Actinomycetes and earthy-musty odorous compounds in brackish fishponds in Tianjin, China
Yong Pan, Lipu Xu, Wenxuan Cao, Shouren Yin, Zongwei Wang and Qiaohong Zhou

ABSTRACT

The presence of the odorous compounds, 2-methylisoborneol (MIB) and geosmin, as well as causative microorganisms in brackish intensive cultivation fishponds in Tianjin, China that had a severe earthy-musty odor were evaluated. The results revealed that MIB was the primary odorous compound present in the Tianjin fishponds, with a concentration ranging from 0.53–5302.7 ng·L$^{-1}$. Furthermore, the concentration of MIB was found to be closely correlated with the gross biomass of actinomycetes in the water, which ranged from 10.67–1528.24 £10^6 cfu·ml$^{-1}$. Therefore, the sequences of the 16SrRNA and morphological characteristics of the actinomycetes in the brackish fishponds were investigated. The results revealed that the actinomycetes in the brackish fishponds included 9 species of common and dominant actinomycetes belonging to 4 genera. Of these genera, Streptomyces were the dominant species, and Streptomyces, Nocardioides and Micromonospora were the most common species in the fishponds evaluated. Next, the ability of each of the isolated Streptomyces to produce MIB was measured under laboratory culture conditions. Streptomyces Sp2 was found to have a strong ability to produce MIB, which indicates that this strain may be the primary source of the earthy-musty odor reported in brackish intensive cultivation fishponds in Tianjin, China.

Key words | actinomycetes, brackish intensive cultivation fishponds, earthy-musty odor, 2-methylisoborneol (MIB)

INTRODUCTION

The presence of an earthy-musty odor in cultivation fishponds is a common problem worldwide, and many studies have reported fishery losses and inconvenience to consumers as a result of such odors (Popovska 1983; Wnorowski 1992; Tucker 2000; Howgate 2004). In addition, several studies have found that these odors are related to the presence of actinomycetes (Persson & Sivonen 1979; Jensen et al. 1994; Sugiura & Nakano 2000; Lanciotti et al. 2003), because some actinomycete species are known to produce the earthy-musty odour compounds MIB and geosmin (Gerber 1979; Schrader & Blevins 1993; Scholler et al. 2002; Zaitlin et al. 2003a; Howgate 2004). However, different actinomycetes have different abilities to produce MIB and geosmin; therefore, some species may produce more odorous compounds than others (Gerber 1968, 1969; Wood et al. 1983; Sugiura & Nakano 2000; Howgate 2004). Accordingly, to remove the earthy-musty odorants from brackish fishponds, it is important to identify the organisms that are primarily responsible for the odor and determine their odorous compounds producing ability.

Tianjin is a main aquaculture base in North China that is comprised of a culture area of 43,000 hectares and has an annual fish yield of 350,000 tons. Its aquatic products
are primarily consumed by China’s main provinces including Beijing, Hebei, Liaoning, Jilin, Heilongjiang and Inner Mongolia. Because of its unique geographical location, the aquaculture water in Tianjin fishponds is brackish, with a salt concentration of 0.2–0.5%. Therefore, intensive high-density cultivation of fish is conducted in this region. However, episodes in which unpleasant earthy-musty odors were emitted from the fishponds in Tianjin have occurred in the last few decades, and this odor has caused great commercial losses.

To determine the cause of the earthy-musty odor emitted by the fish ponds, we continuously monitored Tianjin brackish water fishponds over several years using HSPME coupled with GC-MS (Headspace-Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry) to determine which compounds were responsible for the odor. The results revealed that MIB was the primary odorous compound, and that this compound was common in fishponds throughout Tianjin. Furthermore, the results of the HSPME-GC-MS analysis revealed that the concentration of MIB was thousands of times higher than that of other odorous compounds present in the fishponds. In addition, we found that the microorganisms responsible for production of the odorous compounds were actinomycetes, and that their biomass was significantly correlated with the concentration of MIB.

Therefore, the species of actinomycetes present in the fishponds evaluated in this study was determined so that the greatest producer of odorous compounds could be identified. To accomplish this, we selected 3 representative fishponds of different sizes that had serious odor problems. The composition of odorous compounds and the biomass of actinomycetes in these 3 ponds were then measured from the spring of 2006 to the winter of 2007. In addition, the 16SrRNA of species of actinomycetes isolated from the 3 fish ponds was amplified and sequenced. Next, the ability of each of the isolated species to produce odorous compounds was evaluated under laboratory culture conditions. To the best of our knowledge, this is the first study to identify and evaluate odor producing actinomycetes isolated from brackish fishponds. The results of this study can be used as a base for future studies to determine methods to eliminate the severe earthy-musty odor emitted from Tianjin fishponds.

**MATERIALS AND METHODS**

**Materials**

**Sampling**

Samples were collected from three different sized fishponds in Tianjin, China that were emitting strong odors in April, July and October of 2006 and January of 2007. One liter samples were collected from 0.5 m below the water surface from each quarter of each pond. The four samples from each pond were then mixed thoroughly and one liter aliquots were used for subsequent analysis. The basic information pertaining to each of these three fishponds is presented in Table 1.

**Instruments**

A TGL-16 desktop high speed refrigeration centrifuge was obtained from Beijing Changliu Scientific Instruments Co., Ltd (Beijing, China). In addition, a Thermo-Hybaid PCR machine was purchased from Eppendorf, (Hamburg, Germany). Furthermore, a type DYY-10 electrophoresis machine was purchased from Beijing Liuyi Instrument Factory (Beijing, China). Additionally, a TM3400 documentation system was obtained from the Alpha Innotech Corporation (San Leonardo, CA, USA). A trace 2000 type GC-MS chromatograph column was obtained from Thermo-Finnigan LLC (Madison, CT, USA). Finally, an HSPME syringe (2 cm-30/30 µm DVD/Carboxen/PDMS) and Magnetic Stirrers (Corning Model PC-420) were purchased from Supelco (Bellefonte, PA, USA).

**Reagents**

Chromatographic grade normative geosmin with a concentration of 100 µg ml⁻¹ and a purity of 99.9%, MIB with a concentration of 1 mg ml⁻¹ and a purity of >98% and IBMP and IPMP with a purity of 99.9% were used for this study. IPMB and MIB were purchased from Sigma Chemical Co. (USA), while Geosmin and IBMP were purchased from Supelco Company (Bellefonte, PA, USA). In addition, chromatographic grade methanol was purchased from Beijing Chemical Reagents Company (Beijing, China). Additionally, analytical grade NaCl that was
obtained from Beijing Chemical Reagents Company (Beijing, China) was purified by heating it to 450°C in a muffle furnace for 4 h prior to use.

Finally, distilled water was produced using a water purifier (Gallo Milibo Company, France) and Taq enzyme was obtained from Biotechnology Company (China).

Methods

Earthly-musty odorous compounds determination

Water samples were placed in 125 ml airtight glass bottles and then stored at 4°C until analysis. The concentrations of geosmin and MIB in all samples were then determined within 7 days using HSPME coupled with GC-MS (Lin et al. 2002).

Actinomycetes biomass determination

To determine the biomass of actinomycetes in the samples, 0.1 ml of 0.5% phenol solution was added to a 10 ml water sample and then mixed thoroughly by inverting the samples repeatedly. Next, 10-fold serial dilutions were performed to dilute the sample to $10^{-2}$, $10^{-3}$ and $10^{-4}$, respectively, with 3 replicates being made for each sample. Samples were then plated onto Gao’s medium (Yuan 1977). The plates were then inverted and cultured for 7d at a temperature of 28°C. The concentration of actinomycetes in each sample was then calculated.

Actinomycetes identification

Strain isolation and observation. Sample water from fishponds was serially diluted and then streaked for isolation on Gao’s medium (Yuan 1977). The samples were then cultured in the dark at 25°C for 28 days, after which the isolated cultures were purified and visually characterized.

DNA extraction and PCR. Genomic DNA was extracted and amplified by PCR using the method described by Xu et al. (Xu et al. 2003). Next, 10 µL of the amplified 16 SrRNA was subjected to electrophoresis in a 1.0% agarose gel at 90 V for 1.5 h. The gel was then photographed under ultraviolet light at 260 nm using the Documentation System.

16 SrRNA sequencing. Sequencing was conducted using the following primers: A, 5’-AGAGTTTGATCCTGCGCTT- CAG-3’; B, 5’-TTAAAGGTGATCCAGCGCCA-3’; C, 5’- AGGTTGCGCTGTTG-3’. Sequencing was conducted by BGI Life Tech Co., Ltd. using an ABI PRISM 3730DNA

Table 1  Basic situation of the experimental ponds

<table>
<thead>
<tr>
<th>Ponds</th>
<th>Area (hectare)</th>
<th>Depth (m)</th>
<th>Main culture species</th>
<th>Time</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>DO (mg·L⁻¹)</th>
<th>Salinity (%)</th>
<th>COD (mg·L⁻¹)</th>
<th>NH₄⁻ – N (mg·L⁻¹)</th>
<th>TN (mg·L⁻¹)</th>
<th>TP (mg·L⁻¹)</th>
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<td>2</td>
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<td>11.0</td>
<td>8.0</td>
<td>10.8</td>
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<td>24.4</td>
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<td>25.0</td>
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<td>10.3</td>
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<td>7.5</td>
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<td>20.0</td>
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</table>
Analyzer and BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

**Data analysis.** The sequencing results were used to select known 16SrDNA sequences of related actinomycetes from the GenBank and EMBL databases using the BLAST search software. The sequences were then aligned using the CLUSTAL W1.8 software, after which an evolutionary tree was constructed with the Mega 3.0 software using the Neighbor-Joining method and the Kimur double parameter corrective mode, with the Bootstrap value being calculated based on 1,000 replications.

**Determination of the actinomycetes odorous compounds producing ability**

Suspensions of 6 species of *Streptomyces* were made and then used to inoculate Gao’s liquid medium (1 × 10^5 ind/L, 170 rpm/min) (Yuan 1977). The samples were then cultured for 9 days at 30°C in the dark with shaking. Fifty millilitres of the fermented liquid was then centrifuged for 10 min at 4,000 rpm. A 2–20 ml aliquot of the supernatant was then collected and suspended in 50 ml of distilled water. Next, the MIB content produced by each species of *Streptomyces* was determined. The biomass of each species of *Streptomyces* was then calculated based on their dry weight, which was determined by drying 50 ml of fermented liquid in a 105°C oven for 1 h.

**Statistical analysis**

Correlations between MIB concentration and the parameters including actinomycetes biomass, phytoplankton biomass, pH, DO, temperature and salinity were examined by Pearson’s rank correlation test, and when the p value was below 0.05, the linear regression between the MIB concentration and parameters was regarded as significant.

**RESULTS**

**Odorous compounds concentration**

MIB and geosmin were found to be present in all of the brackish fishponds evaluated in this study, with concentrations of 0.53–5302.7 ng·L⁻¹ and 0.29–12.1 ng·L⁻¹ being observed, respectively. However, in most months and most ponds the content of MIB was much higher than that of geosmin, as indicated by the highest-value being 1,000 times higher for MIB than for geosmin. In addition, the maximum value of MIB was 5302.7 ng·L⁻¹, which is 183 times higher than the olfact for humans. Finally, the MIB concentration of 4 samples exceeded the human olfact value (29 ng·L⁻¹) (Weng 1993), while the concentration of geosmin was generally lower, with only 1 sample containing concentrations greater than the olfact for humans (10 ng·L⁻¹) (Weng 1993)(Table 2).

**Actinomycetes biomass**

The biomass of the actinomycetes in fishponds in Tianjin is shown in Table 2. The total biomass of actinomycetes in the fishponds was 10.67–1528.24 × 10⁶ cfu·ml⁻¹. Correlation analysis revealed that there was a significant positive correlation between the biomass of actinomycetes and the concentration of MIB.

**Isolated actinomycetes species**

**Morphology and culture characteristics of isolated actinomycetes**

Actinomycetes were isolated from every pond, and a total of 9 species were identified based on their culture characteristics. The 9 identified species belonged to the following 4 genera: *Micromonospora*, *Nocardioides*, *Streptomyces* and *Actinomadura*.

Colonies of Sp7 were small, smooth and had a single basal hypha that ruptured into short lengths after 10 hours of culture. It was up to the characteristics of *Nocardioides*. Sp8 had thick substrate mycelia and rare aerial mycelia; therefore, it was identified as *Actinomadura*. Sp9 was identified as *Micromonospora* based on the presence of well developed substrate mycelia, a single spore in the inner substrate mycelia and the absence of aerial mycelia. The remaining 6 strains isolated in this study exhibited traits common to *Streptomyces*, which included well developed substrate mycelia, long spore chains in aerial mycelia, and spore chains that could polarize into spores (see Table 3 and Figure 1).
Table 2 | Actinomycetes species and biomass and odorous compounds concentration in the ponds

<table>
<thead>
<tr>
<th>Sampling ponds</th>
<th>Biomass of actinomycetes</th>
<th>Biomass of Streptomyces</th>
<th>Biomass of Nocardioides</th>
<th>Biomass of Micromonospora</th>
<th>Biomass of Actinomadura</th>
<th>MIB/actinomycetes (Biomass)</th>
<th>Geosmin ng·L⁻¹</th>
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<tr>
<td>1</td>
<td>0.041</td>
<td>0.0734</td>
<td>0.027</td>
<td>0.374</td>
<td>0.08</td>
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<td>0.0167</td>
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</tbody>
</table>

Frequency of every strain
The sequences of Sp1–Sp9, which were all approximately 1,400 bp, were compared with known sequences of *Streptomyces, Nocardioides, Micromonospora,* and *Actinomadura* present in the GenBank database, and an evolutionary tree was then constructed. (Figure 2) As can be seen in the evolutionary tree, these 9 strains belonged to 4 different branches corresponding to *Streptomyces, Nocardioides, Micromonospora,* and *Actinomadura.* These findings were consistent with the identifications that were made based on the morphological evaluations.

Species of actinomycetes in ponds in Tianjin

The results revealed that there were 4 genera and 9 species of actinomycetes isolated from ponds in this study. Among these were 6 species of *Streptomyces,* 1 species of *Nocardioides,* 1 species of *Micromonospora* and 1 species of *Actinomadura.* Specifically, 6 species of actinomycetes belonging to 3 genera were isolated from pond #1, 7 species of actinomycetes belonging to 4 genera were isolated from pond #2, and 7 species of actinomycetes belonging to 4 genera were isolated from pond #3 (Table 2).

The appearance frequency and biomass of the individual actinomycetes are shown in Table 2. Frequency analysis of the actinomycetes revealed that, Sp1, Sp2, Sp6, Sp7 and Sp9 were present in each of the ponds. In addition, frequency analysis indicated that Sp1 was present in 10 of the 12 samples evaluated, making it the most common species in the 3 ponds. This was followed by Sp7, which was present in 9 samples, and then by Sp2, Sp9, and Sp6, which were present in 7, 7 and 6 samples, respectively.

When the numbers of genera in the ponds were evaluated, *Streptomyces* was found to be the most dominant species in 75% of the ponds and to represent 60% of the total biomass. Of the remaining genera of actinomycetes isolated in this study, *Micromonospora* had the largest biomass, while *Actinomadura* had the lowest.

Isolated *Streptomyces’* MIB producing ability

The ability of *Streptomyces* to produce odorous compounds, which is described in detail in Table 4, was as follows: Sp2 > Sp3 > Sp5 > Sp4 > Sp1 > Sp6. And the MIB
Figure 1 | Photos of the isolated Streptomyces (× 100).

Figure 2 | Evolutionary tree constructed based on the 16S rDNA sequences.
Table 4 | Production rate of MIB by 6 Streptomyces isolated from the ponds

<table>
<thead>
<tr>
<th>Number of strains</th>
<th>Concentration of MIB in fermented liquid (ng/ml)</th>
<th>Dry weight of actinomycetes in 50 ml fermented liquid</th>
<th>Production rate of MIB in fermented liquid (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp1</td>
<td>14.3</td>
<td>0.123</td>
<td>$5.81 \times 10^3$</td>
</tr>
<tr>
<td>Sp2</td>
<td>5570.0</td>
<td>0.137</td>
<td>$2.03 \times 10^6$</td>
</tr>
<tr>
<td>Sp3</td>
<td>776.0</td>
<td>0.456</td>
<td>$8.51 \times 10^4$</td>
</tr>
<tr>
<td>Sp4</td>
<td>19.8</td>
<td>0.139</td>
<td>$7.12 \times 10^3$</td>
</tr>
<tr>
<td>Sp5</td>
<td>72.4</td>
<td>0.139</td>
<td>$2.60 \times 10^4$</td>
</tr>
<tr>
<td>Sp6</td>
<td>2.6</td>
<td>0.172</td>
<td>$7.56 \times 10^2$</td>
</tr>
</tbody>
</table>

concentrations produced by Sp2, that is, 5,570 ng/ml, were the highest and it was higher significantly than previous reports which was $60 \mu g/L$ (Sugiura & Nakano 2000) and 8 to 2,337 ng/L (Zaitlin et al. 2003b), respectively.

**DISCUSSION**

**MIB was the primary odorous compound in Tianjin fishponds and was significantly correlated with the biomass of actinomycetes**

The results of this study confirmed the results of our previous study, which indicated that MIB was the primary odorous compound in Tianjin fishponds. The results were similar to the results of a study conducted on fishponds in Donghu of Wuhan, China (Xu et al. 1999). Furthermore, the MIB content in Tianjin ponds was higher than that of many other water bodies including Donghu Lake in Wuhan (10–317 ng L$^{-1}$) (Xu et al. 1999), the Arno River in Italy (0–3.1 ng L$^{-1}$) (Lanciotti et al. 2003), Lake Kasumigaura in Japan (max. 90 ng L$^{-1}$) (Sugiura & Nakano 2000), and reservoirs in Arizona in the United States (max. 300 ng L$^{-1}$, normal 20–80 ng L$^{-1}$) (Westerhoff et al. 2005).

However, the results of this study differed from the results of studies conducted on Beijing fishponds in China (Xu et al. 2007), the Arno River in Italy (Lanciotti et al. 2005) and Lake Kasumigaura in Japan (Sugiura & Nakano 2000) which indicated that the geosmin concentration was higher than that of MIB. Taken together, these findings indicate that there is a difference in the primary odor-producing compounds in different areas and waters.

On the other hand, the results of the statistical analysis revealed that there was a significant correlation between MIB content and the entire biomass of actinomycetes, but not the biomass of blue algae, and the total biomass of actinomycetes in the fishponds in Tianjin is generally higher than that of West Lake in Hangzhou, China ($35 \times 10^6$ cfu·ml$^{-1}$) (Wu et al. 1999), Donghu Lake in Wuhan ($4.6–7 \times 10^6$ cfu·ml$^{-1}$) (Xu et al. 1999), Lake Kasumigaura in Japan ($0–8$ cfu·ml$^{-1}$) (Sugiura & Nakano 2000), Elbow River in Canada ($0–257$ cfu·ml$^{-1}$) (Zaitlin et al. 2003b), and the Arno River in Italy ($120–17,800$ cfu·ml$^{-1}$) (Lanciotti et al. 2003), which indicate that actinomycetes are closely related to the generation of the odorous compound MIB in Tianjin fishponds.

**Common species and dominant species of actinomycetes in Tianjin fishponds**

The results of this study revealed that actinomycetes were popular in brackish intensive cultivation fishponds in Tianjin, and that Streptomyces, Nocardioides and Micromonospora were the most common species, while Streptomyces was the most dominant species. The common species of actinomycetes were similar to the species observed in the Arno River in Italy (Lanciotti et al. 2003), in which the common species were found to be Streptomyces, Micromonospora and Nocardia, but differed from those of studies conducted on plateau lakes in Yunnan, China (Jiang & Xu 1984, 1996) which Rhodococcus and Nocardia were the most common and dominant species.

It is important to note that the dominant species of actinomycetes observed in Tianjin fishponds was the same as that observed in Danish fishponds (Klausen et al. 2005). Further investigations comparing the structure of actinomycetes in brackish fishponds and other waters deserved to be conducted.

**Relationship between odorous compounds and species of actinomycetes in Tianjin fishponds**

The results of our study revealed that the concentration of MIB was closely related to the actinomycetes in the fishponds. The previous studies revealed that there was strain specificity in the ability of actinomycetes to produce odorous
compounds (Yagi et al. 1987; Aoyama 1990; Schrader & Blevins 1993). In this study, Streptomyces, Micromonospora and Actinomadura, which are typical actinomycetes associated with odorous compounds, were all found in Tianjin fishponds (Gerber 1968, 1969; Wood et al. 1983). When the density was considered, there were fewer actinomycetes found in ponds in Tianjin than in plateau lakes; however, the dominant species of actinomycetes in the ponds of Tianjin were Streptomyces (75% of the ponds had dominant species of Streptomyces, and Streptomyces in 60% of the ponds accounted for more than 50% of the total biomass). It has been reported that the earthy-musty odor in fishponds in Tianjin are more severe than in plateau lakes in Yunnan. These findings supported the results of other studies (Gerber 1968, 1969; Wood et al. 1983) which indicated that the odorous compounds producing ability of Streptomyces is even greater than that of other genera.

In addition, the dominant species observed in our study were different from those observed in other lakes and rivers, but were the same as those observed in Danish fishponds. This finding indicates that identical dominant species may be associated with the severe earthy-musty odor that is commonly associated with fishponds.

**Sp2, an actinomycete isolated from fishponds in Tianjin, China, has a strong ability to produce odorous compounds**

As shown in Table 3, Sp1, Sp6, Sp7 and Sp9 produced both high and low rates of MIB, which indicates that they have a general capacity to produce odorous compounds. Conversely, Sp6 and Sp9 were only found to produce low levels of MIB, even though the biomass of Sp9 was very high, indicating that Sp9 is able to produce only a small amount of MIB. Conversely, Sp2 only produced a high level of MIB. Specifically, when the MIB producing ability was observed under laboratory conditions, the MIB concentrations produced by Sp2 was higher significantly than previous reports, and in this study Sp2 produced 7–2,000 times more MIB than the other species, while Sp1 and Sp6 produced only a small amount of MIB. Taken together, these findings indicate that Sp2 may be the primary source of the severe earthy-musty odorants found in fishponds in Tianjin.

**CONCLUSION**

Overall, the results of this study demonstrated that actinomycetes are common in brackish intensive cultivation fishponds in Tianjin, China. In addition, the most common actinomycetes were found to be Streptomyces, Nocardioides and Micromonospora, and Streptomyces was the most dominant species. Furthermore, the primary odorous compound was found to be MIB. The concentration of MIB was found to be correlated with the biomass of actinomycetes, as well as with individual species of actinomycetes, especially Sp2. Therefore, Sp2 should be studied further to confirm that it is the source of the earthy-musty odor emitted from brackish intensive cultivation fishponds in Tianjin, China and to determine a method to eliminate it.

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