Musty odor producing benthic cyanobacteria in the Tama River (Japan) and identification of species by genetic analysis

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ABSTRACT

In upstream reaches of the Tama River, musty odor substance (2-methylisoborneol (2-MIB)) began to be detected in 2008 and the concentration has been increasing thereafter. Then, in 2012, 2-MIB in raw water of a water treatment plant reached 210 ng/L. It was suspected that cause of musty odor was benthic cyanobacteria (*Phormidium*) which attached to stones on the riverbed. However, identification of the benthic *Phormidium* by microscopic observation had been difficult, thus genetic analysis was carried out. In genetic analysis, almost full-length 16S ribosomal DNA and internal transcribed spacer region gene of benthic *Phormidium* strains, which include the strain isolated from the Tama River, a strain in the Yoshino River (350 km away from the Tama River) and standard strains, were sequenced and compared. From homology search of these sequences, 2-MIB producing *Phormidium* in the Tama River was classified into *Phormidium autumnale*. Furthermore, it was found that *P. autumnale* in the Tama River was the same species in the Yoshino River. In addition, the result of *in vitro* cultivation shows that the *P. autumnale* inhabiting the Tama River thrives and produces more 2-MIB at high temperature, but it can grow and produce 2-MIB even at low temperature.

Key words | 2-methylisoborneol, benthic cyanobacteria, genetic analysis, *Phormidium*

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INTRODUCTION

In recent years, customer demands regarding drinking water quality have been increasing. Among them, taste and odor have become major concerns about the drinking water. Tastes and odors occur in water from a variety of sources, most notably algae, actinomycetes, organic and inorganic sulfides, and industrial contaminants, such as phenols (American Water Works Association 2012). The majority of all biologically caused tastes and odors in drinking water characterized worldwide are caused by microbial production of geosmin and 2-methylisoborneol (2-MIB) (Jűttner & Watson 2007). Geosmin and 2-MIB are earthy/musty compounds and have very low odor detection thresholds (below 10 ng/L). In addition, geosmin and 2-MIB are relatively stable in water and cannot be removed by conventional water treatment. Excessive growth of certain types of cyanobacteria in source waters can lead to the production of taste and odor substances such as geosmin and 2-MIB. Cyanobacteria species, particularly filamentous forms, produce more than 25% of all known off-flavor compounds (Smith *et al.* 2008).

Typical off-flavor problems of drinking water in Japan are musty odor production by cyanobacterial blooms, which are caused by eutrophication of water source reservoirs. In Tokyo, musty odor has occurred in waters of the Tone River system. The Edo River, one of the branches of the Tone, has some inflowing small streams. These streams

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were nearly stagnant and had become polluted by rapid urbanization of the basin since the 1970s. As a result, musty odor producing cyanobacteria bloomed and 2-MIB was produced massively (Hosaka *et al.* 1995). The Kanamachi purification plant of Tokyo Waterworks, which draws water from the Edo River, had attempted to remove it by powdered activated carbon, but it had not been sufficient to remove it to below the threshold concentration. Therefore, Tokyo Waterworks decided to install an advanced treatment system with ozonation and biological activated carbon filtration in 1992 in the expectation of more effective and stable removal (Murano & Nishino 1994; Muramoto *et al.* 1995). In addition to Kanamachi, Tokyo Waterworks had introduced the advanced treatment system to all the purification plants in the Tone River system by 2014.

On the other hand, in the upstream reaches of the Tama River, which is also used for a source of drinking water by Tokyo Waterworks, 2-MIB began to be detected in 2008 and the concentration increased thereafter. Then, in September 2012, 2-MIB in raw water of a water treatment plant reached 210 ng/L, which was 20 times higher than the drinking water quality standard in Japan (10 ng/L). Since 2-MIB was detected in a wide range of the Tama River and the concentration became higher in the downstream, the cause of 2-MIB generation was thought to be different from planktonic cyanobacteria in reservoirs, which is well known as a source of musty odor in Japan. As a result of investigation of the riverbed, it was suspected that the cause of musty odor generation was benthic cyanobacteria (*Phormidium*) which attached to stones on the riverbed. However, very few studies of 2-MIB producing benthic *Phormidium* in Japan (Sugiura *et al.* 1998) were available. Therefore, we identified the species of benthic *Phormidium* in the Tama River and investigated the characteristics of the production of the musty odor substance.

MATERIALS AND METHODS

Investigation of riverbed

Tama River is a river whose headwater is located in Yamanashi Prefecture, flowing up to Tokyo, and its total length is 138 km. Ogouchi Dam and Reservoir are located at a point 89.4 km from the river mouth. The water from the Ogouchi Reservoir is taken at Hamura Weir at the 53.9 km point from the mouth and transferred to Ozaku and Higashimurayama Purification Plant and used as the raw water (Figure 1). Also, the water taken at the Hamura Weir was transferred and stored in Murayama and Yamaguchi Reservoirs.

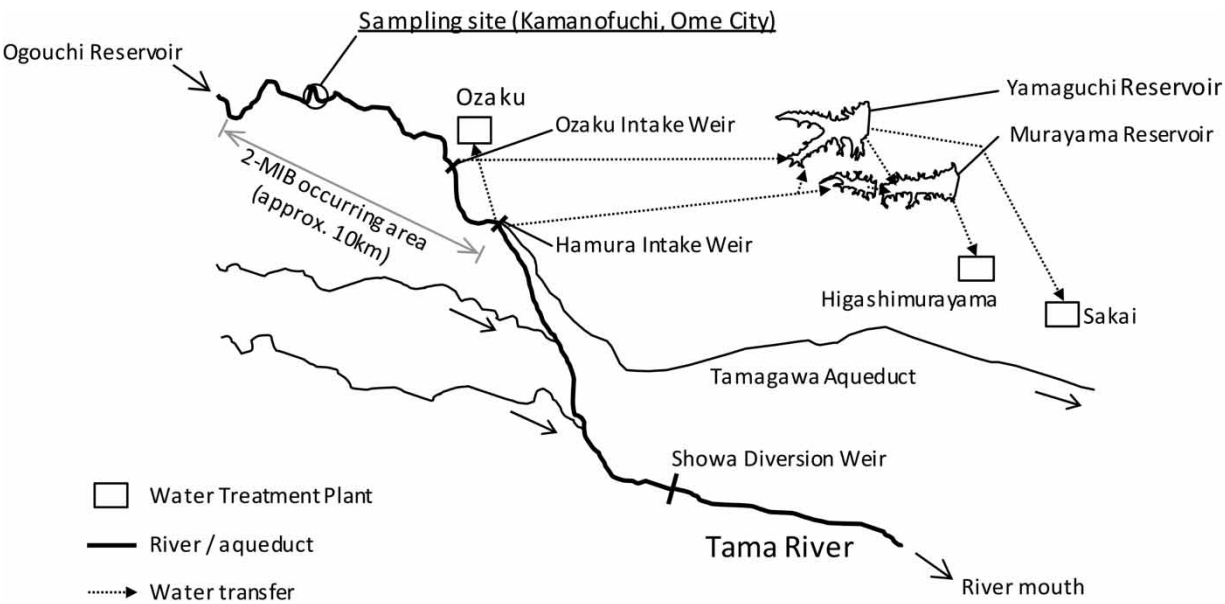

Figure 1 | Sampling sites in the Tama River.

To investigate the cause of musty odor in the upstream reaches of the Hamura Weir, the changes in concentration of 2-MIB in the river were first investigated. As a result, while the 2-MIB was not detected at all in the Ogouchi Reservoir, it was detected at approximately 10 km upstream of the weir, and it increased as the river goes downstream. These results indicated that 2-MIB occurs widely in this area. Therefore, we investigated in the riverbed the possibility of benthic cyanobacteria that produced 2-MIB. Stones covered the riverbed of the Tama River in this area, thus algal mats on the stones were investigated. The Kamanofuchi Park of Ome City (about 7 km upstream of the Hamura Weir) was chosen for sampling (Figure 1) and stones on the riverbed in the Tama River were collected to obtain the algal body by brushing. The algal body was collected from three stones by scrubbing with a brush a 25 cm^2 area of each. Then, the collected algal body was diluted to 300 mL with ultra-pure water as sample. Filamentous cyanobacteria in 50 μL of sample was observed by a microscope and counted in units of 100 μm. In addition, 2-MIB concentration of 40 mL of sample was measured by purge and trap GC/MS (P&T: O.I. Analytical 4660, GC: Agilent 6890, MS: Agilent 5973). Moreover, filamentous cyanobacteria and other kinds of algae were cultured in CT medium (Watanabe & Ichimura 1977) and the ability of 2-MIB production was confirmed. Also, we attempted to culture actinomycetes based on the method described in the literature (Japan Water Works Association 2011b), since there was a possibility of musty odor generation by actinomycetes.

Identification by genetic analysis

As a result of investigation of the riverbed, it was suspected that the cause of musty odor generation was benthic cyanobacteria (*Phormidium*). However, identification of the species of the benthic *Phormidium* strain isolated from the Tama River by microscopic observation was difficult, thus genetic analysis was carried out.

Sample collection

Collected samples are shown in Table 1. Samples of benthic *Phormidium* were collected at the site described above in August 2011. The algal body of *Phormidium* was cultured in CT medium, and then cultured algal body was used as a sample (*P.* sp. 2011 Tama River). In addition, three of the stored samples, which morphologically resembled the sample described above, were used. In the past, these stored samples were collected in the watershed or purification plant of the Tama River system. They were collected from riverbed stone in the interval up to the Hamura Weir from Showa Weir in 2008 (*P.* sp. 2008 Tama River). Others were collected in the Murayama Reservoir and the

Table 1 | List of the strains used for genetic analysis

Group	Sample name	Description
Standard strain of CCALA	*P. autumnale* CCALA 143	Musty odor was not observed
	P. cf. *autumnale* CCALA 145 (geosmin)	Geosmin was observed after 20 days of culture in CT medium
	P. autumnale CCALA 697	Musty odor was not observed
	P. favosum CCALA 882	Musty odor was not observed
Objective of this study (collected from riverbed)	*P.* sp. (2008, Tama River, 2-MIB)	Collected at downstream of the Hamura Weir in 2008, 2-MIB producing
	P. sp. (2011, Tama River, 2-MIB)	Collected at upper reaches of the Hamura Weir in 2011, 2-MIB producing
Comparison	*P.* sp. (2005, Murayama Reservoir, 2-MIB)	Collected at Murayama Reservoir (which intakes raw water from Hamura Weir) in 2005, 2-MIB producing
	P. sp. (2008, Higashimurayama PP, geosmin)	Collected at Higashimurayama Purification Plant (which intakes raw water from Hamura Weir), geosmin producing
	P. autumnale (Yoshino River, 2-MIB)	Collected at the Yoshino River, Nara Prefecture, 2-MIB producing

Higashimurayama Purification Plant. Moreover, a gene extraction sample of 2-MIB producing *P. autumnale* which was collected at the Yoshino River, Nara Prefecture (350 km away from Tokyo) by Nara Waterworks was used. For comparison, standard strains from the Culture Collection of Algal Laboratory, Czech Academy of Sciences (CCALA; http://ccala.butbn.cas.cz/index.php) were used.

DNA extraction

Extraction of nucleic acids was performed using the DNeasy PLANT kit (QIAGEN Co.) and the protocol was referred to 'Purification of Total DNA from Plant Tissue (Mini Protocol)'.

DNA amplification

Polymerase chain reaction (PCR) amplification of a cyanobacterial 16S rDNA and the 16S-23S internal transcribed spacer (ITS) region was performed based on the method of the reference which identified species of cyanobacteria (Taton *et al.* 2003). PCR products were electrophoresed and it was confirmed that the target areas were amplified. Then, the PCR products were purified. After the purification, a second PCR was performed using several primer sets (forward: 16S27F, 16S378F, 16S1407, and 16S1114F; reverse: 16S781R, 16S784R, 16S1494R, and 23S30R) in the interior of the amplified product to obtain multiple fragments up to 1,000 bases from several 100 bases.

However, the second PCR product of *P. favosum* CCALA 882 was only strain fragments of several 100 bases, so that it was not possible to cover the whole sequence of targeted 16S rDNA and ITS region. Since the sequence of this strain was considered to have a large difference from the other strains, in order to obtain the full-length sequence, we designed new primers based on the fragments of some nucleotide sequences analyzed (Table 2). Also, the reaction conditions and the enzyme used were changed as follows: reaction mixture: 50 μL (total volume), 2 μL of extracted DNA, 5 μL 10× buffer for KOD-plus, 5 μL 2 mM dNTP, 2 μL 2 mM MgSO4, 1 μL KOD-plus, 1.5 μL of each primer. Reaction condition: one cycle of 1 min at 94 °C; 13 cycles of 15 s at 94 °C, 1 min at 68 °C; 26 cycles of 15 s at 92 °C and 30 s at 68 °C; and a final step of 5 min at 68 °C.

Table 2 | Designed primer sets for *P. favosum* CCALA 882 in second PCR

No. of set	Primer	Sequence (5′ → 3′)
1	16S700F	Ggtggagcggtgaaatgcgt
	16S1100R	Tgacttgacgtcatccccacctt
2	16S1100F	Tactgcggtgaatccgtt
	23S-R	Gtcccacttgtgatcttccaac

Sequencing and analysis

Fragments obtained were subjected to sequence analysis by ABI3130 Sequencer (Applied Biosystems, Inc.). Then, to confirm similarity, the obtained DNA sequences of nine cyanobacteria were analyzed by ClustalW (http://www.genome.jp/tools/clustalw/). Since these strains are morphologically very close, it was considered that comparison of 16S rDNA could not give enough information to estimate similarities between the strains. Therefore, it was decided to compare the entire length of the 16S-23S ITS region and 16S rDNA obtained by the sequence of about 2,000 bases in the analysis. A phylogenetic tree was created by a combination of NJ-plot (http://pbil.univ-lyon1.fr/software/njplot.html) and the ClustalW.

Characteristics of 2-MIB production

To investigate the temperature dependence of the 2-MIB production and proliferation of the strain isolated from the Tama River (*P.* sp. (2011, Tama River, 2-MIB)), culture experiments were carried out. After transferring the strain to the CT medium, an experiment was carried out under the condition of a 12 h light–dark cycle and light intensity of 3,000 lux, using a shaking incubator (TAITEC BR-43FH). Samples were shake-cultured for 3–6 weeks each at a temperature of 6 °C (the typical water temperature in winter), 14 °C (intermediate), 22 °C (the typical water temperature in summer), and we measured algal body number, the dissolved and total 2-MIB concentration every week. Production of 2-MIB was evaluated by 2-MIB amounts in algal body in order to avoid the influence of 2-MIB in culture medium (2-MIB which was emitted from algal body to the medium) which decreased by decomposition or volatilization.

RESULTS AND DISCUSSION

Investigation of riverbed

Benthic cyanobacteria collected from the riverbed stones were of thick and fine filamentous type. Among them, the fine type was estimated as *Homoeothrix janthina* that do not produce 2-MIB from the morphological characteristics. It was considered that the thick filamentous cyanobacteria had caused the 2-MIB since 2-MIB concentrations and count value of samples are proportionally related. The results of comparing the amount of 2-MIB and the count values of the *Phormidium* during 10 months from the start of the survey are shown in Figure 2. Since the amount of 2-MIB became higher as the count of thick filamentous cyanobacteria was large, it was estimated that the thick filamentous cyanobacteria is the cause of 2-MIB. Further, it was confirmed that the thick cyanobacteria produced 2-MIB by culturing in CT medium. *Achnanthes* genus and *H. janthina*, which dominated the algal assemblage, were also isolated and cultured, but production of 2-MIB could not be confirmed. Further, culture was performed based on the method of culturing actinomycetes (Japan Water Works Association 2011b), and it was confirmed that actinomycetes were not detected.

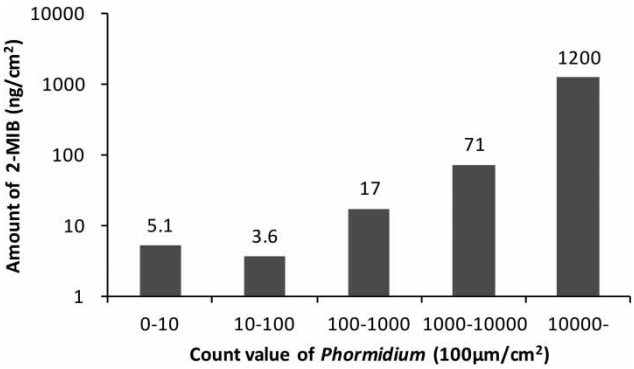

Figure 2 | Comparison of the amount of 2-MIB and the count value of the *Phormidium* during 10 months from the start of the survey.

Figure 3 shows a micrograph of the *Phormidium* which was estimated to be the cause of 2-MIB occurrence. The characteristics of the shape are as follows: it does not form akinete or heterocyst, and has a sheath, the width is about 7 μm and the length 4–5 μm, there is no constriction between cells, the tip of the trichomes are curved, and calyptra is present. From these features, this cyanobacterium was considered to be close to *P. autumnale*. However, it was very similar to both the geosmin-producing strain (shown in Table 1 as *P.* sp. (2008, Higashimurayama PP, geosmin)) and 2-MIB-producing strain (shown in Table 1 as *P.* sp. (2005, Murayama Reservoir, 2-MIB)) by comparing morphologically with our stored strain; also, some literature has classified *P. autumnale* as geosmin producing (Japan Waterworks Association 2011a). Since further identification by

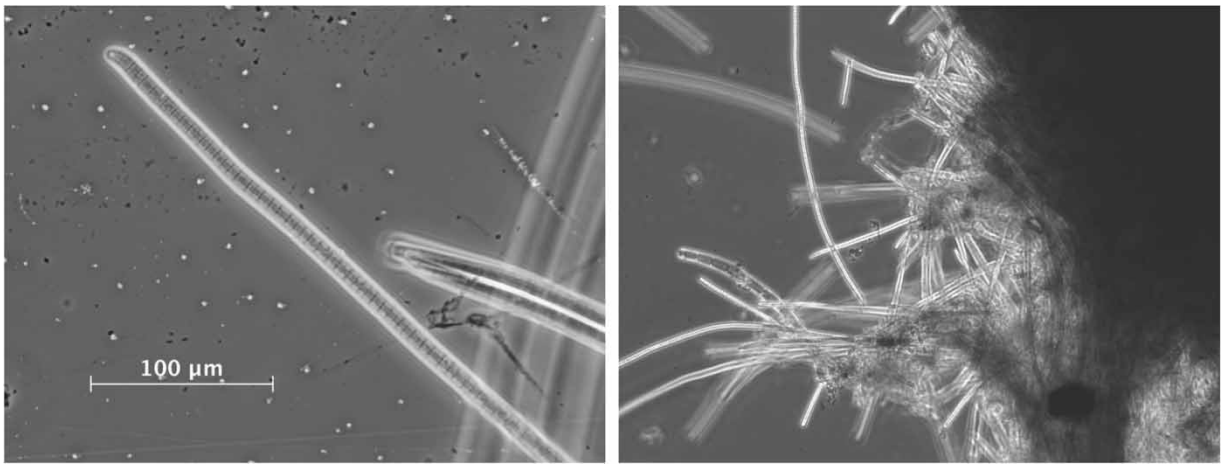

Figure 3 | Micrograph of *Phormidium* which was estimated to be the cause of 2-MIB occurrence. Left: isolated and cultured strain; right: collected mat from the riverbed stone in the Tama River.

microscopic observation was difficult, we attempted to identify species by genetic analysis.

Identification by genetic analysis

The results of analysis of homology with ClustalW for nine strains of *Phormidium* are shown in Table 3. Homology of 99.7% was obtained between *P.* sp. (2011, Tama River) and *P.* sp. (2008 Tama River). For the calculations of homology, we included the ITS region in which variation is larger than the 16S rDNA region in order to reveal the diversity within species. Since the results of homology including the ITS region were high, it was suggested these could be the same species.

Results of the obtained sequence of 2,000 bases for the standard strains are shown in Table 3 in comparison with strains collected at the Tama River. The standard strainS, *P. autumnale* CCALA143, 145, and 697, showed homology from 89.7 to 95.1% with *P.* sp. (2008, Tama River, 2-MIB).

In the case of *P. favosum* CCALA 882, the difference from other gene sequences was large, so it was suggested that *P. favosum* CCALA 882 is a distant species compared with the others. By comparing the DNA sequence of the Tama River and the Yoshino River (*P. autumnale* (Yoshino River, 2-MIB)), very high homology (99.9%) was obtained.

A phylogenetic tree of these obtained sequences is shown in Figure 4. Since the strains collected at the Tama River and *P. autumnale* taken at the Yoshino River and standard strains form a group apart from the standard strain of *P. favosum*, it was supposed the strains taken at the Tama River could be classified as *P. autumnale*. In addition, the strains of the Tama River formed the same group as *P.* sp. (2005, Murayama Reservoir, 2-MIB) and were placed at a position very close to the strain of the Yoshino River. Since *P.* sp. (2005, Murayama Reservoir, 2-MIB) was collected at the Murayama Reservoir in 2005, it was concluded that *P. autumnale* had existed in the Tama River at least since 2005. Furthermore, it was found that *P. autumnale* in the Tama River was the same species as those collected in the Yoshino River.

Table 3 | Similarity to *P.* sp. (2008, Tama River, 2-MIB)

Comparison objective	Number of different bases	Similarity (%)
P. autumnale (Yoshino River, 2-MIB)	3	99.9
P. sp. (2011, Tama River, MIB)	6	99.7
P. sp. (2005, Murayama Reservoir, 2-MIB)	19	99.1
P. autumnale CCALA 697	97	95.1
P. cf. *autumnale* CCALA 145 (geosmin)	102	95.0
P. sp. (2008, Higashimurayama PP, geosmin)	192	90.4
P. autumnale CCALA 143	207	89.7
P. favosum CCALA 882	316	84.2

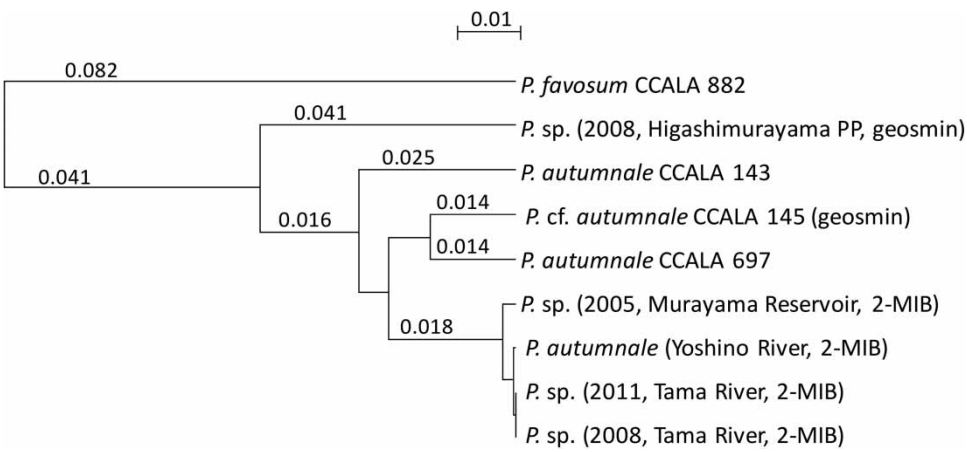

Figure 4 | Phylogenetic tree of obtained sequences.

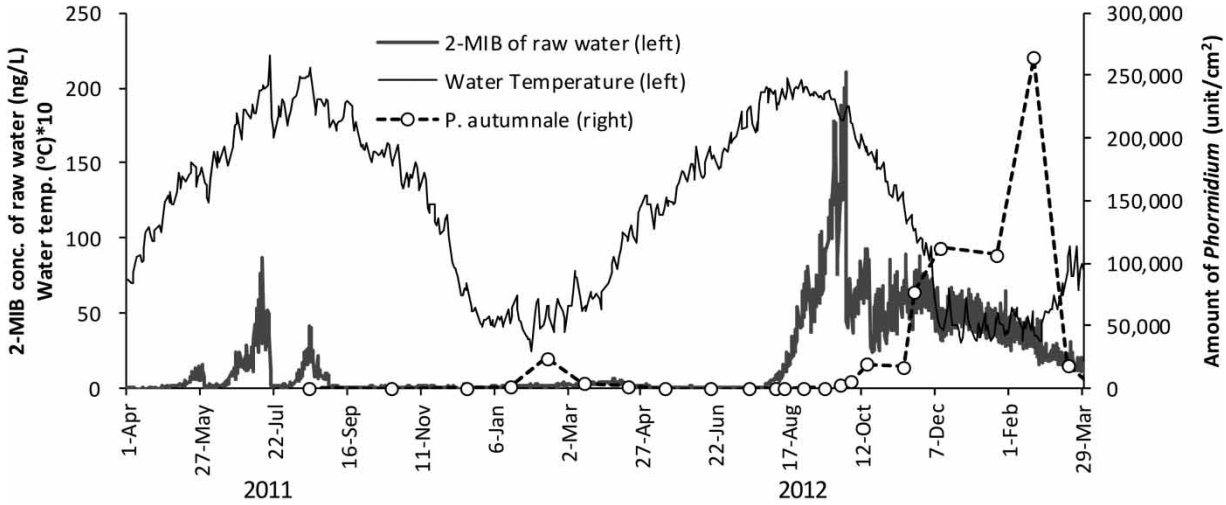

Figure 5 | Total number of *P. autumnale* collected at the Tama River and 2-MIB concentration and temperature of raw water of Ozaku Purification Plant.

Characteristic of 2-MIB production

The total number of *P. autumnale* collected at the sampling site and 2-MIB concentration of raw water of Ozaku Purification Plant are shown in Figure 5.

While the 2-MIB concentration was high in the summer, the *P. autumnale* produced a larger number in winter than in summer. From these results, it was considered that the *P. autumnale* is able to grow also in winter, but produces large amounts of 2-MIB during summer as compared to winter. Therefore, we investigated the temperature dependence of 2-MIB production and growth rate of algal cells of the *P. autumnale* by culture at different temperatures.

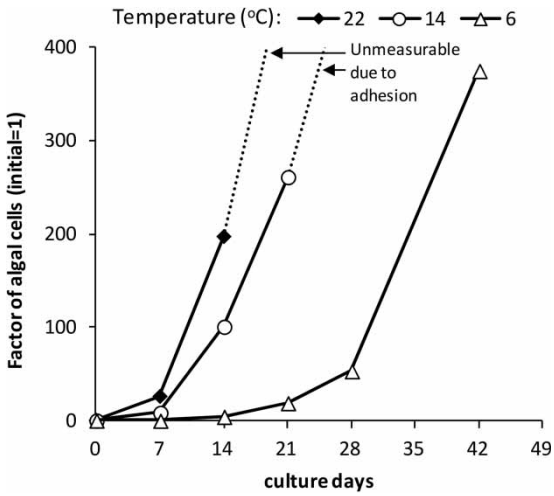

Figure 6 | Change in the number of algal cells during the batch culture experiments.

Temperature dependence of algal growth

Figure 6 shows the change in the number of algal cells per week during the batch culture experiments. Since accurate counts of algal cells had become difficult as algal bodies proliferated and adhered to each other, algal actual numbers were larger than the measured value. Also, measurement was not possible after 14 days at 22 °C and 21 days at 14 °C. Growth rate was larger in the order of 22, 14, 6 °C, and the number of algal cells after 14 days culture at 22 ° was about 40 times that at 6 °C. However, algal bodies grew gradually even at 6 °C, and reached 350 or more times the initial concentration after the 42 days of culture. Thus, it has been confirmed that the *P. autumnale* strain found in the Tama River has high growth rate in the summer, but it can grow slowly in the winter.

Temperature dependence of 2-MIB production

Figure 7 shows the change in the 2-MIB amount per unit body in algal cells (ng/100 μm) during the batch culture experiments. The amount of 2-MIB in algal unit cells was approximately constant at the 14 °C and the 6 °C cultures, but it was increased significantly at 22 °C. From

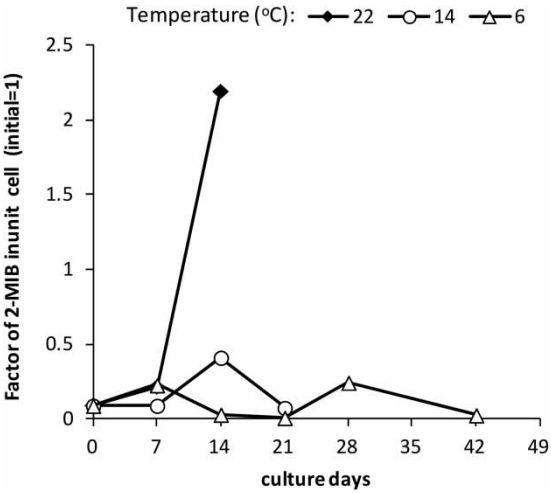

Figure 7 | Change in the 2-MIB amount per unit body in algal cells (ng/100 μm) during batch culture experiments.

these results, it was found that the *P. autumnale* strain can produce 2-MIB at low temperature. However, 2-MIB production increases significantly in the summer.

CONCLUSION

We investigated the cause of 2-MIB occurrence in the Tama River. It was found that benthic *Phormidium* was the source of 2-MIB; however, identification of the species of the *Phormidium* by microscopic observation had been difficult and therefore genetic analysis was carried out. In genetic analysis, in addition to *Phormidium* in the Tama River, musty odor producing benthic *Phormidium* strain in the Yoshino River, Nara Prefecture, which had been reported as the cause of 2-MIB, and standard strain of CCALA were used. Approximately full-length (about 2,000 bp) 16S ribosomal DNA region gene and ITS region were sequenced. From homology search of these sequences, 2-MIB producing *Phormidium* in the Tama River could be classified into *P. autumnale*. It was concluded that *P. autumnale* had existed in the Tama River at least since 2005. Furthermore, it was found that *P. autumnale* in the Tama River was the same species as those collected in the Yoshino River.

In addition, as a result of investigation of the occurrence in the Tama River and *in vitro* cultivation of *P. autumnale*, it was found that *P. autumnale* inhabiting the Tama River thrives and produces more 2-MIB at high water temperature (22 °C), but it can grow and produce 2-MIB even at low water temperature (6 °C).

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