analyzed by bomb calorimeter (Parr-1271, Parr company, USA). Determination of yttrium oxide content in feed using ICP-MS inductively coupled plasma mass spectrometer (7900, Agilent, USA). Crude protein (CP) was assessed by an automated Kjeldahl analyzer (KD-310, OPSIS, Sweden). Gross energy was analyzed by bomb calorimetry (Parr-1271, Parr company, USA). Apparent digestibility coefficients of nitrogen and

gross energy were calculated as: 1- (Y_2O_3 in feed × nutrient or energy in feces) / (Y_2O_3 in feces × nutrient or energy in feed). DP:DE was calculated as g digestible CP / MJ digestible energy. DP:DE values for each of the 6 diets are shown in Table 1.

2.7 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).

2.8 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.

3 RESULTS AND DISCUSSION

3.1 Occurrence of microbes that synthesize components causing muddy flavour and oxidize nitrogen in the RAS water, and concentration of geosmin and 2-MIB in the rearing water.

The concentration of geosmin in water from the depuration tank rose from trace amounts (5.1 ng L^{-1}) at the onset of depuration to 45.9 ng L^{-1} at the end of the 10-day period. The water in the RAS tank with fish contained 169 ng geosmin L^{-1}, and the water in the drum filter contained 184 ng geosmin L^{-1}. Corresponding values for 2-MIB were 21.4 ng L^{-1} at the end of depuration, while the concentrations of 2-MIB were 45.4 ng L^{-1} in the RAS tank and 41.3 ng L^{-1} in the drum filter.

The diversity indices, as assessed by of high-throughput sequencing, revealed 698 microbial species in rearing tank water, and 729 species in the water of the drum filter. 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 both in the rearing tanks and in the drum filter, accounting for 54% of the total bacteria in both (Figure 1 a). Actinobacteria, accounting for 1.0 % of bacteria in the water of the drum filter and 1.2 % in the tanks with fish, as well as Cyanobacteria accounting for 0.03% in the drum filter and 0.05% in the RAS tanks are the most probable sources for geosmin and 2-MIB. These levels are consistent with previous observations that Actinobacteria and Cyanobacteria can cause intense off-flavours, even when these bacteria represent low (0.007-0.9%) proportions of the total bacteria (Lukassen et al., 2017). In Figure 1 (b), nitrobacteria were also assessed. *Nitrosomonas*, which oxidizes ammonia to nitrite, represented 0.7 %, in the drum filter and 1.1 % in the rearing tanks. Corresponding values for *Nitrospira*, which further oxidizes nitrite to nitrate, were 1.0 % in the drum filter and 1.5 % in the tanks with fish. Although the nitrate nitrogen does not directly affect the geosmin and 2-MIB levels in the water (Schrader, Davidson, & Summerfelt, 2013), low nitrogen level in the water might be an efficient way to control the growth of nitrogen-dependent actinobacteria and cyanobacteria (Cottingham, Ewing, Greer, Carey, & Weathers, 2015; Dai et al., 2018; Saadoun, Schrader, & Blevins, 2001). The observed differences indicate that the fish rearing tanks are prioritized water sampling points for controlling off-flavour in RAS.

3.2 Accumulation of geosmin and 2-MIB in dorsal and ventral tissues

At onset of the depuration, the lipid concentration in dorsal tissues was lean, only 19% of the lipid content in ventral tissue. The dorsal tissue also had lower concentration of 2-MIB (31% of that in ventral tissue) and geosmin (19%) (Table 2). Lipid concentration both in dorsal and ventral tissues were significantly related to dietary lipid concentration. No significant correlation between dietary protein or lipid concentrations and 2-MIB or geosmin were seen in ventral tissue, and no significant interactions were evident.

The main effect of dietary protein level on 2-MIB and geosmin concentration in dorsal tissue, however, was highly significant (Table 2). The concentration of 2-MIB in this tissue decreased from an average of 0.69 to 0.44 μg kg^{-1} when dietary protein level increased from 420 to 490 g

276 kg^{-1}. Similarly, the concentration of geosmin decreased from 5.54 to 2.96 µg kg^{-1}. This significant
277 response to protein, and corresponding lack of response to lipid in the feeds may be due to preferred
278 binding to lean tissues, reflecting the water-soluble features of 2-MIB and geosmin (Ikai et al.,
279 2003).
280
281 A more reasonable explanation is that the various diets provide different amounts of substrate for
282 microbial growth. The two main sources of nutrients for bacterial growth from crude protein are
283 indigestible nitrogenous components and endogenous nitrogen, both being excreted in feces. Also,
284 amino acids are deaminated and ammonia is excreted in situations where the essential amino acid
285 composition is imbalanced, or protein is in excess. The ratio between digestible protein and
286 digestible energy (DP:DE) is a good measure for the latter (Einen and Roem, 1997). DP:DE values
287 ranging from 20.6 to 25.6 g DP $(MJ\ DE)^{-1}$ were seen in the diets (Fig. 1).
288
289 The regressions of DP:DE on 2-MIB and geosmin in dorsal tissue are presented in Fig. 3. Only
290 values from this tissue are presented since the ventral tissues were not significantly affected by the
291 DP:DE ratio of the feeds. Increasing DP:DE was accompanied by a linear decrease on 2-MIB, with
292 moderately high determination (R^2 = 0.55). The concentration of geosmin also decreased when
293 DP:DE increased, and the response was best described (R^2 = 0.98) by a 2nd degree curvilinear
294 regression. The response was steep when increasing DP:DE from 20.6 to 24.3 g MJ^{-1}, while only
295 a marginal response was seen by a further increase in DP:DE to 25.6 g MJ^{-1}.
296
297 The DP:DE ratio was linearly and highly determined (R^2 = 0.90) by dietary crude protein
298 concentration (Table 3). Dietary ash and the analytical residue both accounted for an R^2 at 0.48,
299 while the determination of dietary lipid on DP:DE was only R^2 = 0.37. This indicates that dietary
300 protein played an important role in limiting accumulation of 2-MIB and geosmin in lean tissues
301 than lipids, minerals and various indigestible organic dietary components contributing to the
302 analytical residues. This finding should be subject to further investigation. The results were in
303 keeping with previous findings of a positive correlation between lipids in feed and tissues, and a
304 negative correlation between dietary protein and tissue lipids (Santinha, 1999). Intake of dietary
305 crude protein during the first 12 weeks of feeding on dorsal tissue 2-MIB was significantly (R^2 =
306 0.83) explained by a 2nd degree polynomial regression. Dietary gross energy intake was not

significantly ($R^2 = 0.05$) related to tissue 2-MIB concentration. Simultaneously, the determination of crude protein intake on dorsal tissue geosmin was moderate ($R^2 = 0.38$), while that of dietary energy intake was low ($R^2 = 0.23$).

The ratio of 2-MIB on geosmin was slightly higher at the beginning of the 10-day period with clean water and fasting, than after depuration (Fig. 4). This indicates that 2-MIB was removed from the tissues more efficiently than what was the case with geosmin, in keeping with the hypothesis of a previous review (Rurangwa & Verdegem, 2015).

As can be seen from Table 2 and the clusters in Fig. 5, the tissue lipid content affected the accumulation of both geosmin and 2-MIB. Prior to depuration, the lean dorsal tissue contained 7.6 µg kg^{-1} geosmin and 0.9 µg kg^{-1} 2-MIB, distributed within a narrow range. The concentration of geosmin in the fatty ventral tissue ranged from 11.1 to 35.3 µg kg^{-1}, while that of 2-MIB ranged from 0.8 to 2.7 µg kg^{-1}. Thus, the results in Table 2 and Fig. 5 (a and c) confirm that the fatty ventral tissue accumulated off-flavour components to a much higher degree than the lean dorsal tissue. ANOVA also revealed a significant ($P < 0.001$) difference in lipid, geosmin, and 2-MIB levels between dorsal and ventral tissues. This higher capacity of storing 2-MIB and geosmin in fatty tissues distribution of lipid and muddy flavours in fish is consistent with previous findings on barramundi (*Lates calcarifer*) (Percival, Drabsch, & Glencross, 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. 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 (*Oncorhynchus mykiss*) (Mikael A. Petersen, Hyldig, Strobel, Henriksen, & Jørgensen, 2011). The concentrations of geosmin and 2-MIB in the rearing water seemed to be a 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, Poole, Kirchhoff, & Forde, 2009).

3.3 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 hours of treatment (Peter B. Johnsen & Lloyd, 1992; Robertson, Jauncey, Beveridge, & Lawton, 2005; Rohani, Normah, Zahrah, Utama, & Saadiah, 2009). For example, the concentrations of muddy flavours can decrease to below the detection threshold after 7 days (Mikael A. 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 18.7 μg kg^{-1}, while that of the dorsal tissue had values between 0.6 and 7.4 μg kg^{-1} (Figure 3 b and 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 was between 0.2 and 0.9 μg kg^{-1}. The clusters of geosmin were clearly separated from those of 2-MIB before depuration. After depuration these clusters were overlapping. This was mainly due to higher concentration of geosmin than that of 2-MIB. Reduction in the ventral clusters was the highest. The concentration of both geosmin and 2-MIB in ventral tissue decreased by nearly 50%). This indicates that rate of depuration may be dose dependent, with higher rate of removal with high tissue concentration. This result is 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 (*Salmo salar*) (Davidson et al., 2014), although Rurangwa and Verdegem (2015) suggested that the depuration rate of geosmin should be slower than that of 2-MIB. Clearance of both geosmin and 2-MIB during depuration was slower in the lean dorsal than in the fatty ventral tissue. This indicates that the rate of depuration is more efficient from fatty than lean tissues.

Both geosmin and 2-MIB were initially found at only low levels in the depuration pond water (Figure 2), and the concentrations of both were significantly increased when the depuration period was completed. The combination of fasting fish and adding fresh water 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. 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, Lloyd, & Zimba, 2004). Previous experiments on fatty fish, such as Arctic charr

(*Salvelinus alpinus*) (Houle et al., 2011), also indicated that high lipid content in the fish complicates purging if only fresh water is employed. When Davidson (2014) tried disinfecting the depuration tank with hydrogen peroxide (H_2O_2) prior to purging fish, a rapid reduction of geosmin and 2-MIB levels in Atlantic salmon was observed. This indicates that the pre-treatment of depuration tanks or water with environmentally friendly oxidants might be useful to increase the efficiency of purging of fatty fish when by the combined use of fasting and clean freshwater.

4 CONCLUSIONS

Japanese seabass, kept in a Recirculated Aquaculture System (RAS) were fed 6 diets with different contents of protein and lipid for 15 weeks. Accumulation of the muddy flavor components geosmin and 2-MIB in was decreased by increasing ratio of digestible protein on digestible energy (DP:DE). The decrease was linear for 2-MIB and curvilinear for geosmin with reduced accumulation by increasing the CP:DE ratio from 20.6 to 24.3 g DP per MJ DE. Understanding the underlaying mechanisms for the dependence of high DP:DE to reduce tissue accumulation of geosmin and 2-MIB requires more research. Both geosmin and 2-MIB were accumulated at higher rates in fatty ventral tissues than in lean dorsal tissues. Ten days purging by keeping the fish in freshwater and fasting them resulted in similar rates of removal of geosmin and 2-MIB. This depuration was not sufficient to produce high-quality fish, and this purging procedure should be combined with other means to reduce geosmin and 2-MIB-producing *Actinobacteria* and *Cyanobacteria* in water of the fish rearing tanks of the RAS.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (Grant No: 31502182), National Key R&D Program of China (Grant No:2019YFD0900203), Provincial Key Research and Development Program of Zhejiang (Grant No: 2019C02048), and Public Welfare Technical Applied Research Project of Zhejiang Province (LGN19C190001).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Qiang Lu and Yuan Zou were involved in data analysis, and manuscript writing. Yuexing Zhang designed the experiment, obtained funding and was involved in writing and manuscript revisions. Liying Huang, Haokun Liu and Xiaolong Yin provided essential reagents and materials, and were involved in manuscript revisions. Qiang Lu, Yuan Zou and Zhiyong Dong conducted the experiment. Trond Storebakken was involved in design, manuscript writing and revisions.

DATA AVAILABILITY STATEMENT

The authors confirmed that the data supporting the results in this study are presented in tables and figures in this published article.

REFERENCES

Burr, G. S., Wolters, W. R., Schrader, K. K., & Summerfelt, S. T. (2012). Impact of depuration of earthy-musty off-flavors on fillet quality of Atlantic salmon, *Salmo salar*, cultured in a recirculating aquaculture system. *Aquacultural Engineering, 50*, 28-36. https://doi.org/10.1016/j.aquaeng.2012.03.002

Cottingham, K. L., Ewing, H. A., Greer, M. L., Carey, C. C., & Weathers, K. C. (2015). Cyanobacteria as biological drivers of lake nitrogen and phosphorus cycling. *Ecosphere, 6*(1), art1. https://doi.org/10.1890/ES14-00174.1

Dai, Z., Su, W., Chen, H., Barberán, A., Zhao, H., Yu, M., . . . Xu, J. (2018). Long-term nitrogen fertilization decreases bacterial diversity and favors the growth of Actinobacteria and Proteobacteria in agro-ecosystems across the globe. *Global Change Biology, 24*(8), 3452-3461. https://doi.org/10.1111/gcb.14163

Davidson, J., Schrader, K., Ruan, E., Swift, B., Aalhus, J., Juarez, M., . . . Summerfelt, S. T. (2014). Evaluation of depuration procedures to mitigate the off-flavor compounds geosmin and 2-methylisoborneol from Atlantic salmon Salmo salar raised to market-size in recirculating aquaculture systems. *Aquacultural Engineering, 61*, 27-34. https://doi.org/10.1016/j.aquaeng.2014.05.006

Dickschat, J. S., Bode, H. B., Mahmud, T., Müller, R., & Schulz, S. (2005). A novel type of geosmin biosynthesis in myxobacteria. *The Journal of Organic Chemistry, 70*(13), 5174-5182. https://doi.org/10.1021/jo050449g

Dickschat, J. S., Nawrath, T., Thiel, V., Kunze, B., Müller, R., & Schulz, S. (2007). Biosynthesis of the off-flavor 2-Methylisoborneol by the myxobacterium nannocystis exedens. *Angewandte Chemie International Edition, 46*(43), 8287-8290. https://doi.org/10.1002/anie.200702496

Ding, L., Zhang, L., Wang, J., Ma, J., Meng, X., Duan, P., . . . Sun, Y. (2010). Effect of dietary lipid level on the growth performance, feed utilization, body composition and blood chemistry of juvenile starry flounder (*Platichthys stellatus*). *Aquaculture Research, 41*(10), 1470-1478. https://doi.org/10.1111/j.1365-2109.2009.02440.x

Einen, O. & Roem, A.J. (1997) Dietary protein:energy ratios for Atlantic salmon in relation to fish size, feed utilization and slaughter quality. *Aquaculture Nutrition*, 3, 115-126. https://doi.org/10.1046/j.1365-2095.1997.00084.x

Frank, D., Poole, S., Kirchhoff, S., & Forde, C. (2009). Investigation of sensory and volatile characteristics of farmed and wild barramundi (*Lates calcarifer*) using gas chromatography−olfactometry mass spectrometry and descriptive sensory analysis. *Journal of Agricultural and Food Chemistry, 57*(21), 10302-10312. https://doi.org/10.1021/jf902030y

González, S., Flick, G. J., O'Keefe, S. F., Duncan, S. E., McLean, E., & Craig, S. R. (2006). Composition of farmed and wild yellow perch (*Perca flavescens*). *Journal of Food Composition and Analysis, 19*(6), 720-726. https://doi.org/10.1016/j.jfca.2006.01.007

Grimm, C. C., Lloyd, S. W., & Zimba, P. V. (2004). Instrumental versus sensory detection of off-flavors in farm-raised channel catfish. *Aquaculture, 236*(1), 309-319. https://doi.org/10.1016/j.aquaculture.2004.02.020

Houle, S., Schrader, K. K., Le François, N. R., Comeau, Y., Kharoune, M., Summerfelt, S. T., . . . Vandenberg, G. W. (2011). Geosmin causes off-flavour in arctic charr in recirculating aquaculture systems. *Aquaculture Research, 42*(3), 360-365. https://doi.org/10.1111/j.1365-

2109.2010.02630.x
Howgate, P. (2004). Tainting of farmed fish by geosmin and 2-methyl-iso-borneol: a review of sensory aspects and of uptake/depuration. *Aquaculture, 234*(1), 155-181. https://doi.org/10.1016/j.aquaculture.2003.09.032
Ikai, Y., Honda, S., Yamada, N., Onuma, S., Tomita, B., Kawamura, N. & Miyazaki, Y. (2003). Determination of geosmin and 2-methylisoborneol in water using solid phase extaction and headspace-GC/MS. *Journal of the Mass Spectrometry Society of Japan*, 51 (1), 174-178. https://doi.org/10.5702/massspec.51.174
Jiang, J., He, X., & Cane, D. E. (2007). Biosynthesis of the earthy odorant geosmin by a bifunctional Streptomyces coelicolor enzyme. *Nature Chemical Biology, 3*(11), 711-715. https://doi.org/10.1038/nchembio.2007.29
Johnsen, P. B., & Lloyd, S. W. (1992). Influence of fat content on uptake and depuration of the off-flavor 2-Methylisoborneol by channel catfish (*Ictalurus punctatus*). *Canadian Journal of Fisheries and Aquatic Sciences, 49*(11), 2406-2411. https://doi.org/10.1139/f92-266
Johnsen, P. B., Lloyd, S. W., Vinyard, B. T., & Dionigi, C. P. (1996). Effects of temperature on the uptake and depuration of 2 - methylisoborneol (MIB) in channel catfish *Ictalurus punctatus*. *Journal of the World Aquaculture Society, 27*(1), 15-20. https://doi.org/10.1111/j.1749-7345.1996.tb00589.x
Li, M., Mi, T., Yu, Z., Ma, M., Zhen, Y. (2020). Planktonic bacterial and archaeal communities in an artificially irrigated estuarine wetland: diversity, distribution, and responses to environmental parameters. *Microorganisms*, 8(2), 198. https://doi.org/10.3390/microorganisms8020198
Lindholm-Lehto, P. C., & Vielma, J. (2019). Controlling of geosmin and 2-methylisoborneol induced off-flavours in recirculating aquaculture system farmed fish—A review. *Aquaculture Research, 50*(1), 9-28. https://doi.org/10.1111/are.13881
Lukassen, M. B. Off-flavour producing bacteria in aquaculture. Aalborg Universitetsforlag, 2017. 53 p. (Ph.d.-serien for Det Ingeniør- og Naturvidenskabelige Fakultet, Aalborg Universitet). https://doi.org/10.5278/vbn.phd.eng.00015
Lukassen, M. B., Saunders, A. M., Sindilariu, P. D., & Nielsen, J. L. (2017). Quantification of novel geosmin-producing bacteria in aquaculture systems. *Aquaculture, 479*, 304-310. https://doi.org/10.1016/j.aquaculture.2017.06.004
Luo, G., Xu, J., Teng, Y., Ding, C., & Yan, B. (2010). Effects of dietary lipid levels on the growth, digestive enzyme, feed utilization and fatty acid composition of Japanese sea bass (*Lateolabrax japonicus* L.) reared in freshwater. *Aquaculture Research, 41*(2), 210-219. https://doi.org/10.1111/j.1365-2109.2009.02319.x
Lylloff, J. E., Mogensen, M. H., Burford, M. A., Schlüter, L., & Jørgensen, N. O. G. (2012). Detection of aquatic streptomycetes by quantitative PCR for prediction of taste-and-odour episodes in water reservoirs. *Journal of Water Supply: Research and Technology-Aqua, 61*(5), 272-282. https://doi.org/10.2166/aqua.2012.006
Martins, P., Cleary, D. F., Pires, A. C., Rodrigues, A. M., Quintino, V., Calado, R., Gomes, N. C. (2013). Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for Scophthalmus maximus and Solea senegalensis. *PloS one*, 8(11), e80847. https://doi.org/10.1371/journal.pone.0080847
Percival, S., Drabsch, P., & Glencross, B. (2008). Determining factors affecting muddy-flavour taint in farmed barramundi, *Lates calcarifer*. *Aquaculture, 284*(1), 136-143. https://doi.org/10.1016/j.aquaculture.2008.07.056

Petersen, M. A., Hyldig, G., Strobel, B. W., Henriksen, N. H., & Jørgensen, N. O. G. (2011). Chemical and sensory quantification of geosmin and 2-Methylisoborneol in rainbow trout (*Oncorhynchus mykiss*) from recirculated aquacultures in relation to concentrations in basin water. *Journal of Agricultural and Food Chemistry, 59*(23), 12561-12568. https://doi.org/10.1021/jf2033494

Reyon, D., Tsai, S. Q., Khayter, C., Foden, J. A., Sander, J. D., Joung, J. K. (2012). FLASH assembly of TALENs for high-throughput genome editing. *Nature biotechnology*, 30(5), 460-465. https://doi.org/10.1038/nbt.2170

Robertson, R. F., Jauncey, K., Beveridge, M. C. M., & Lawton, L. A. (2005). Depuration rates and the sensory threshold concentration of geosmin responsible for earthy-musty taint in rainbow trout, *Onchorhynchus mykiss*. *Aquaculture, 245*(1), 89-99. https://doi.org/10.1016/j.aquaculture.2004.11.045

Rohani, A. C., Normah, O., Zahrah, T., Utama, C. C., & Saadiah, I. (2009). Quality of fish fillet from pond-raised red tilapia and its utilisation in the development of value-added product. *J. Trop. Agric. and Fd. Sc, 37*(2), 153-161.

Rurangwa, E., & Verdegem, M. C. J. (2015). Microorganisms in recirculating aquaculture systems and their management. *Reviews in Aquaculture, 7*(2), 117-130. https://doi.org/10.1111/raq.12057

Saadoun, I. M. K., Schrader, K. K., & Blevins, W. T. (2001). Environmental and nutritional factors affecting geosmin synthesis by Anabaena SP. *Water Research, 35*(5), 1209-1218. https://doi.org/10.1016/S0043-1354(00)00381-X

Santinha, P. J. M. (1999). Effects of the dietary protein : lipid ratio on growth and nutrient utilization in gilthead seabream (*Sparus aurata* L.). *Aquaculture nutrition, v. 5*(no. 3), pp. 147-156-1999 v.1995 no.1993. https://doi.org/10.1046/j.1365-2095.1999.00107.x

Schrader, K. K., Davidson, J. W., Rimando, A. M., & Summerfelt, S. T. (2010). Evaluation of ozonation on levels of the off-flavor compounds geosmin and 2-methylisoborneol in water and rainbow trout *Oncorhynchus mykiss* from recirculating aquaculture systems. *Aquacultural Engineering, 43*(2), 46-50. https://doi.org/10.1016/j.aquaeng.2010.05.003

Schrader, K. K., Davidson, J. W., & Summerfelt, S. T. (2013). Evaluation of the impact of nitrate-nitrogen levels in recirculating aquaculture systems on concentrations of the off-flavor compounds geosmin and 2-methylisoborneol in water and rainbow trout (*Oncorhynchus mykiss*). *Aquacultural Engineering, 57*, 126-130. https://doi.org/10.1016/j.aquaeng.2013.07.002

Schrader, K. K., & Dennis, M. E. (2005). Cyanobacteria and earthy/musty compounds found in commercial catfish (*Ictalurus punctatus*) ponds in the Mississippi Delta and Mississippi–Alabama Blackland Prairie. *Water Research, 39*(13), 2807-2814. https://doi.org/10.1016/j.watres.2005.04.044

Schulz, S., Fuhlendorff, J., & Reichenbach, H. (2004). Identification and synthesis of volatiles released by the myxobacterium *Chondromyces crocatus*. *Tetrahedron, 60*(17), 3863-3872. https://doi.org/10.1016/j.tet.2004.03.005

Suurnäkki, S., Gomez-Saez, G. V., Rantala-Ylinen, A., Jokela, J., Fewer, D. P., & Sivonen, K. (2015). Identification of geosmin and 2-methylisoborneol in cyanobacteria and molecular detection methods for the producers of these compounds. *Water Research, 68*, 56-66. https://doi.org/10.1016/j.watres.2014.09.037

Tucker, C. S. (2000). Off-flavor problems in aquaculture. *Reviews in Fisheries Science, 8*(1), 45-88. https://doi.org/10.1080/10641260091129170

Wang, Z., Xu, Y., Shao, J., Wang, J., & Li, R. (2011). Genes associated with 2-Methylisoborneol biosynthesis in cyanobacteria: isolation, characterization, and expression in response to light. *PLOS ONE, 6*(4), e18665. https://doi.org/10.1371/journal.pone.0018665

Westerhoff, P., Nalinakumari, B., & Pei, P. (2006). Kinetics of MIB and geosmin oxidation during ozonation. *Ozone: Science & Engineering, 28*(5), 277-286. https://doi.org/10.1080/01919510600892836

Zhang, T., Xue, Y., Li, Z., Wang, Y., Yang, W., & Xue, C. (2016). Effects of ozone on the removal of geosmin and the physicochemical properties of fish meat from bighead carp (*Hypophthalmichthys nobilis*). *Innovative Food Science & Emerging Technologies, 34*, 16-23. https://doi.org/10.1016/j.ifset.2016.01.001

558 TABLE 1 Main chemical compositions of the experimental diets (in dry matter)
559

Proximate Composition, kg^{-1}	Diet no. (Protein/Lipid) D1 42/15	D2 42/18	D3 45/15	D4 45/18	D5 49/15	D6 49/18
Dry matter, g	924	933	934	931	926	928
Crude protein, g	423	411	458	452	494	496
Crude lipid, g	152	185	154	187	141	174
Ash, g	73	71	72	72	74	72
Gross energy, MJ	22.4	23.1	22.5	23.0	22.5	23.2
DP/DE[1], g MJ^{-1}	22.0	20.6	23.7	22.6	25.6	24.3

560 [1]DP/DE is ratio of digestible protein on digestible energy.

TABLE 2 The 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 (42/15)	13.5^{bc}	0.67^{ab}	4.76^{b}	70.3	1.71	19.1
D2 (42/18)	20.8^{a}	0.73^{a}	6.58^{a}	72.6	1.70	20.3
D3 (45/15)	12.4^{bc}	0.55^{bc}	3.03^{c}	55.6	2.45	29.8
D4 (45/18)	14.3^{b}	0.42^{c}	3.47^{c}	80.6	1.76	21.1
D5 (49/15)	10.1^{c}	0.47^{c}	3.08^{c}	46.9	1.84	17.4
D6 (49/18)	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	12.21	0.36	4.08
Two-way ANOVA						
P-value						
Protein	**< 0.01**	**< 0.01**	**< 0.001**	0.62	0.49	0.16
Lipid	**< 0.05**	0.26	0.30	**< 0.05**	0.30	0.65
Protein * Lipid	0.23	0.46	0.16	0.37	0.74	0.35
Main effect of crude 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

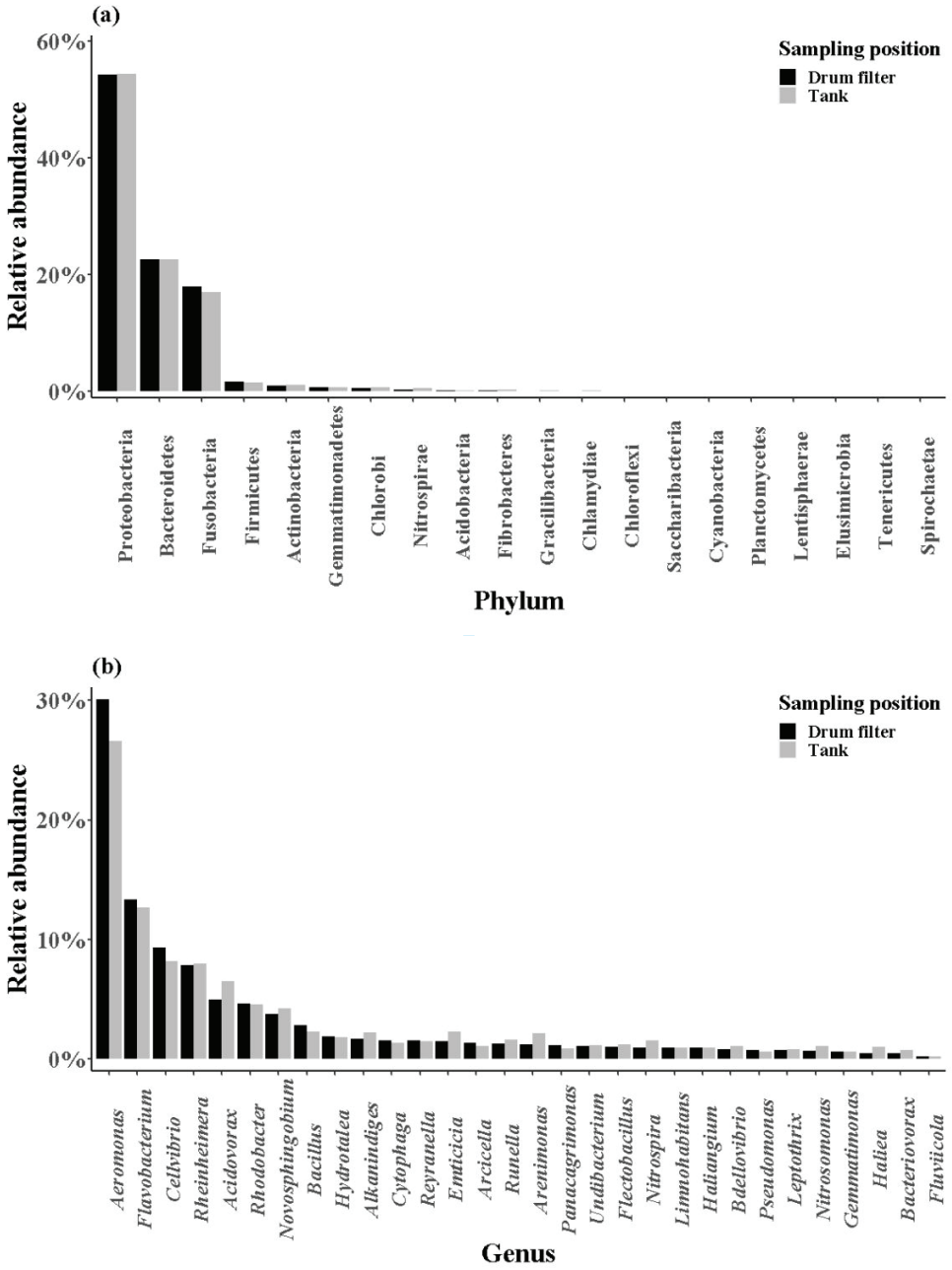
[1]Means of three replicate tanks. Means in each column with different superscripts are significantly different ($P < 0.05$). [2]s.e.m., standard error of means. [3]420, 450, and 490 g kg^{-1} of dietary crude protein. [4]150 and 180 g kg^{-1} of dietary lipid.

568 TABLE 3 Regression analysis of dietary macronutrient concentrations or analytical residue on
569 DP:DE ratio
570

Component, x	Regression, DP:DE=	R^2
Crude protein (CP)	22.4 + 18.7x	0.895
Lipid	32.4 - 0.056x	0.37
Ash	62.7 + 1.19x	0.48
Residue[1]	31.7 - 0.036x	0.48

571
572 [1] Dry matter – (CP + lipid + ash)
573

574



575

576

577 FIGURE 1 Relative abundance of bacteria community structures of phylum (a) and genus (b) in
578 water sampled from the drum filter (black bars) and in the raising tanks (grey bars) with fed fish.
579

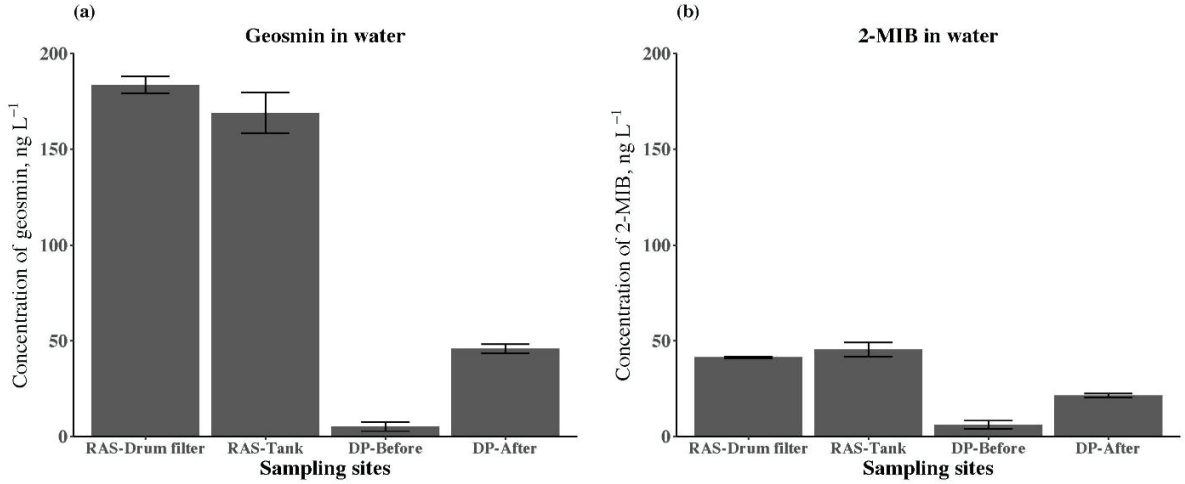


FIGURE 2 Concentrations of geosmin and 2-MIB in the water. DP-Before and DP-After show water from depuration tank before onset of the depuration period and after the depuration period, respectively. Data presented as mean ± SD

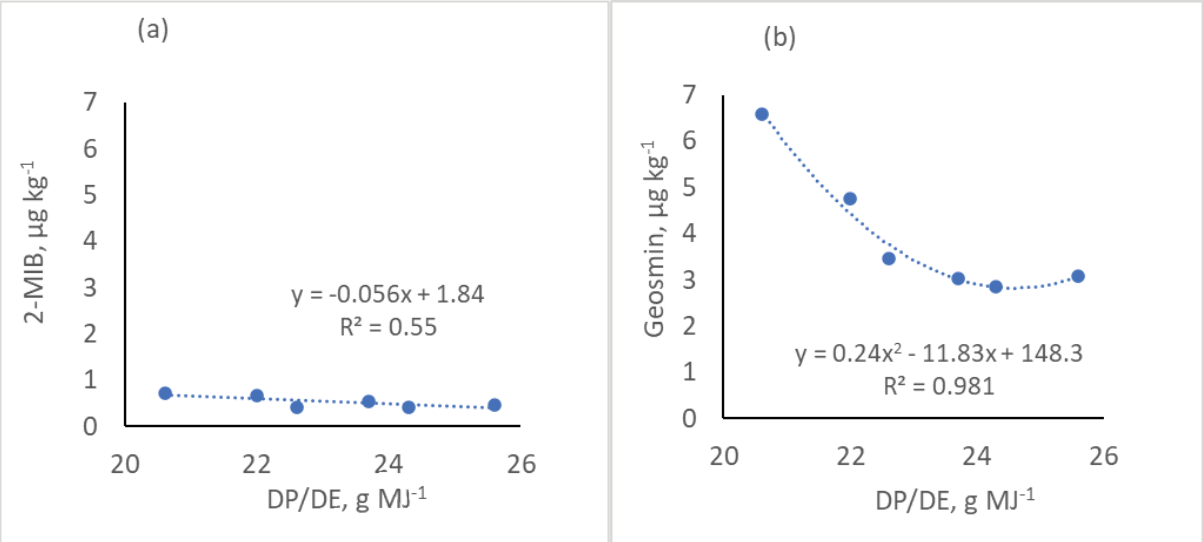
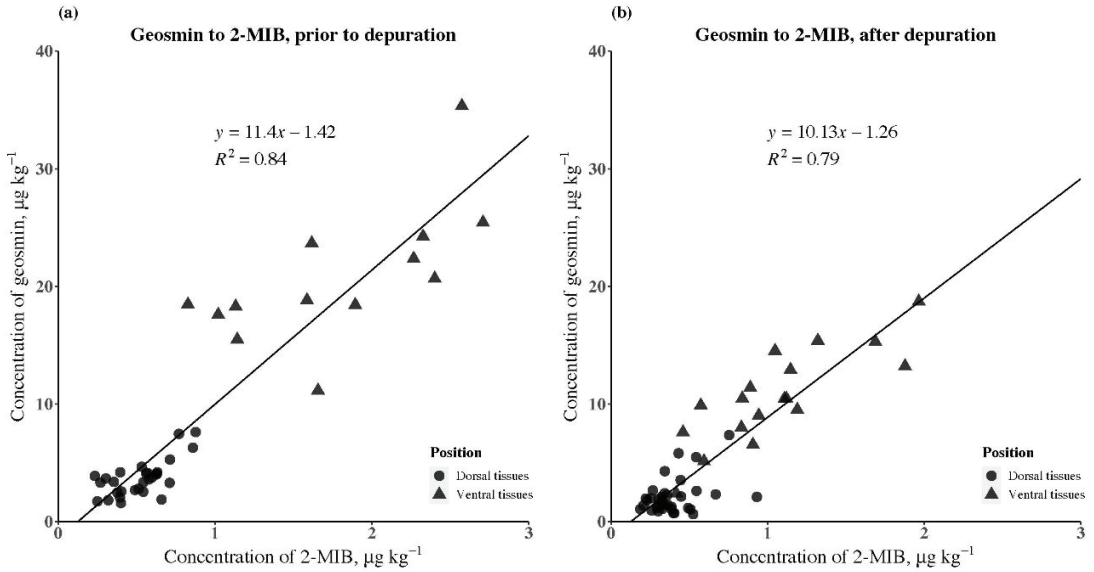


FIGURE 3 Regression analysis of the ratio between digestible protein and digestible energy (DP/DE) on the concentration of 2-MIB (a) and geosmin (b) in dorsal tissues of Japanese seabass.

591



592
593

594 FIGURE 4 Scatter plots of geosmin related to 2-MIB in Japanese seabass, before (a) and after (b)
595 depuration.

596

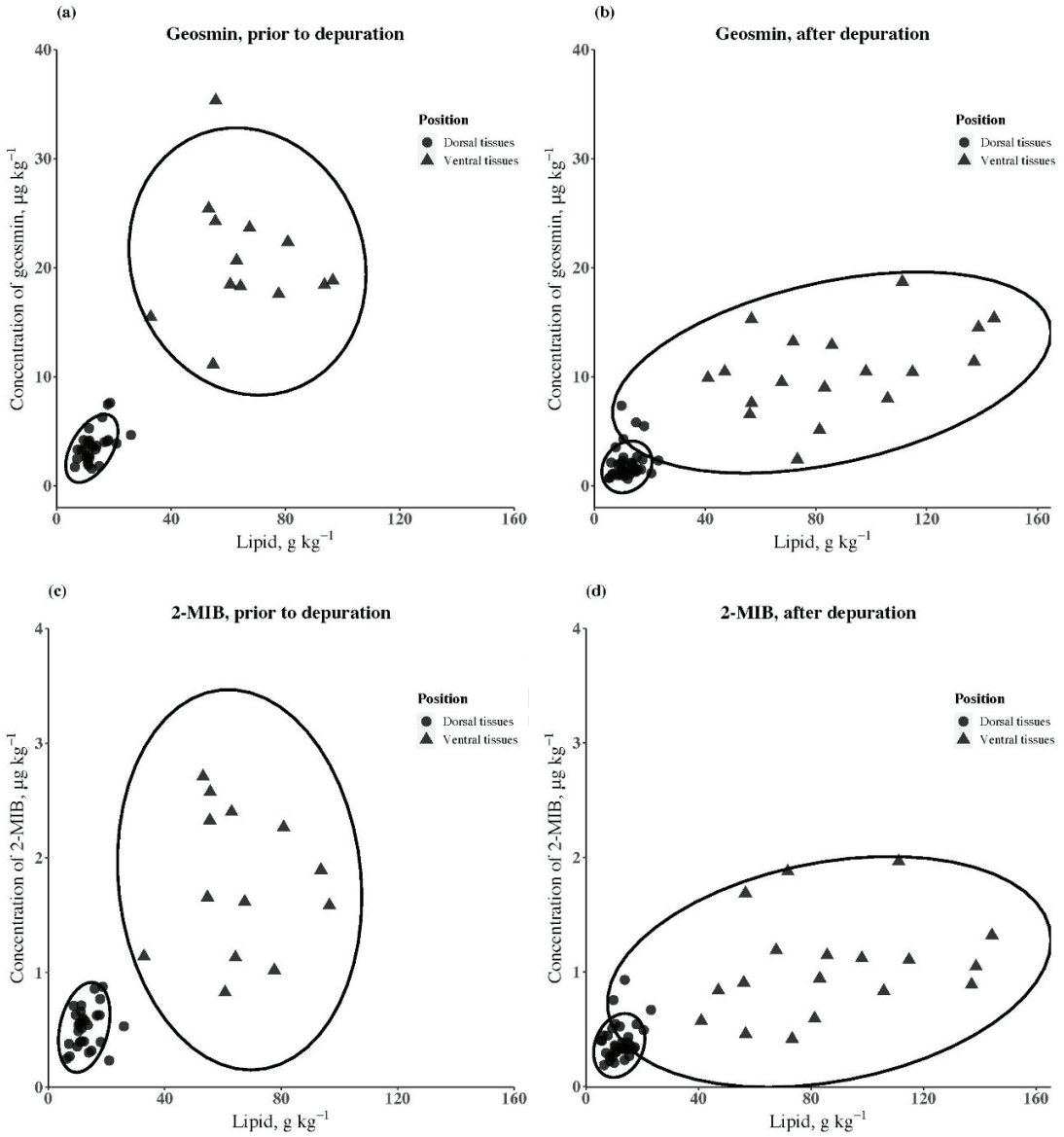


FIGURE 5 Scatter plots of geosmin and 2-MIB related to lipids in fish