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Development of a liquid chromatography atmospheric pressure chemical ionization mass spectrometry method for determining off-flavor compounds and its application toward marine recirculating aquaculture system monitoring and evaluation of aeration as a depuration approach

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ABSTRACT

The off-flavor compounds geosmin and 2-methylisoborneol (2-MIB) are well-known to impact the quality of farmed freshwater fish species, but little is known about off-flavors in marine aquaculture. To begin addressing this knowledge gap, a method for determining geosmin and 2-MIB using LC with atmospheric pressure chemical ionization (APCI) MS detection was developed. While 2-MIB was readily detected using LC-APCI/MS, geosmin exhibited on-column degradation that was independent of column chemistry and could not be eliminated. Optimized conditions were identified that balanced the separation and ionization efficiency of 2-MIB and geosmin while minimizing geosmin degradation, but the overall method sensitivity for geosmin was reduced by the on-column losses. The method was used with direct aqueous injections to determine the volatilization rates of geosmin and 2-MIB at ppb levels during aeration under laboratory conditions in both salt water and pure water to simulate marine and fresh water aquaculture, respectively. The volatilization rates of both compounds were 30% faster in salt water than in fresh water with or without aeration, but aeration was found to enhance the rate by a factor of 2.5 in both water types. The LC-APCI/MS method was combined with stir bar sorptive extraction (SBSE) to achieve greater sensitivity for determining off-flavors in recirculating aquaculture system (RAS) water. Using SBSE-LC-APCI/MS, the LODs for geosmin and 2-MIB were 70 ng/kg (part per trillion) and 6 ng/kg, respectively. The on-column losses resulted in a relatively high LOD for geosmin that renders this method unsuitable for determining geosmin at the low ng/kg levels expected in RAS. SBSE using both grab water samples and an in-situ diving unit were used to evaluate 2-MIB levels in the culture water of two separate marine RAS that were supporting the growth of European sea bass but had differing levels of water treatment. 2-MIB was readily detected using both SBSE approaches in the RAS with less sophisticated treatment when the animal stocking density was at its highest (50 kg/m^3) but was not detected in the more sophisticated RAS regardless of stocking density. Geosmin was not detected in either system, but the results were inconclusive given its higher LOD. These limited results suggest that the anaerobic water treatment components, present only in the more sophisticated RAS, maintained the level of 2-MIB below the LOD.

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1. Introduction

Farming is one of the earliest forms of biomanufacturing and is used to generate food products to meet critical dietary needs for carbohydrates, fats, and animal protein. While seafood is an important source of dietary protein, seafood production has lagged other types of farming and still relies heavily on nature to produce animals, which are then wild-caught. Traditional fishing approaches are unsustainable due to factors such as environmental stress, over-fishing, and the need to support growing human populations.

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Land-based recirculating aquaculture systems (RAS) exist in various configurations and are a promising technology for biomanufacturing fish protein in a sustainable way that minimizes water use and environmental impact from both chemical and biological sources. While RAS is commonly used for hatcheries, it is becoming increasingly used to grow fish from juvenile to market size because of its ability to support high stocking densities. Fish growth in RAS is due to control of food, temperature, and water quality, which is maintained through a multi-step process that can include treatment of ammonia, solids, CO_2, and other harmful byproducts [1–3]. When used with such sophisticated water treatment components, up to 99.5% of the water can be reused in RAS.

A major challenge for fish (products) raised in RAS is musty or 'earthy' off-flavors caused by geosmin and 2-methylisoborneol (2-MIB), which are secondary metabolites of certain algae and bacteria that proliferate in aerobic, organic-rich conditions [4]. Of the two compounds, 2-MIB is the primary off-flavor compound in RAS and is present in all aerobic treatment components [4], with concentrations frequently above odor threshold concentrations of low ng/L or ng/kg (parts per trillion) [5]. The presence of off-flavor compounds in fish meat can devalue the final product and negatively impact public perception of the fish-farming industry. While geosmin and 2-MIB have been studied in freshwater species cultured in RAS including trout [6–8] and salmon [9], little is known about off-flavor compounds in marine RAS species.

Fish manufacturers using RAS currently have limited options for removing off-flavors in their products. Since the amount of off-flavor compounds in the fish flesh is in equilibrium with the aqueous concentrations, the industry generally relies on the low-technology depuration process of placing fish in large volumes of clean water to promote off-flavor purging from the fish flesh prior to harvest [10]. This process is not sustainable due to the large amount of clean water required, the lack of measurement tools to monitor its effectiveness, as well as the costly time delays in getting products to market.

Depuration of off-flavor compounds in RAS using chemical disinfectants [11–13], sludge adsorption [14,15], or ultrasonically-induced cavitation [16] have also been reported. Of these approaches, disinfection with ozone is commonly used in RAS to treat organic carbon, turbidity and taste and odor compounds including geosmin and 2-MIB [12,13], but higher ozone doses required for effectiveness can cause mortality to cultured fish and to microbes in the biofilter [12].

Aeration is another potential depuration technology that can be cost-effective and relatively easy to implement in RAS. Aeration using a column packed with pristine, high surface area media was demonstrated to be effective in reducing off-flavors in fresh water in both laboratory studies and on- farm [17]. In another study in which aeration was evaluated in fresh water RAS as a stand-alone treatment with and without 'dirty' high surface area media, off-flavors were significantly reduced, but the reduction was less with the media due to biofilm formation [11]. While aeration offers promise, its effectiveness has not been demonstrated in marine aquaculture.

To address some of these identified knowledge gaps, we sought to understand and address the issue of off-flavor compounds in marine aquaculture using controlled laboratory experiments as well as measurements in marine RAS. Our first aim was to develop an analytical method centered on 2-MIB, the primary off-flavor compound in RAS, utilizing LC-MS. To the best of our knowledge, off-flavor compounds are most frequently determined with GC in combination with analyte preconcentration [18]. LC-MS methods can offer comparable sensitivity to GC methods but offer the advantage of being compatible with direct injection of aqueous samples, which was required to support our second aim. Our second aim was to gain a better understanding of aeration, without the use of high surface area media, as a potential technology for off-flavor depuration. We studied the rates of volatilization of geosmin and 2-MIB during aeration under controlled laboratory conditions in both salt water and pure water to simulate marine and fresh water aquaculture, respectively. Our final aim was to couple the developed LC-MS method with stir bar sorptive extraction (SBSE), a well-documented preconcentration approach [19–23], to determine off-flavor compounds in two separate RAS, a nursery system with aerobic water treatment, and a more sophisticated grow-out system with both aerobic and anerobic treatment loops. We utilized SBSE in grab water samples as well as a novel in-situ approach in which the stir bars were placed in a diving unit suspended in the RAS. The results from both SBSE approaches were compared for culture water samples collected from both the tank and the moving bed bioreactor, which was continuously vigorously aerated to move the floating media.

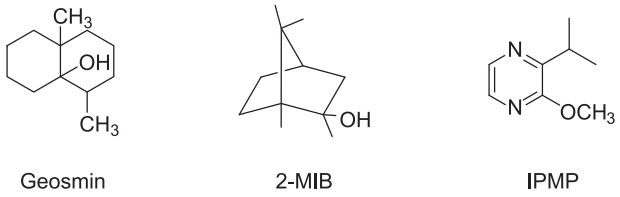

Fig. 1. Structures of the off-flavor compounds geosmin, 2-MIB, and IPMP (IS).

2. Materials and methods[1]

2.1. Reagents

Supelco TraceCERT Certified Reference Materials (CRMs) for (+/−) geosmin and 2-methylisoborneol (CRM47525) and 2-isopropyl-3-methoxypyrazine (IPMP; CRM47527-U) with nominal concentrations of 100 µg/mL in methanol were purchased from Millipore Sigma (St. Louis, MO, USA). IPMP was used as an internal standard based on a method reported by the U.S. Geological Survey [24]. The structures of 2-MIB, geosmin, and IPMP are presented in Fig. 1. Methanol and water (J.T. Baker Brand, HPLC grade) used to prepare solutions and chromatographic mobile phases were purchased from Avantor Performance Materials (Center Valley, PA, USA). Crystal Sea Marinemix was used to prepare simulated seawater and matrix-matched calibrants and was purchased from Marine Enterprises International, LLC. (Baltimore, MD, USA).

2.2. Optimized LC-APCI/MS method for 2-MIB and geosmin

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC with an SL series single quadrupole mass spectrometer and an atmospheric pressure chemical ionization (APCI) source was used to measure off-flavor compounds. The following optimized method conditions were used for the aquaculture monitoring and remediation technology studies. An Agilent Zorbax Eclipse Plus C18 rapid resolution HT (high-throughput) analytical column with 1.8 µm particles and dimensions of 4.6 mm × 50 mm was used. A mobile phase comprised of water (A) and methanol (B) flowing at 0.8 mL/min was used with the following gradient conditions: 0 min to 0.3 min, hold at 40% A, 60% B (v/v); 0.3 min to 6 min, linear gradient to 15% A, 85% B (v/v); 6 min to 10 min, hold at 15% A, 85% B (v/v). The column temperature was maintained at 45 °C, and an

[1] Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

injection volume of 15 µL was used. A post run time of 10 min was used between each injection to ensure equilibration to the initial conditions of the gradient.

The method utilized APCI/MS detection with positive polarity and stepped, selected ion monitoring (SIM) based on retention order as follows: *m/z* 153 for IPMP from 1.5 min to 4 min; *m/z* 151 for 2-MIB from 4 min to 6.4 min; and *m/z* 165 for geosmin from 6.4 min to 10 min. The LC flow was diverted to waste until 1.5 min to ensure salts from salt water samples were diverted to waste and not introduced to the mass spectrometer. For the detection of IPMP and 2-MIB, a gain setting of '1' on the electron multiplier was used, whereas for the geosmin segment, a gain of '10' was used to enhance detection. Other, optimized APCI/MS parameters for detection included: drying gas flow, 8.0 L/min; nebulizer pressure, 207 kPa (30 psi); drying gas temperature, 250 °C; vaporizer temperature, 500 °C; capillary voltage, +2000 V; corona current, 8 µA; and fragmentor, 50 V.

2.3. Aeration time-course experimental conditions

To evaluate aeration as a potential technology for off-flavor removal, the rate at which volatilization of geosmin and 2-MIB occurred in both pure laboratory water and in simulated seawater was studied under controlled laboratory conditions. To prepare the simulated seawater solution, 18 g of marine mix was added to 500 g of HPLC-grade water. The solution was sonicated and heated at 40 °C for 1 h, but not all components of the marine mix were soluble. While the gravimetric concentration was estimated to be 36 g/kg (parts per thousand), the value estimated with a portable handheld refractometer was 30 g/kg after the insoluble components settled overnight. The solution was stored at 4 °C when not in use to preserve freshness. An internal standard solution of nominally 0.62 µg/g IPMP was gravimetrically prepared by diluting 500 µL of the CRM solution to 100 mL with methanol. A stock solution of nominally 10 µg/g geosmin and 2-MIB was gravimetrically prepared by diluting 1 mL of the combined geosmin and 2-MIB CRM solution to 10 mL total with water. From the stock solution, matrix-matched working solutions were prepared gravimetrically with either HPLC-grade water or simulated seawater to be nominally 180 ng/g (parts per billion, ppb) geosmin and 2-MIB by diluting 750 µL of the stock solution to 50 mL. Matrix-matched calibrants for the LC-APCI/MS measurements were then prepared gravimetrically by weighing between 90 µL and 270 µL of the working solutions and 90 µL of the internal standard into a 2-mL vial with a 400 µL insert; the total volume for all calibrants was 360 µL, and the balance of the volume was comprised of water or simulated seawater. The internal standard was added to improve the reliability of the quantitation and to compensate for any potential changes to the solution such as evaporation. LC-APCI/MS measurements were calibrated using an average relative response factor (RRF) obtained using an internal standard approach to quantitation from the measurement of the pure water ($N = 2$) or simulated seawater calibrants ($N = 3$).

The aeration experiments for the pure water and saltwater conditions were conducted separately due to the labor-intensive protocol for sampling and the need to collect many data points early in the time- course. For both the pure water and saltwater conditions, duplicate samples were prepared for the aeration studies and for use as controls (i.e. not aerated). The aeration test samples and controls were prepared identically to the previously-described matrix-matched working solutions containing nominally 180 ppb geosmin and 2-MIB. The concentrations were chosen to balance between levels that were realistic in aquaculture systems versus what could be directly measured using the LC-APCI/MS method without a concentration step. Prior to starting the aeration experiments, $t = 0$ subsamples were gravimetrically prepared in a LC vial with a 400 µL insert by weighing 270 µL of the test aeration or control sample and 90 µL of the internal standard solution, vortex-mixing, and placing into the thermostatted autosampler for subsequent LC-APCI/MS analysis.

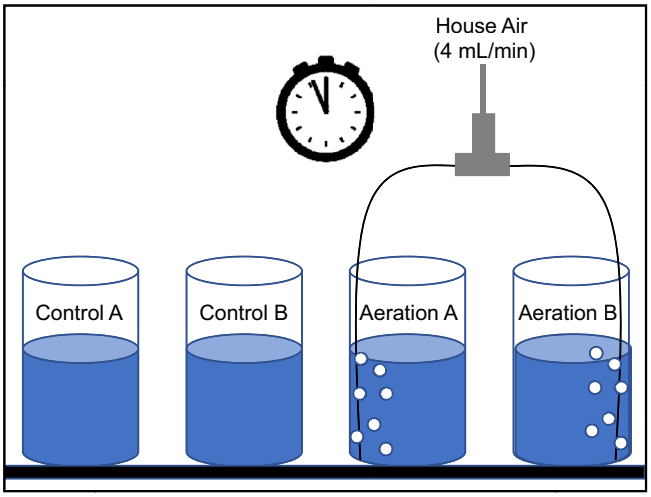

Fig. 2. Experimental set-up for aeration experiments of off-flavor compounds in pure water and simulated seawater.

The samples for aeration and the controls were then transferred to pre-weighed 100 mL beakers that were weighed again after transfer and covered with parafilm until the experiment was started. Samples were aerated using a single, house air line regulated to 700 kPa (100 psi) and split into two streams with a tee fitting and nylon tubing cut to equal lengths; one tubing line was placed in the bottom of each of the two beakers (Fig. 2). The timer for the experiment started when all four beakers were uncovered, and the air flow was started. The air flow was estimated as the rate at which water was displaced from a graduated cylinder filled with water that was inverted in a large crystallizing dish that was also partially filled with water. Using this approach, the air flow rate was measured to be approximately 4 mL/min for each of the two nylon air lines. The experiments were conducted at room temperature, or 23 °C.

The concentrations of geosmin and 2-MIB were monitored in each of the aerated and control samples over time by drawing a single, 270 µL subsample from each beaker approximately hourly at first, then at longer intervals later in the time course. At each sampling point, the exact time was recorded, and the masses of the subsample and the beaker containing the sample were recorded to correct the measured concentrations for volume losses due to subsample extractions and evaporation, respectively. The test subsamples were gravimetrically prepared as described previously for the $t = 0$ subsamples and were analyzed using the optimized LC-APCI/MS method with direct aqueous injections to determine the change in geosmin and 2-MIB concentrations over the course of the experiment. The time course was monitored until the concentrations dropped near the LOQ for geosmin (≈1 ppb), which corresponded to 24 h for the aerated samples (6 sampling points) and 48 h for the control (8 sampling points) for the pure water samples, and 7 h for the control and aerated salt water samples (5 sampling points) due to the faster observed volatilization rates.

2.4. Evaluation of SBSE-LC-APCI/MS performance

Ten (10) polydimethylsiloxane (PDMS)-coated Twister stir bars of four different geometries were used to perform stir bar sorptive extraction (SBSE) experiments and were purchased from Gerstel

(Linthicum, MD, USA). The stir bars had the following dimensions of film thickness x length (number), respectively: 0.5 mm × 10 mm (4), 1.0 mm × 10 mm (2), 0.5 mm × 20 mm (2), and 1.0 mm × 20 mm (2). The stir bars were conditioned prior to first use and after each subsequent use as follows: sonicated in water for 15 min; soaked in methanol for 2 h, sonicated for a final 15 min; dried overnight in a glass dish covered with foil; baked in a laboratory oven at 200 °C for a minimum of 2 h under nitrogen in an open-tube stainless steel column (dimensions 10 cm × 4.6 mm); cooled to room temperature under nitrogen; and transferred to sealed glass vials to prevent accumulation of airborne contaminants.

The performance of the stir bars for semi-quantitative measurements was evaluated prior to testing SBSE for monitoring in RAS. To determine if there was an effect of film length or thickness on extraction efficiency and reproducibility, the stir bars ($N = 1$ for each dimension) were tested by extracting equivalent concentrations of 2-MIB, geosmin, and IPMP (IS) in a pure water matrix (350 ng/kg) in capped vials while maintaining the same sample volume (10 mL), stirring time (2 h), and methanol back- extraction solvent volume (200 µL) for direct comparability of the results. Each stir bar back-extract was measured using the optimized LC-APCI/MS method, and RRFs based on an internal standard approach to quantitation were calculated and compared. The RSD of the RRFs calculated across all stir bar dimensions was 7% for both geosmin and 2-MIB. This good reproducibility was not surprising given the RRF is based on a ratio and does not reflect the absolute amounts of the off-flavors absorbed. However, it was observed that the peak areas for the off-flavor compounds and the internal standard were not dependent on stir bar length (2 cm vs. 1 cm) or on film thickness (0.5 mm vs. 1.0 mm) using this experimental procedure, which could be due to the stir rate, small sample volume, or constant extraction time of 2 h being insufficient to promote sorption into the longer or thicker films. Given that the RRF values were irrespective of stir bar dimension, the stir bars were used interchangeably for calibrants and RAS samples that were prepared using an analogous extraction procedure.

To estimate the method limit of quantitation (LOQ) and limit of detection (LOD), additional extractions of more dilute solutions of geosmin and 2-MIB (70 ng/kg) were performed and the results combined with those from the higher-level solutions (350 ng/kg). The LOQ ($S/N = 10$) and LOD ($S/N = 3$) were estimated to be 240 ng/kg and 70 ng/kg for geosmin and 20 ng/kg and 6 ng/kg for 2-MIB, respectively. The higher LOQ and LOD were expected for geosmin and indicate SBSE-LC-APCI/MS is not suitable for determining this compound at the sensitivity required for RAS analysis. However, 2-MIB is known to be the primary off-flavor compound in RAS, and the LOD of 6 ng/kg is sufficient for RAS monitoring but is slightly less than the ≈1 ng/kg LOD typically reported for GC–MS methods [18].

2.5. RAS configuration and culture conditions

An intensive grow-out trial of the commercially-important species European sea bass (*Dicentrarchus labrax*), commonly called 'Bronzini' in the US market, was conducted at the Aquaculture Research Center (ARC) within the Institute of Marine and Environmental Technology (IMET) in Baltimore, MD. All aspects of the grow-out were in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Juvenile sea bass were provided from the IMET in-house breeding program and were cultured over a period of approximately 12 months to market size (450 g). During the 12-month period, fish were cultured in two RAS that were best-suited to their size to ensure animal growth and health and are described below.

The nursery system (RAS 1) was used to culture fish from a mass of approximately 10 g to 50 g. RAS 1 contained a 4.1 m^3 culture tank (3.5 m^3 actual volume), a microscreen drum filter for solid removal, a foam fractionation unit for dissolved organics removal, a moving bed biofilter (MBB) for ammonia removal, and a total system volume of 4.5 m^3. The hydraulic retention time (HRT) of the tank water was maintained at 30 min, and the water was recirculated through the waste treatment components. The following tailored culture conditions were maintained: salinity, 15 g/kg; temperature, 26 °C; and photoperiod, 16 h of light.

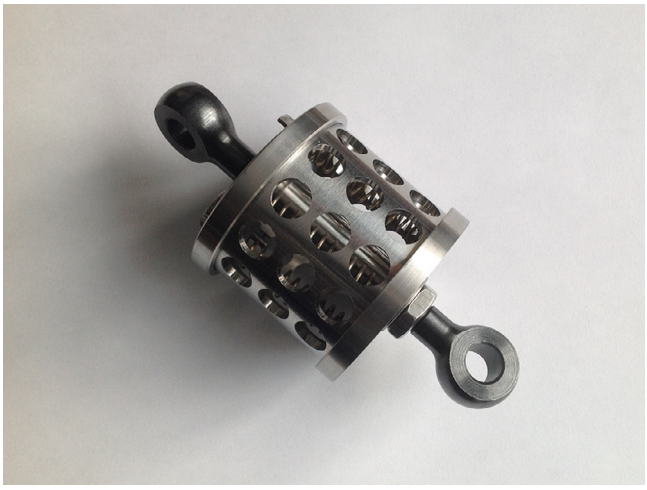

Fig. 3. Diving unit used for SBSE 2 procedure.

The intensive grow-out system (RAS 2) was used to culture fish from a mass of approximately 50 g to the final mass of 450 g. RAS 2 was a fully-contained, near zero-discharge system, comprised of two paired 12 m^3 culture tanks (11 m^3 actual volume) with two treatment loops. The aerobic waste treatment loop processed 99% of the system flow and contained a microscreen drum filter for solid removal, a foam fractionation unit for dissolved organics removal, a CO_2 stripper, a MBB for ammonia removal, and a low head oxygenator. The anaerobic loop processed 1% of the system flow and contained a sludge collection tank, a nitrate removing reactor, and an anaerobic digester for fish sludge removal. The total system volume was 41 m^3. The HRT of the tank water was 30 min, and the turnover rate was 48 times a day through the aerobic reactor loop. The tailored culture conditions for RAS 2 were identical to those for RAS 1.

2.6. SBSE conditions for RAS measurements

A diver unit specifically designed for holding up to four stir bars for in-situ SBSE experiments was also purchased from Gerstel (Fig. 3). The diver was conditioned prior to use by soaking in methanol for 2 h, followed by sonication for 15 min, air drying, and wrapping in aluminum foil.

The RAS were evaluated primarily for 2-MIB (given the high LOD for geosmin) using two SBSE approaches to extract and concentrate the low levels of off-flavor compounds expected in the culture water samples. For the first approach, SBSE 1, a diver unit containing up to 4 stir bars was suspended in either the RAS tank or MBB water for 2 h for in-situ, semi-quantitative determinations. Given the 30 min HRT in each RAS, there were approximately four turnovers of the system water during the extraction period. After 2 h, the stir bars were removed from the diver, rinsed with distilled water, and placed in a 2 mL vial with a 400 µL glass insert that was then capped and stored at −20 °C until back-extraction in the laboratory.

The second SBSE procedure was used for quantitative determinations. SBSE 2 utilized grab water samples from the RAS tank

and MBB water that were collected using 50 mL oven-cleaned, capped amber glass bottles, and stored at 4 °C until analysis. To analyze the grab water samples, 10 mL subsamples were weighed into capped receiving vials. For calibrating SBSE 2, matrix-matched working calibrants were prepared gravimetrically to mimic the grab water samples. First, a stock calibrant solution was prepared gravimetrically to be nominally 800 ng/kg of each 2-MIB and geosmin in HPLC- grade water. The working calibrants were then prepared by gravimetrically combining 5 mL of the stock calibrant and 5 mL of the 30 g/kg simulated seawater mix to yield a total volume of 10 mL, a salinity of 15 g/kg, and nominal concentrations of 2-MIB and geosmin of 400 ng/kg. One stir bar was placed in each grab water sample and working calibrant and stirred for 2 h. The stir bars were removed, rinsed with HPLC water, patted dry with a lint-free wipe, and placed in a 2 mL vial with a 400 µL insert to prepare for back-extraction.

The same process was used to back-extract the stir bars used for the in-situ samples, grab water samples, and working calibrants. A solution of 30 ng/g (ppb) IPMP in methanol was prepared gravimetrically for use as the combination internal standard and stir bar back-extraction solvent. Next, 200 µL of the IPMP solution was gravimetrically added to each 2 mL vial containing a stir bar. The stir bars were ultrasonically extracted for 15 min and removed from the vials with a magnet. The back-extracted samples were then analyzed using the optimized LC-APCI/MS method. Calibration was achieved using an internal standard approach to quantitation using RRFs calculated for each analyte. The RRFs were then used to calculate the absolute amount of 2-MIB in each RAS sample (ng) as well as the concentrations of the two analytes (ng/kg) in the grab water samples (where the sample mass was known).

3. Results and discussion

3.1. Development of an LC-APCI/MS method for geosmin and 2-MIB

The structures of geosmin and 2-MIB lack chromophores and are not determinable with absorbance detection at the required sensitivity, rendering MS as the most viable detection option. Preliminary experiments were performed with a triple quadrupole mass spectrometer and electrospray ionization to test detectability using a high-sensitivity, state of the art instrument. Using a mobile phase comprised of water, methanol, and formic acid and a scan range from *m/z* 125 to *m/z* 250 in both positive and negative modes, no detectable signals were obtained for either geosmin or 2-MIB, indicating that electrospray ionization is not suitable for these compounds. However, both molecules contain hydroxy functional groups that generally form ions due to water loss when APCI is used in positive mode with mass spectrometric detection. Therefore, method detection was centered around the use of positive-mode LC-APCI/MS.

Further experiments were performed on a single-quadrupole MS system due to the ease of tuning, parameter optimization, and direct ion monitoring for APCI/MS detection. A conventional octadecyl- silica (C18) column with 5 µm core-shell particles and dimensions of 150 mm × 4.6 mm, a methanol- water mobile phase flowing at 1.0 mL/min, a column temperature of 20 °C, and positive-mode scanning over the range from 125 *m/z* to 350 *m/z* were used initially. The peak for 2-MIB revealed an abundance of signal and a mass spectrum with the expected primary ion at *m/z* 151 corresponding to the loss of water molecule $[M_r - H_2O + H]^+$. Like 2-MIB, the internal standard (IPMP) exhibited facile ionization using APCI, but yielded a primary ion at *m/z* 153 representing $[M_r + H]^+$. Conversely, geosmin revealed two, low-abundance broad peaks, the spectra for which had a range of ions but predominant ions at *m/z* 163, which is not in agreement with the predicted ion due to water loss at *m/z* 165. Subsequent analysis of geosmin using selected ion monitoring (SIM) at *m/z* 163 revealed an array of closely-spaced peaks of modest intensity, whereas SIM at *m/z* 165 revealed a single peak of low intensity. The results suggested that geosmin was experiencing reactivity with the stationary phase leading to a distribution of detected products. It was speculated that a lower column temperature could reduce the apparent reactivity. However, when the experiment on the C18 column was repeated at a colder temperature of 6 °C, similar results were obtained.

To test if another column chemistry would yield different results, both the geosmin and 2-MIB solutions were analyzed using the LC-APCI/MS approach with a pentafluorophenylpropyl (PFP) stationary phase bonded to 2.7 µm core-shell particles (other dimensions same as C18). Both geosmin and 2-MIB exhibited similar behavior on the PFP column as on the C18 column: the geosmin had two broad, low-intensity peaks with mass spectra revealing a range of ions including the predominant *m/z* 163 ion, and 2-MIB had a single, high-intensity peak with primary ion at *m/z* 151. The chromatographic behavior of the two off-flavor compounds was irrespective of the two column chemistries investigated.

To confirm that the observations correlated with column interactions and not an issue with the chemical integrity of the CRM, the geosmin solution was analyzed using a conventional GC–MS method. The GC- MS analysis was performed using a capillary column with a 5%-phenyl-methylpolysiloxane bonded phase of 0.25 µm thickness and with dimensions of 15 m × 0.25 mm. The GC chromatogram of the geosmin solution revealed a single albeit broad peak that was expected given the relative polarity of the molecule for GC analysis. The mass spectrum was searched against the NIST mass-spectral library and was in alignment with geosmin or related isomers that have indistinguishable mass spectra. The 2-MIB was also analyzed using GC–MS for comparison and revealed a similar peak shape but had a definitive mass spectral hit with the NIST library.

Since the viability of the geosmin CRM solution was confirmed using GC–MS, a final experiment was conducted with flow injection analysis (FIA) using the LC-APCI/MS approach. The CRM solution of geosmin was injected, bypassing the column, and analyzed using APCI/MS in scan mode from *m/z* 150 to *m/z* 350. The resulting mass spectrum revealed only 2 peaks: a predominant peak at *m/z* 165, expected for the water loss ion, and a second smaller peak at *m/z* 166, representing the C^{13} analog. The FIA experiment confirmed that the solution of geosmin was a single-component of the proper identity (supporting the GC result), the expected chemical ionization behavior was due to loss of water, and geosmin was degrading through interactions with analytical columns, regardless of phase chemistry.

While FIA of the geosmin solution yielded the required detectability, the use of a chromatographic column is essential for the analysis of marine aquaculture matrix samples due to the presence of salts and other impurities that require separation. Subsequent method development involved mitigating on- column losses for geosmin using a short, high throughput column with a rapid gradient and optimizing MS detection for geosmin while balancing the detectability of 2-MIB and the internal standard, IPMP. The column selected was a C18 with 1.8 µm particles and dimensions of 4.6 mm × 50 mm. The methanol-water gradient was optimized for composition, flow rate, and column temperature to minimize geosmin retention while ensuring separation from 2-MIB and IPMP. Likewise, the APCI single quadrupole MS detection parameters were optimized for corona current, drying gas flow, drying gas temperature, fragmentor voltage, electron multiplier gain, nebulizer pressure, vaporizer temperature, and capillary voltage. The optimized instrumental parameters are those described in Section 2.2.

Once satisfactory detection was obtained using the single quadrupole MS system, the chromatography conditions were transferred back to the MS/MS instrument to compare the sensitivity of the two systems. Parameters were optimized to achieve the largest peak area for geosmin, and included: drying gas flow, 11 L/min; nebulizer pressure, 138 kPa (20 psi); drying gas temperature, 290 °C; vaporizer temperature, 500 °C; capillary voltage, +4500 V; corona current, 4 µA; and cell accelerator, 0 V. For detection, the MS/MS system was evaluated in both SIM mode using the same ions as for the single quadrupole method and multiple reaction monitoring (MRM) mode using the following transitions: IPMP *m/z* 153 → 138, collision energy (CE) 20; 2-MIB *m/z* 151 → 95, CE 10; and geosmin *m/z* 165 → 95, CE 10. For both modes, the delta electron multiplier voltage was set to +400 for geosmin to enhance the signal, whereas IPMP and 2-MIB required no adjustment. The SIM mode yielded higher peak areas and a better S/N than the MRM approach and was comparable to the performance on the single MS system. Given these results, all experiments were performed using the optimized conditions on the single MS system due to ease of use and the better flexibility for ion switching in the same chromatographic run. The performance of the method was determined when the separation was combined with the SBSE sample preparation and is described in Section 2.4.

3.2. Volatilization rates of geosmin and 2-MIB during aeration

The primary 'technology' for removing off-flavor compounds from fish meat in the aquaculture industry is placing fish in clean water for approximately a week to allow for depuration. This approach adds lengthy delays in getting the fish to market, in addition to being wasteful of freshwater resources. Other, more reliable and sustainable approaches are needed.

Aeration takes advantage of the inherent volatility of the off-flavor compounds and was evaluated as a potential technological solution to off-flavor removal. To test the viability of aeration, the rates of volatilization of geosmin and 2-MIB were evaluated at ppb levels in a controlled laboratory environment in both pure and salt water to simulate fresh and marine aquaculture conditions as described in the Experimental Section. The concentrations of geosmin and 2-MIB were determined over time using direct aqueous injections with LC-APCI/MS analysis that was calibrated with an internal standard method of quantitation. Chromatograms of a salt water calibrant solution containing geosmin, 2-MIB, and IPMP as well as a salt water sample that had been aerated for 7 h are presented in Fig. 4.

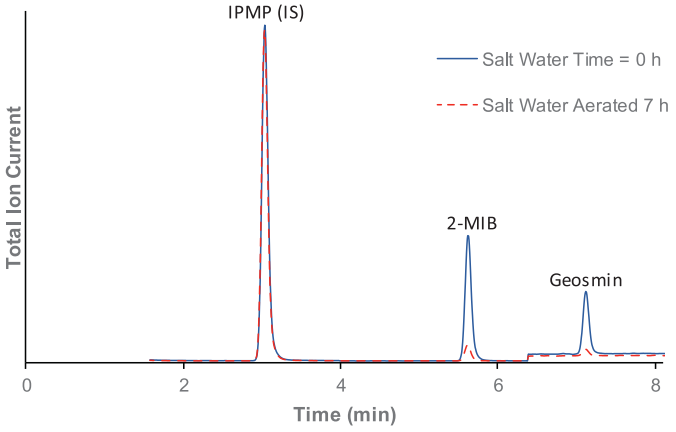

Fig. 4. Representative chromatograms using the optimized LC-APCI/MS method. Chromatograms of a calibrant and salt water sample 'a' after 7 h of aeration.

The concentrations of geosmin and 2-MIB in solution decreased over time during aeration and in the controls, as expected. To determine the rates of volatilization, the natural logarithm (ln) of the measured concentration for both the geosmin and the 2-MIB were plotted as a function of time. The graphical results for geosmin and 2-MIB in the aerated and control pure water samples are presented in Fig. 5. The experimental results are linear, indicating first-order processes for the evaporation of geosmin and 2-MIB in both the aerated and control samples. There is also reasonable overlap of the lines for the duplicate samples under each set of conditions, which is indicative of experimental reproducibility. The experimental half-life ($t_{1/2}$) for each sample was calculated as $t_{1/2} = \ln(2)/-k$, where k is the slope of the line. The half-life results are also presented in Fig. 5. The results show remarkably similar results for geosmin and 2-MIB, with $t_{1/2}$ values of approximately 3 h and 8 h for the aerated samples and control samples, respectively. These limited data suggest that aeration enhances the volatility of these off-flavor compounds from pure water by a factor of approximately 2.7.

To investigate the possibility that the compounds were degrading in solution rather than volatilizing, subsamples of a control ($t = 24$ h) and a pure water sample that had been aerated for 21 h were analyzed using the LC-APCI/MS method with positive mode scanning over the range from *m/z* 100 to *m/z* 500. The full scan did not reveal the presence of new products, indicating the geosmin and 2-MIB were volatilizing as expected.

The graphical results for geosmin and 2-MIB in the aerated and control salt water samples are presented in Fig. 6. Like the pure water results, the evaporation from salt water was also indicative of a first- order process for both geosmin and 2-MIB, with good experimental reproducibility. Also, like the pure water results, the $t_{1/2}$ values are very similar for both geosmin and 2-MIB in the salt water and in the controls, with values of approximately 2 h and 5 h, respectively. These limited data suggest that aeration enhances the volatility of these off-flavor compounds from salt water by a factor of approximately 2.5, a similar enhancement to that observed in the pure water experiment. More notably, the rate of evaporation of the off-flavor compounds is approximately 30% faster from the salt water than the pure water, as indicated by the shorter half lives in both the aerated and control samples. A summary of the average results from the aeration experiments, including an estimate of the experimental reproducibility, is presented in Table 1.

Based on the results from these limited, proof-of-concept studies, aeration enhances the removal of off- flavor compounds from both pure and salt water, but the rate was faster from salt water. Further evaluations of aeration as a remediation technology in marine RAS are warranted, as well as the possibility of adding salt in combination with aeration to enhance off-flavor removal from freshwater species just prior to harvest. A caveat is that the rate of volatilization could be lower at the initial concentrations anticipated in RAS (low ng/kg) than those observed in our study where the initial concentrations were in the ppb range, as suggested by the results reported by Lalezary et al. for air- stripping off-flavor compounds from drinking water [25]. Such investigations were beyond the scope of the present study.

3.3. Determination of 2-MIB in RAS using SBSE-LC-APCI/MS

Both the SBSE 1 (in-situ) and SBSE 2 (grab samples) LC-APCI/MS procedures were evaluated for their utility in determining 2-MIB in RAS tank and MBB water and to identify potential trends in concentration during the grow-out of European sea bass from juvenile to harvest stages. The approaches were also used to monitor for geosmin, but negative results were anticipated given its high LOD using SBSE-LC-APCI/MS.

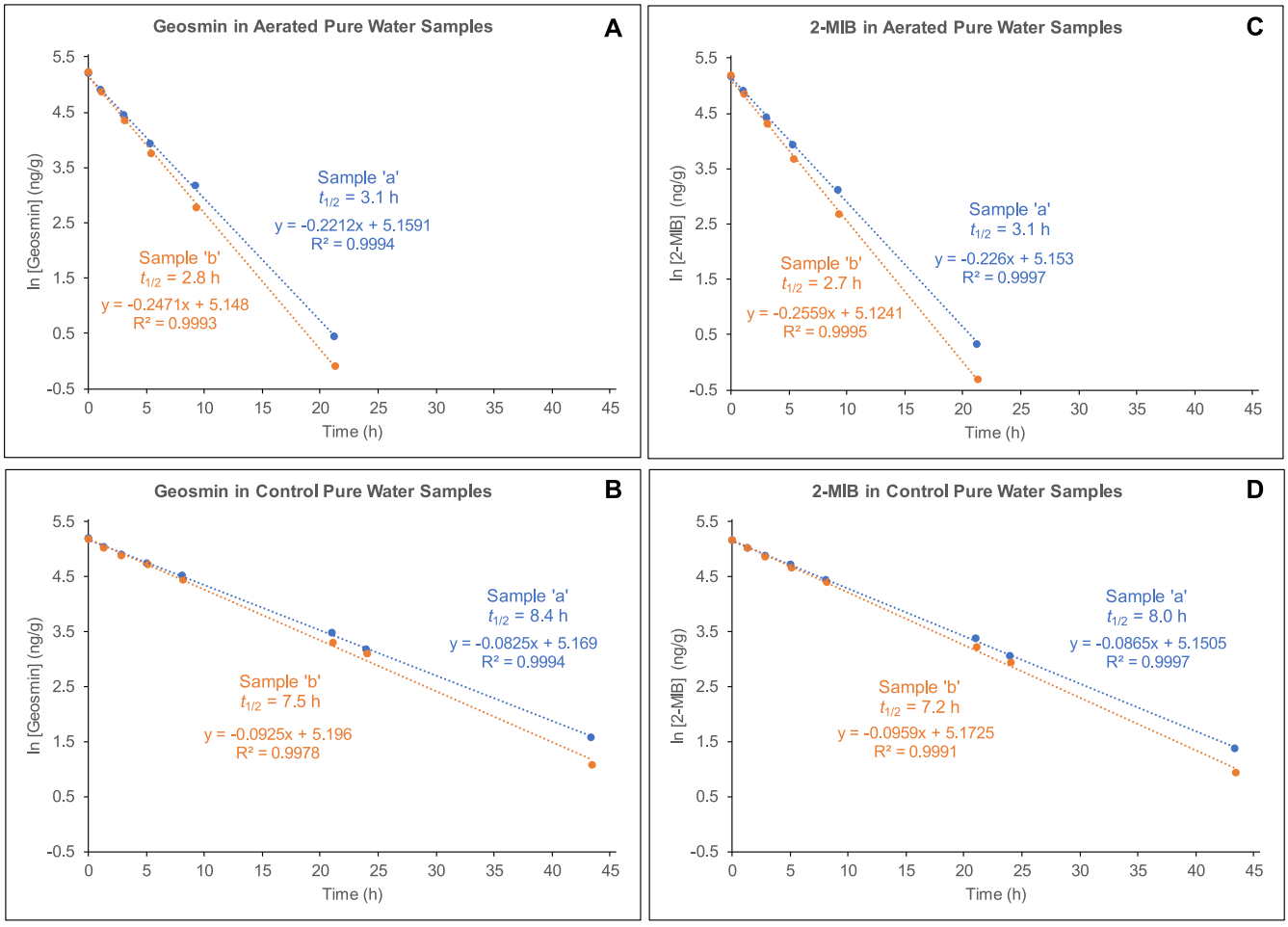

Fig. 5. Effects of aeration on geosmin and 2-MIB in pure water. Plots of ln concentration versus time in pure water for: geosmin in aerated samples (A) and control samples (B) and 2-MIB in aerated samples (C) and control samples (D). Each pane shows results for the duplicate experiments (sample 'a' and 'b') including the equation of the line and the corresponding regression (R^2) value and calculated half-life ($t_{1/2}$).

Table 1
Summary of results from aeration experiments. Results for -*k* and $t_{1/2}$ represent the average of the duplicate samples for each experimental condition. The reproducibility of the measurements is estimated as the SD of the experimental half-lives.

Sample conditions	Geosmin			2-MIB		
	-*k*	$t_{1/2}$ (h)	SD (h)	-*k*	$t_{1/2}$ (h)	SD (h)
Pure water - control	0.0875	7.9	0.6	0.0912	7.6	0.6
Pure water - aerated	0.2342	3.0	0.2	0.2410	2.9	0.3
Salt water - control	0.1423	4.9	0.1	0.1302	5.3	0.1
Salt water - aerated	0.3366	2.1	0.2	0.3056	2.3	0.2

RAS 1, the nursery system, was evaluated one time for off-flavor compounds in both the MBB and tank water just prior to fish transfer to the grow-out system when the stocking density was at its highest, 50 kg fish per m^3 of culture water. Duplicate grab water samples (a and b) were collected from each the MBB and tank water (4 total) at the same time the in-situ experiments were conducted so that the results from the two SBSE approaches could be directly compared. Four stir bars representing the different dimensions were placed in the diving unit for both the MBB and tank water experiments (8 total), but the 1 cm × 1.0 mm stir bars were evaluated qualitatively as blanks and extracted without the internal standard. The LC-APCI/MS results for the blanks revealed that IPMP was not present in the RAS water and hence not a source of quantitative bias. In addition, 2-MIB was detected in the blanks, but geosmin was not. Because the LOD for geosmin is much higher, its presence or absence in RAS 1 was inconclusive.

The 6 remaining in-situ stir bars as well as the 4 grab samples were then processed for quantitative determinations of 2-MIB using the internal standard method. The results for 2-MIB (ng) in the RAS 1 tank and MBB water, obtained from the in-situ stir bars as well as the grab samples, are presented in Fig. 7, panels A and B, respectively. The data profiles are similar for both the tank and MBB water samples when panels A and B are visually compared to each other and indicate several trends in the data. When the results are compared within a panel (A or B), the absolute amounts of 2-MIB (ng) were higher in the in-situ samples than in the grab samples. Since SBSE is an equilibrium extraction process, it is plausible that the higher sorption for the in-situ experiments can be explained through environmental factors such as the significantly larger water volume (4500 L vs. 10 mL), the flow dynamics in the system, and the higher water temperatures at the time of the extraction (26 °C vs. 22 °C). Also, when the results are

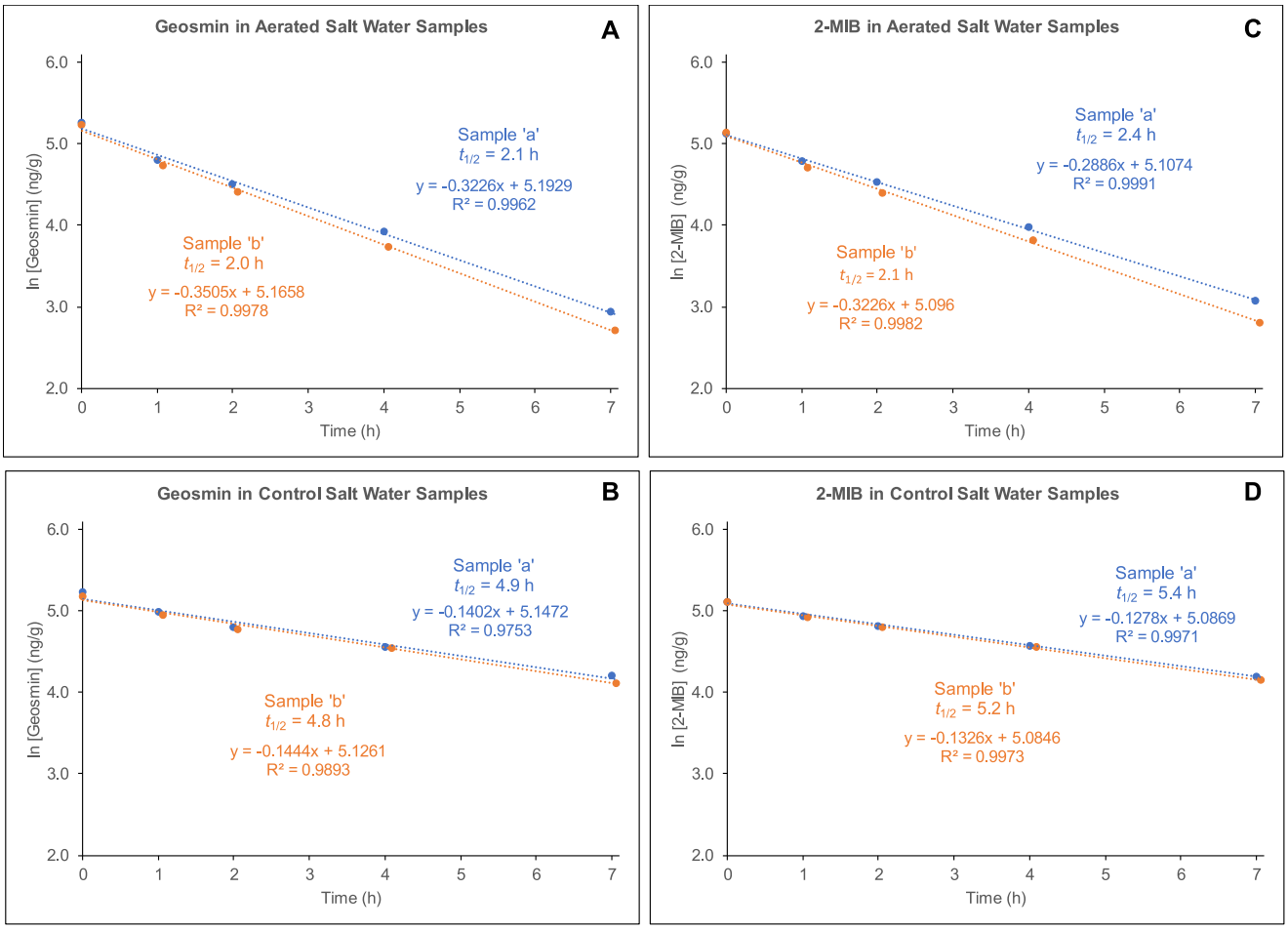

Fig. 6. Effects of aeration on geosmin and 2-MIB in salt water. Plots of ln concentration versus time in simulated seawater for: geosmin in aerated samples (A) and control samples (B) and 2-MIB in aerated samples (C) and control samples (D). Each pane shows results for the duplicate experiments (sample 'a' and 'b') including the equation of the line and the corresponding regression (R^2) value and calculated half-life ($t_{1/2}$).

compared within a panel, the absolute amount of 2-MIB was higher for the 2 cm stir bars than the 1 cm stir bars for the in-situ samples. The higher 2-MIB in the 2 cm stir bars is expected given the larger sorptive surface area, but the enhancement was not a factor of 2, which could represent some variability in the film length during manufacturing. However, the in-situ results contrasted the results for the controlled, laboratory-based extractions, where the performance of the stir bars was independent of the film dimension. These limited results indicate the 2 cm stir bars have more capacity when used in-situ and are a better choice for concentrating the off-flavor compounds for semi-quantitatively monitoring 2-MIB levels in RAS. Lastly, when the concentrations of 2-MIB determined in the duplicate grab water samples (presented as text insets in ng/kg) are compared within a panel, the 'b' samples have slightly lower concentrations than the 'a' samples. Each of the tank and MBB grab 'a' samples were analyzed in the laboratory 4 d after collection, whereas the 'b' samples were analyzed 7 d after collection. The 3-day difference in processing was most notably due to the limited number of stir bars and the time required for stir bar cleaning and reconditioning between experiments. The slightly lower results for the 'b' samples likely represent a real trend of the 2-MIB degrading over time, even though the grab samples were stored at 4 °C. These results suggest that grab samples should be analyzed as soon as possible after collection to get the most reliable concentration results.

When the overall results in Fig. 7A and B are compared to each other, the levels of 2-MIB are consistently higher (≈10% to 15% based on the grab sample results) in the MBB water than in the tank water. 2-MIB is not uniformly distributed in the system, which could be due to the higher surface area for bacteria to adhere and create biofilm in the MBB (≈175 m^2) than in the tank (≈10.5 m^2) in combination with the aerobic and organic-rich conditions that stimulate the proliferation of 2-MIB-producing bacteria in the MBB community [1,5]. The enhanced production of 2-MIB likely offsets the volatilization of 2-MIB from the vigorously-aerated MBB and supports the observations of Davidson et al. that media with significant biofilm formation hinder the effectiveness of aeration [11]. Since the fish were moved from RAS 1 right after this set of measurements was made, no additional studies were completed to reconcile the source of the concentration differences in the MBB and the tank water.

RAS 2, the intensive grow-out system, was cleaned and allowed to equilibrate before the fish were transferred to the system in small batches. When the stocking density was very low (1 kg/m^3), just after some of the fish were transferred to RAS 2, the system was evaluated for off-flavor compounds using both SBSE 1 and SBSE 2 using an analogous approach to that used for RAS 1. When the stir bars were analyzed, there was no detectable 2-MIB or geosmin in any of the samples. Since RAS 2 had just been initialized and there were few fish in the system, the result seemed consistent with the low biological activity in the system. To test

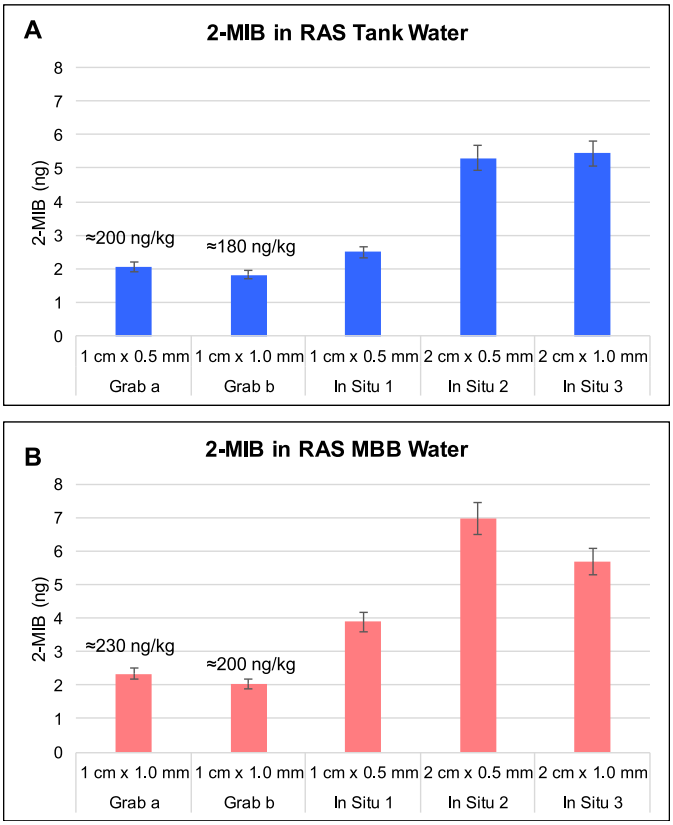

Fig. 7. Results for 2-MIB in RAS 1. Comparison of results for 2-MIB (ng) using SBSE 1 (in-situ experiments with a diver) and SBSE 2 (grab samples) procedures as a function of stir bar dimensions for both A: tank water samples and B: moving bed biofilter (MBB) water samples. For each experiment $N = 1$, and the error bars were estimated at 7% of the 2-MIB concentration and reflect the uncertainty in the calibration only. Concentrations of 2-MIB (ng/kg) were also calculated and are revealed above the bars for the grab sample data.

a hypothesis that the off-flavor compound concentrations would trend with stocking density and/or feed loads, RAS 2 was evaluated 3 additional times over the next 8 months when the stocking densities were 19 kg/m^3, 35 kg/m^3, and 55 kg/m^3 and the daily feed loads were 7.8 kg, 13.0 kg, and 16.4 kg, respectively. The latter conditions represented the highest stocking density and daily feed load achieved in RAS 2 before the fish were harvested. For the evaluations at these higher stocking densities, only SBSE 2 (grab samples) was used, since it is the more quantitative approach, but neither geosmin nor 2-MIB were detected in any of the samples. While the detection of geosmin was not anticipated due to its high LOD, it was a surprise that 2-MIB was below its LOD of 6 ng/kg, particularly at the highest stocking density of 55 kg/m^3, which exceeded the stocking density of 50 kg/m^3 when 2-MIB was readily detected in RAS 1. We speculate that the anaerobic water treatment components used only in RAS 2 (i.e., anaerobic sludge digestion and nitrate removal reactors) maintained the level of 2-MIB in the system water below its LOD. Guttman and van Rijn similarly reported that geosmin and 2-MIB levels were reduced by sludge-mediated chemical and physical absorption and biological degradation when the fish culture water was recirculated through anaerobic sludge basins [1,12]. While it is unclear if the 2-MIB concentrations were trending with stocking density over the time course because the levels were below the LOD, the results suggest that off-flavor compounds might not be a major issue for marine aquaculture when sophisticated systems like RAS 2, a fully-contained, near zero-discharge system with anaerobic solid and nitrate treatment reactors, are used.

4. Conclusions

An LC-APCI/MS method was developed for the direct analysis of 2-MIB and geosmin in aqueous samples, which is not readily achievable with typical GC methods. 2-MIB was readily detectable using the method, but on-column degradation reduced the sensitivity for determining geosmin. Even with the losses for geosmin, the method was applicable for determining both compounds at low ppb levels (geosmin LOQ ≈ 1ppb) with direct injections of aqueous samples and was used to evaluate aeration as a potential technology for reducing off-flavor compounds in water. Using controlled laboratory conditions that simulated marine and freshwater aquaculture, the volatilization rates of both geosmin and 2-MIB were faster in salt water than in freshwater, but aeration without high surface area media enhanced the rates in both water types. The results from these model studies indicate that aeration is a potential technology worth further consideration for off-flavor removal, particularly in marine RAS.

To achieve the enhanced sensitivity required for detection of off-flavor compounds at the low part per trillion (ng/kg) levels expected in RAS, the LC-APCI/MS method was combined with SBSE to extract and concentrate 2-MIB and geosmin, with resulting LODs of 6 ng/kg and 70 ng/kg, respectively. While the on-column degradation and high LOD render the method unsuitable for determine geosmin at the requisite levels, it is fast and sensitive for determining 2-MIB, which is likely to be the major off-flavor compound present in all aerobic treatment components in RAS. SBSE was used with both grab water samples for quantitative determinations and with a diving unit, which was a quick and easy way to extract 2-MIB directly from RAS (in situ) for semi-quantitative assessments. The SBSE approaches were combined with the LC-APCI/MS method to determine 2-MIB in this work, but SBSE is also compatible with GC methods, where it could be used to determine both geosmin and 2-MIB in other aquaculture or drinking water applications where both compounds are of concern.

SBSE-LC-APCI/MS was used to further understand the impact of aeration and water treatment on 2-MIB levels in two separate marine RAS that were culturing European sea bass. The 2-MIB levels were determined in water samples collected from both the moving bed bioreactor (MBB), which was vigorously aerated and contained high surface media, and the culture tank of each system. In RAS 1, which contained only aerobic treatment components, the 2-MIB levels were found to be ≈10% to 15% higher in the MBB than in the tank water. In the MBB, the volatilization of 2-MIB from the aeration process is likely outpaced by the enhanced production of 2-MIB by certain species present in the biofilms on the aeration media. Therefore, for marine RAS that have only aerobic treatment, the aeration process may need to be operated separately without high surface area media to more successfully reduce off-flavor compounds. In RAS 2, which contained both aerobic treatment components and an anaerobic treatment loop, 2-MIB was not detected in either the MBB or the tank water. These limited results suggest that the inclusion of anaerobic treatment processes is effective at reducing off-flavors in marine RAS.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Mary Bedner: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Writing - original draft, Visualization. **Keiko Saito:** Conceptualization, Methodology, Resources, Writing - review & editing.

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