Effects of Inoculated Bacillus subtilis on Geosmin and 2-Methylisoborneol Removal in Suspended Growth Reactors Using Aquacultural Waste for Biofloc Production

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Introduction

Geosmin and 2-methylisoborneol (2-MIB) are two of the most common taint compounds that adversely affect the quality of aquacultural animals. In the present study, 94% of geosmin and 97% of 2-MIB in suspended growth reactors producing bioflocs (SGRs) with aquaculture waste were removed after inoculation with Bacillus subtilis, significantly higher than that of control SGRs (70% of geosmin and 86.4% of 2-MIB). The lowest concentrations of geosmin and 2-MIB achieved in the effluent of the SGRs were 2.43 ± 0.42 ng/l and 2.23 ± 0.15 ng/l, respectively. The crude protein content of the bioflocs produced in the SGRs was 35 ± 4%. The NH4+-N and NO2--N concentrations in the effluent of the reactors were 1.13 ± 0.21 mg/l and 0.42 ± 0.04 mg/l, respectively. These results suggest that inoculated with Bacillus subtilis, SGRs have a better performance to reuse the nitrogen in fish waste and to remove geosmin and 2-MIB from the culture water efficiently.

Keywords: Geosmin, 2-methylisoborneol, biofloc technology, Bacillus subtilis, aquaculture waste
solid waste, and yield zero discharge in RASs [28].

*Bacillus subtilis* is widely used as a probiotic bacterium in aquaculture [31]. Inoculating SGRs with *Bacillus subtilis* can improve biofloc formation and nitrogen removal in aquaculture systems using solid waste [20]. It was reported that a bioactivated carbon filter seeded with *Bacillus subtilis* was capable of removing 2-MIB at mg/l levels [11, 18, 30]. Hence, a good performance of removing geosmin and 2-MIB is anticipated with the application of bioflocs.

In the present study, the efficiency of geosmin and 2-MIB removal in SGRs inoculated with *Bacillus subtilis* was investigated. The potential to develop a convenient and efficient technology for removing geosmin and 2-MIB in RAS water as a bypass unit is expected.

**Materials and Methods**

**RAS and Fish Waste**

The RAS culturing *Scortum barcoo* (Recirculating Aquaculture Engineering and Technology Laboratory, China) was equipped with three fish tanks (the volume of each tank was 1 m$^3$), a solid/liquid separator, a cold/heat control, three biofilters, and two circulating pumps (Fig. 1). The system is recirculated at a rate of 24 times per day. A compressed air blower is used to aerate the water. The stocking density of *Scortum barcoo* was approximately 34.5±0.7 kg/m$^3$. Fish were fed with a commercial diet containing 44% crude protein, 5% crude fiber, 18% ash, 4% calcium, 1.2% phosphorus, 2.5% lysine, and 12% moisture (Suzhou Tong Wei 8912, China). Water quality in terms of dissolved oxygen (DO, 6.00±0.27 mg/l), pH (6.55±0.61), total ammonium nitrogen (NH$_4$+N, 0.05 mg/l), nitrite-nitrogen (NO$_2$-N, 0.02±0.15 mg/l), and nitrate-nitrogen (NO$_3$-N, 10.17±0.48 mg/l) in the RAS was monitored every 2 days. The effluents were pumped from the flow equalizer into a laboratory-scale suspended growth reactor using a peristaltic pump (Fig. 1).

**Laboratory-Scale SGRs**

Six SGRs with a working volume of 10 L (internal diameter: 15.0 cm; liquid-filling height: 100.0 cm; Fig. 1) were used to produce bioflocs. The temperature of the reactors was maintained at 22–26°C. The mixture was thoroughly suspended by placing an air stone at the bottom of the reactor with air supplied from a 138-W air pump (ACO-008, SenSen Co., Ltd., China) operating at a rate of approximately 20 l/min.

**Bacillus subtilis**

The purified isolated bacterium was obtained from the public laboratory of Shanghai Ocean University (China). The strain was subjected to polymerase chain reaction amplification using universal primers specific for a conserved region of the 16S rRNA gene, 7F (5’-AGAGTTTATCTGCCG-3’) and 1492R (5’-GGTTACCTTGTTACGATT-3’). It exhibited high similarities (99%) to *Bacillus subtilis* subsp. (NCBI Reference Sequence) NC014479, and was identified as *Bacillus subtilis*. Before being added to the SGRs, the bacteria were inoculated into 500 ml of liquid medium containing beef extract peptone under sterile conditions and cultured at 37°C for 24 h with agitation [20].

**Experimental Procedure**

**SGR start-up and biofloc production.** The reactors were filled with 10 L of tap water containing 2.0 g/l aquaculture solid waste (containing crude protein (20 ± 4%), crude fiber (27 ± 6%), and ash (18 ± 2%)). The initial total suspended solid (TSS) in each reactor was 2,000 mg/l. The dissolved organic carbon (DOC) to TAN ratio was maintained at >15 (w/w) by the addition of glucose as the carbon source. *Bacillus subtilis* was added to three SGRs (treatment group) to a final density of (3.75±0.12)×10$^8$ cells/ml every 7 days. The three other SGRs were not inoculated with *Bacillus subtilis*. 

![Fig. 1. Schematic diagram of the experimental system.](image-url)
An acclimation period was allowed until the NH4+-N concentration in the reactors was lower than 1.0 mg/l and kept stable [13].

The water quality parameters, including temperature, DO, pH, oxidation-reduction potential (ORP), and concentrations of NH4+-N, NO3--N, NO2--N, DOC, and total nitrogen (TN), were determined at 9:00 am every day. The biochemical analysis of biofloc samples was conducted on days 21 and 30.

**Geosmin and 2-MIB removal in SGRs.** Geosmin (C12H26O) and 2-MIB (C4H9O) used in the current experiment were obtained from Labor Dr. Ehrenstorfer-Schafers (Augsburg, Germany), at 98.5% purity. When the NH4+ concentration was kept stable [13].

The removal rates of geosmin and 2-MIB were tested in triplicates for every initial concentration added for 7 days. Water temperature, DO, pH, NH4+-N, NO3--N, NO2--N, DOC, TN, and concentrations of geosmin and 2-MIB in the water were analyzed every day. At the end of each test, the biofloc samples were subjected to biochemical analysis.

**Analysis methods.**

**Water quality parameters.** Water temperature, pH, and DO were measured using a YSI556 tester (YSI Inc., OH, USA). Water samples were filtered through 0.45 μm filters before determining NH4+-N, NO3--N, NO2--N, and DOC levels (APHA, 2005). DOC was measured using a total organic carbon analyzer (TOC-V, CPH; Shimadzu Seisakusho, Japan). TSS was analyzed according to APHA methods [1].

**Biofloc composition.** Ash content of the bioflocs was determined by filtering the suspension through a glass fiber filter. Next, the crude filter was dried, weighed, and burned in a muffle furnace at 550°C for 4 h; and ash was then cooled and weighed. The crude protein content of the bioflocs was determined by using the Kjeldahl method (Kjeltec 8400; FOSS, Sweden).

**Geosmin and 2-MIB determination.** Geosmin and 2-MIB were quantified using a method of solid-phase microextraction-gas chromatography-mass spectrometry (GC-MS) as described previously [19, 30]. Briefly, the samples (12 ml) of geosmin and 2-MIB in the aqueous phase were first extracted using a solid-phase micro-extraction device, which was equipped with a headspace extraction bottle (15 ml) and a miniature magnetic rotor. The extraction was performed with the addition of 3.6 g sodium chloride for 40 min at 60°C. The fibers were placed in the bottle headspace without contacting the liquid. The samples were analyzed in splitless operation mode at 250°C for 3 min. In the present study, GC-MS was carried out with an HP-5 MS capillary column (30 m × 0.25 mm) and a mass spectrometer. The temperature program was the following: 60°C for 3 min, followed by increasing to 150°C at a rate of 5°C/min, then increasing from 150°C to 250°C at a rate of 15°C/min, and finally holding the temperature at 250°C for 3 min.

The removal efficiency of geosmin and 2-MIB in the SGRs was calculated (%) based on Eq. (1).

\[
\text{Removal efficiency} = \left( \frac{C_{\text{initial}} - C_t}{C_{\text{initial}}} \right) \times 100\% \tag{1}
\]

The removal rates of geosmin and 2-MIB in the SGRs was calculated (ng of geosmin/2-MIB (mg TSS/day)) based on Eq. (2):

\[
\text{Removal rate of geosmin or 2-MIB} = \frac{C_{\text{initial}} - C_t}{\text{TSS}_t} \tag{2}
\]

C_initial (ng/l) indicates the concentration of geosmin/2-MIB added. C_t indicates the concentration of geosmin/2-MIB at the indicated time point. TSS_t (mg/l) indicates the TSS concentration in the SGRs at the indicated time point.

**Data Analysis.**

Data were analyzed using SPSS 17.0 for Windows (SPSS, Inc., IL, USA). Significant difference was considered when the p value was <0.05. The water parameters, biofloc compositions, and concentrations and removal rate of geosmin and 2-MIB between control group and treatment group were subjected to a one-way analysis of variance.

**Results and Discussion.**

**Start-Up of Reactors and Biofloc Yield.**

In a new biofloc system, bacterial community development will normally need a few days [13]. In the present study, the start-up periods for the treatment and the control SGRs were 13 days and 17 days, respectively. The NO3--N concentration in the treatment groups was significantly lower than that in the control groups (Fig. 2), suggesting that more NH4+-N was incorporated into heterotrophic bacteria than was transformed by nitrification [8]. The crude protein content of the bioflocs in the inoculated reactors increased from 20 ± 4% to 30 ± 4%, which was greater than that in the control reactors (p = 0.003) (Fig. 2). The higher protein level in the treatment SGRs also indicated that more nitrogen was transformed into microbial protein. During the first 21 days of the experimental period, the pH value was 6.47–8.22, DO was 5.42–7.4 mg/l, and ORP was -43.5 to -93.2 mV in all reactors.

**Removal of Geosmin and 2-MIB.**

Rapid removal of 2-MIB and geosmin from the water was observed in all SGRs (Fig. 3). The highest removal rates of geosmin and 2-MIB were 62.1 ± 0.9 ng geosmin /g TSS·d and 196 ± 10 ng 2-MIB /g TSS·d in the control SGRs, and 207 ± 10 ng geosmin /g TSS·d and 230 ± 8 ng 2-MIB/g TSS·d in the inoculated SGRs.

Bacillaceae is a primary member of the microbial community in bioflocs and has been shown to degrade
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In addition, bioflocs have greater than 99% porosity [5] and contain rich extracellular polymeric substances [20]. Bioflocs also exhibited chemical or physical sorption abilities [7]. The processes underlying this removal were perhaps mediated by both chemical/physical sorption and biological degradation [9]. The susceptibility of both geosmin and 2-MIB to biological degradation can be attributed to the biofloc structure, which is similar to biodegradable ketones and alicyclic alcohols [25]. It is considered that these compounds can be used by the bacteria as a primary carbon source [11]. Saito et al. [25] found that geosmin was extremely difficult to microbially degrade when geosmin was used as the sole carbon source and the remarkable acceleration of the reaction was realized by the addition of ethanol. In order to induce the heterotrophic bacteria dominated in the SGRs, glucose was added regularly in the current experiment. Therefore, the good removal efficiency of geosmin and 2-MIB is anticipated in the current experiment.

The efficiencies of geosmin and 2-MIB removal in the inoculated reactors were significantly higher than those in control SGRs. At the end of the experiments, 94% of geosmin at initial levels of 1,000 ng/l and 500 ng/l, 92% of geosmin at an initial level of 100 ng/l, 98% of 2-MIB at initial levels of 700 ng/l and 500 ng/l, and 97% of 2-MIB at an initial level of 100 ng/l were removed in the inoculated SGRs. Although a more rapid degradation of geosmin and 2-MIB with lower concentrations of these two compounds has been observed [16], it has been shown that the removal rates of geosmin and 2-MIB do not appear to be affected by the initial concentration of these two compounds [14]. The performance of the treatment reactors reduced both geosmin and 2-MIB to below the level that would be likely to cause taint problems in aquaculture water (Fig. 4) [29].

In the current experiment, although the 2-MIB concentration in the SGRs fluctuated more distinctly than that of geosmin, the amount of 2-MIB removed in the inoculated reactors was significantly greater than that of

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**Fig. 2.** Changes in dissolved inorganic nitrogen during the start-up period of the reactors. (A) NH₄⁺-N. (B) NO₂⁻-N. (C) NO₃⁻-N. (D) The crude protein content of the bioflocs on day 21 (mean ± standard deviation (minimum–maximum)).
geosmin, suggesting that the removal of 2-MIB is more efficient than that of geosmin. These findings differ from earlier results demonstrating that geosmin appears to be degraded more easily than 2-MIB by the bacteria within the sand filters and bioreactors, including *Pseudomonas* sp., *Alphaproteobacterium*, *Sphingomonas* sp., and *Acidobacteriaceae* sp. [15].

The reactor in the present study showed the needed improvement in removal efficiency of these compounds in a less-required time. It can be carried out by increasing the

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**Fig. 3.** Removal rates of geosmin and 2-MIB in water in reactors. 
(A) Geosmin (100 ng/l). (B) 2-MIB (100 ng/l). (C) Geosmin (500 ng/l). (D) 2-MIB (500 ng/l). (E) Geosmin (1,000 ng/l). (F) 2-MIB (700 ng/l) (mean ± standard deviation (minimum–maximum)).
biofloc concentrations and/or the amount of the inoculated *Bacillus subtilis* in the SGRs.

**Performance of SGRs During the 2-MIB and Geosmin Addition Period**

The NH$_4^+$-N, NO$_2^-$-N, and especially NO$_3^-$-N levels were lower in the inoculated reactors than in the control SGRs (Fig. 4). At the end of the experiments, NH$_4^+$-N and NO$_3^-$-N concentrations remained below 2 mg/l and 0.5 mg/l, respectively, in both the control and inoculated reactors (Fig. 5), meeting the water quality requirements for the nitrification process in the biofilters [33]. In addition, the
crude protein contents of the bioflocs in the inoculated group (35 ± 4%) and the control group (29 ± 4%) were higher compared with those in phase 1, showing that adding the off-flavor compounds into the reactors did not adversely influence the crude protein content of the microflocs (Fig. 5).

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References


