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Identification and change in concentration of musty-odor compounds during growth in blue-green algae

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Abstract

The identification of musty-odor compounds and their concentration change during growth in blue-green algae were investigated in three species of *Oscillatoria* and six species of *Phormidium*. Among them, two species, *P*. M-71 and NIES-512, proved to be musty. In *P*. M-71, musty-odor substance was identified as geosmin and in *P*. NIES-512, 2-MIB, on purge/trap GC-MS analysis. In *P*. M-71, the concentrations of geosmin at 24 and 288 hours after initiation of growth were 70 and 240ng・(L culture)$^{-1}$, and 190 and 31 ng・(L culture)$^{-1}$・A_{720}^{-1}, respectively. In contrast to the result in *P*. M-71, 2-MIB in *P*. NIES-512, the concentrations of 2-MIB at 50 and 312 hours were 790ng and 49μg・(L culture)$^{-1}$, and 3.0 and 18pg・(10^5 cells)$^{-1}$, respectively. These results indicated that the concentration of 2-MIB in *P*. NIES-512 was 200 times higher than that of geosmin in *P*. M-71 at late exponential phase of growth and that the activity of 2-MIB synthesis increased as cells grew whereas that of geosmin synthesis decreased during growth.

Effect of growth temperature on 2-MIB synthesis was studied in P. NIES-512. The cells grew between 10-35°C and the concentration of 2-MIB was highest at 25°C. It should be noted that 2-MIB was significantly synthesized even at 10°C. When the cells were incubated under phosphate- and light-limiting condition (s), the concentration of 2-MIB was considerably high at exponential growth phase, and then reduced.

These results suggest that musty odor responsible for 2-MIB should occur even in wintertime and in oligotrophic reservoirs.

Introduction

Phosphate and nitrogen compounds are rich in waterbodies such as lakes and reservoirs where industrial and living wastes flow in and enhance the growth of photoautotrophic microalgae. Among them, waterblooms and scums of blue-green algae might reduce dissolved oxygen and sometimes produce toxins (Codd *et al*., 1989). Blue-green algae sometimes release musty and/or earthy odor (s) into water and impart them to drinking water and fish (Martin *et al*., 1991). In Japan, musty odor occurred in Habu reservoir, Aichi, since 1979 and was complained by the local residents who utilized the water as daily lives (Yamada *et al*., 1985). The pond for breeding catfish eutrophicated by the fish bait in USA was encountered by waterblooms of blue-green algae which imparted earthy odor to catfish flesh and made it unpalatable and unmarketable (Martin *et al*., 1991; Johnsen *et al*., 1996).

The nature of musty or earthy odor is responsible for 2-MIB (2-methyl isoborneol:1,2,7,7-tetramethyl-*exo*-bicycloheptan-2-ol) (Medsker *et al*., 1969) and geosmin (1,10-*trans*-dimethyl-*trans*-9-decalol) (Gerber and Lechevalier, 1965) (Fig. 1). Gerber and Lechevelier (1965) isolated geosmin as earthy-smelling substance from actinomycetes, *Streptomyces griseus* and 2-MIB was detected in blue-green algae as well as in *Streptomyces* by Medsker *et. al.* (1969). As little as 10ng・L^{-1} of geosmin and 5ng・L^{-1} of 2-MIB in water can be detected by olfaction (Dionigi, 1993). The guideline value for both geosmin and 2-MIB in drinking water established in 2003 by Japanese Ministry of Welfare and Labor is less than 10ng・L^{-1}.

It is reported that the members of the musty-odor compound producer are distributed in fungus such as *Basidiobolus, Chaetomium, Penicillium, Streptomyces, Actinomyces*, and *Microbispora* (Diongi, 1993), and in blue-green algae such as *Anabaena, Lyngbya, Oscillatoria, Phormidium*, and *Symploca* (Yagi, 1983). In blue-green

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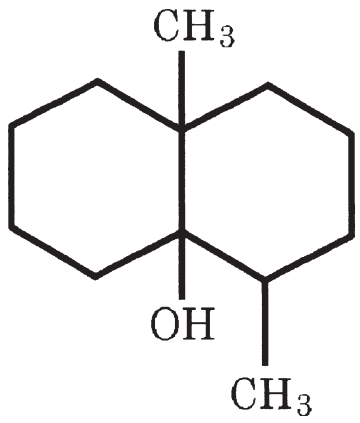
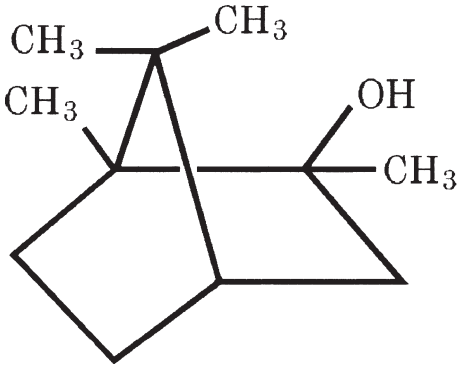


Geosmin(1,10-*trans*-dimethyl-*trans*-9-decalol)



2-MIB(2-methylisoborneol)

Fig. 1 Structure of geosmin and 2-MIB

algae, either geosmin or 2-MIB is synthesized whereas actinomycetes synthesize both musty-odor compounds (Yagi, 1983). It has been believed for a long time that musty odor in reservoirs was responsible for fungi, especially actinomycetes, but they inhabit the mud of ponds and the productivity of these compounds is relatively low. At present, it is evident that musty odor in reservoirs is essentially attributed to blue-green algae (Yagi, 1983).

It is unclear whether the cause of musty odor complained by residents near the rivers into which water reservoirs flow might be due to geosmin and/or 2-MIB. The present study describes the productivity of these musty-odor compounds in blue-green algae under various growth conditions.

Materials and Methods

Organisms and culture conditions

Blue-green algal strains obtained from IAM Culture Collection (presently termed, Institute of Molecular and Cellular Biosciences, The University of Tokyo) and NIES Collection (National Institute for Environmental Studies) were shown in Table 1. They were grown in BG11 liquid medium from which Na_2CO_3 and Na_2SiO_3 were omitted (Allen, 1968) at ca. 23°C under continuous illumination from a bank of fluorescent lamps (Toshiba, 18μE•m^{-2}•s^{-1}, on the surface of the culture flasks).

Growth conditions

The blue-green algae were grown at 25°C under the condition described above to evaluate the relationship between cell density [cells•(mL culture)$^{-1}$)] and absorbance at 720nm (A_{720}). Cell number was measured on

Table 1 Occurrence of musty odor in axenic blue-green algae

Organism	Strain	Musty or earthy odor
Oscillatoria		
neglecta	M-82	−
tenuis	M-243	−
sp.	M-117	−
Phormidium		
ambiguum	M-71	+
foveolarum	M-59	−
molle	M-77	−
mucicola	M-221	−
tenue	NIES-512	+
sp.	M-99	−

counting chamber (Thoma, Hirschmann, Germany) and A_{720}, by spectrophotometer (UV-2100, Shimadzu). Growth constant, k, was calculated by the method described by Kratz and Myers (1955).

The cells of *Phormidium tenue* NIES-512 were grown in a 3L flask containing BG11 medium under continuous illumination at 18μE•m^{-2}•s^{-1} with aerating sterile air through a syringe filter unit (pore size:0.2μm, Dismic-25, Advantec) at 10, 15, 20, and 25°C. An aliquot of cell suspension was withdrawn every twelve or twenty four hours to measure A_{720} value and to analyze a musty -odor compound (s).

P. ambiguum M-71 cells were grown at 25°C and sampled by the same method as described in *P. tenue* NIES-512. Since the cells aggregated too heavily to estimate cell number, A_{720} value in the strain was measured after sonication.

Growth and musty-odor compound analysis of the strain was also determined under phosphate- and/or light-limiting (1μM K_2HPO_4-BG11 medium and/or 3μE•m^{-2}•s^{-1}) conditions.

Analysis of musty-odor compounds

The cell suspensions of nine blue-green algae were examined whether musty odor could be smelled directly through the authors' noses or not (Table 1). Two strains, *P. tenue* NIES-512 and *P. ambiguum* M-71, out of nine, proved apparently to be musty (Table 1).

In both strains, cells were centrifuged at 6,080 x g for 5min at 4°C and the supernatant was passed through glass microfiber filter (GF/A, Whatman, England). The filtrate was gently transferred to a bottle to fill fully in order to exclude air and kept in ice until analysis.

Musty-odor compounds in *P. tenue* NIES-512 and *P. ambiguum* M-71 were collected by purge/trap (Model 4560, OI Company) method and analyzed by GC-MS (Model 5973N, Agilent) described by Johnson *et al*. (1996) at Shizuoka Chemical Analysis Center.

Results and Discussion

Identification of musty-odor compounds in blue-green algae

Three axenic strains of *Oscillatoria* and six axenic strains of *Phormidium* (Table 1) were grown and their musty odor was examined directly by authors' noses since it is reported that man is able to smell musty odor at 5ng•(L culture)$^{-1}$ and 10ng•(L culture)$^{-1}$ for 2-MIB and geosmin, respectively (Diongi, 1993). Among nine strains, two strains, the cell suspensions of *P. ambiguum* M-71 and *P. tenue* NIES-512 were odorous whereas both smell was slightly different from each other (Table 1). Purge/trap and GC/MS analysis revealed that geosmin in *P. ambiguum* M-71 and 2-MIB in *P. tenue* NIES-512 were responsible for their musty-odorous nature (Table 1). In 1985, Yamada *et al* (1985) reported that *P. tenue* NIES-512 produced 2-MIB, but this is the first report that *P. ambiguum* M-71 produces odorous compound, geosmin (Table 1).

Changes in 2-MIB and geosmin concentration during growth

Fig. 2 shows the relationship between cell number and A_{720} for *P. tenue* NIES-512 grown at 25°C under continuous illumination. In lag time phase, exponential phase, and stationary phase during growth, the cell number and their optical density, A_{720} were in proportion (Fig. 2) and gives the formula: Cell No (cells•mL^{-1}) = 3.3 x 10^7 x A_{720} + 1.5 x 10^7 (r=0.98).

The change in concentration of 2-MIB during growth for *P. tenue* NIES-512 was shown in Fig. 3. As the cells grew, the concentration of 2-MIB in cell suspension increased (Fig. 3a), giving the concentration of 46μg•(L culture)$^{-1}$. It is, furthermore, evident that 2-MIB concentration on the basis of cell number also increased along with cell proliferation (Fig. 3b). After

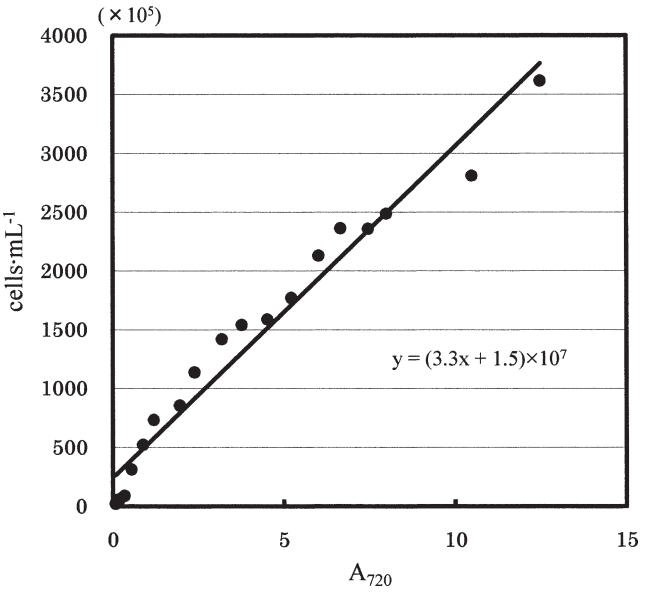


Fig. 2 Cell Number as a function of A_{720} during growth of *Phormidium tenue* NIES-512 at 25°C.
The cells were grown in BG11 medium at 25°C under continuous illumination (18 E•m^{-2}•s^{-1}), and cell number and A_{720} were measured at intervals.

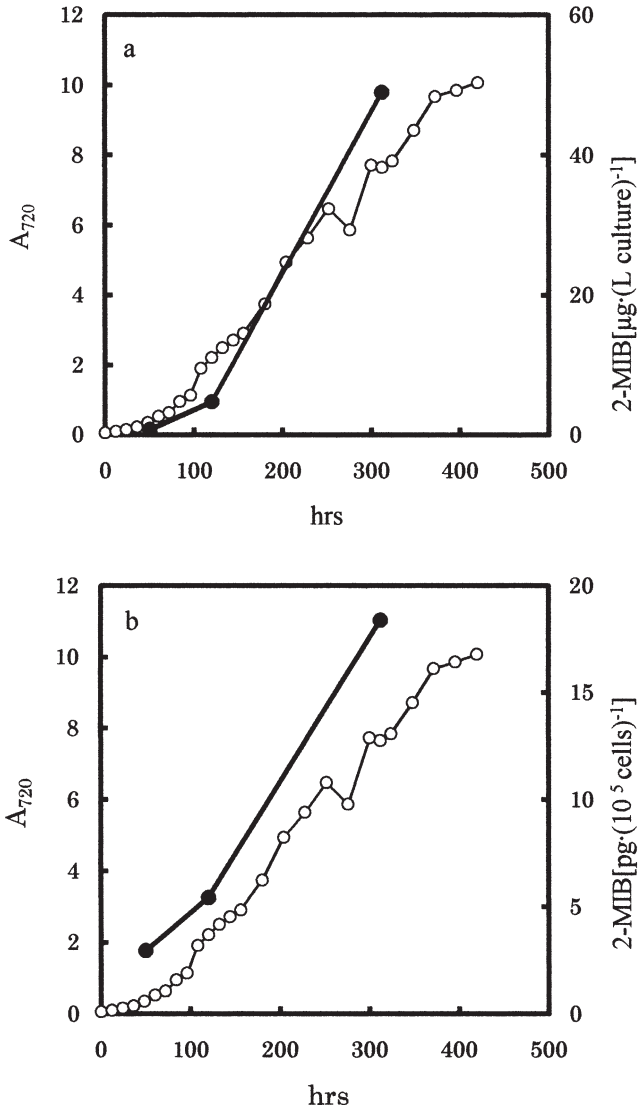


Fig. 3 Time dependency of 2-MIB concentration during growth of *Phormidium tenue* NIES-512.

The cells grown under the same condition as described in Fig. 2 were withdrawn at intervals, and 2-MIB and cell number were measured.

a The concentration of 2-MIB was expressed as $\mu g \cdot (L\ culture)^{-1}$.

b The concentration of 2-MIB was expressed as $pg \cdot (10^5\ cells)^{-1}$.

312 hours of growth, i. e. late exponential growth phase, 2-MIB concentration reached $18pg \cdot (10^5\ cells)^{-1}$ (Fig. 3b). These results indicate that 2-MIB biosynthesis is more active in the cells of late exponential-stationary phase than in those of exponential phase. This transition pattern of 2-MIB production during growth is very typical of secondary metabolism in plants.

Since the cells of geosmin producer, *P. ambiguum* M-71, in a batch culture aggregated tightly during growth, they were treated with sonication before geosmin analysis. It was not possible to count a cell number owing to the invisibility of septa inside trichome of *P. ambiguum* M-71 under phase contrast microscope. Time course of geosmin concentration during growth of *P. ambiguum* M-71 is presented in Fig. 4. Geosmin concentration in cell suspension based on the volume of the culture gradually increased up to $240ng \cdot (L\ culture)^{-1}$ at 288 hours (Fig. 4a). On the other hand, the concentration showed a marked decline on the basis of A_{720}, giving the

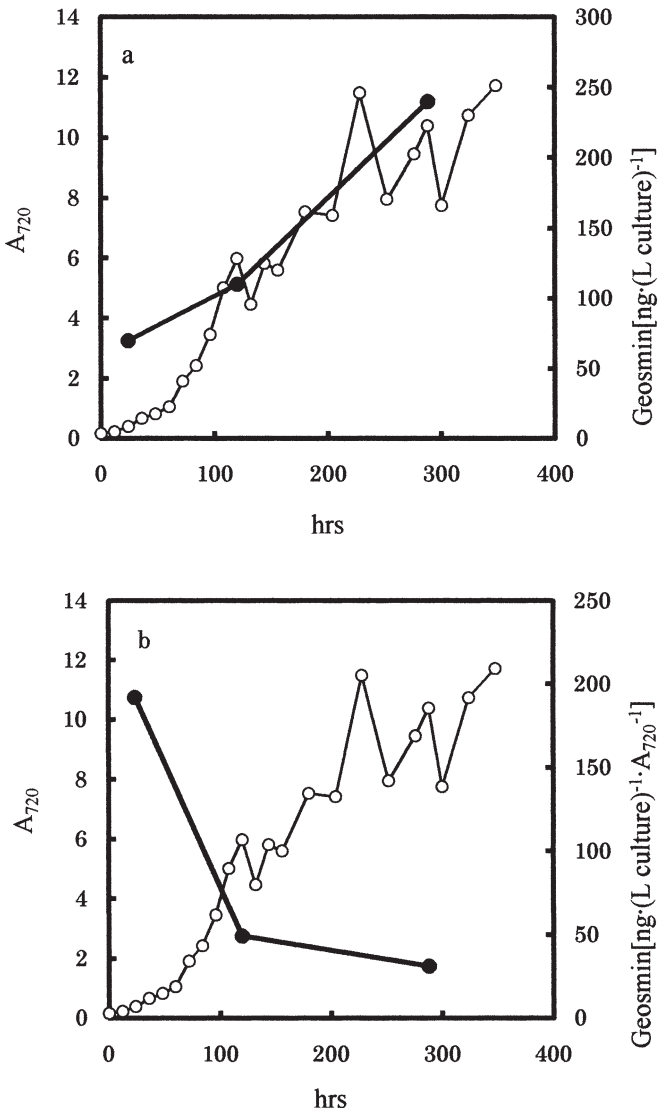


Fig. 4 Time dependency of geosmin concentration during growth of *Phormidium ambiguum* M-71.

The cells grown under the same condition as in *Phormidium tenue* NIES-512 (Fig. 2) were withdrawn at intervals, and geosmin and A_{720} were measured.

a The concentration of geosmin was expressed as $ng \cdot (L\ culture)^{-1}$.

b The concentration of geosmin was expressed as $ng \cdot (L\ culture)^{-1} \cdot A_{720}^{-1}$.

concentrations of 190 and 31ng•(L culture)$^{-1}$•A_{720}^{-1} at 24 and 288 hours, respectively (Fig. 4b).

The concentration of 2-MIB on the basis of a volume of cell suspension in *P. tenue* NIES-512 was ca. 10 times as high as that of geosmin in *P. ambiguum* M-71 at lag phase (Figs. 3a and 4a), furthermore, at late exponential phase, the former was ca. 200 times as high as that of the latter (Figs 3b and 4b) and the sensitivity of olfaction to geosmin and 2-MIB in human are almost the same, suggesting that 2-MIB rather than geosmin should be responsible for complaints on malodor and taste of drinking water. It is possible that the transition pattern of geosmin and 2-MIB observed during growth in the present study could be common in other blue-green algae which produce these off-flavor compounds, but more detailed investigations in many blue-green algae should be done to certify this hypothesis.

Effect of growth temperature on 2-MIB production

Fig. 5 shows the growth curves of *P. tenue* NIES-512 at 10, 15, 20, 25, 30, and 35°C under continuous illumination. The strain grew well at 25 and 30°C, and even did at 10°C although the growth rate was very low (Fig. 5). In Fig. 6, growth constant as a function of growth temperature was plotted. Although *P. tenue* NIES-512 could grow at various temperatures studied here, they grew much faster at 25 and 30°C than in any other temperatures.

Effect of temperature on the synthesis of 2-MIB in *P. tenue* NIES-512 was shown in Fig. 7. Since 2-MIB is extremely volatile at 30 and 35°C, it is probable to underestimate the concentration of 2-MIB in cell suspension of *P. tenue* NIES-512. Experiments were, there fore, made between 10 and 25°C. At 10, 15, and 20°C, 2-MIB concentration increased slightly (Fig. 7). In contrast to this, 2-MIB concentration in *P. tenue* NIES-512 strikingly increased at 25°C. This result indicates that the problems of musty-odor synthesized by blue-green algae would be more eminent from late spring to early autumn in Japan. It should be emphasized that 2 -MIB is yet synthesized even at 10°C.

Effect of phosphate and light on 2-MIB production

It is well recognized that phosphate as well as inorganic nitrogen regulates the growth of photoautotrophic microorganisms. Fig. 8 shows the effect of phosphate- and/or light-limiting condition on 2-MIB production in *P. tenue* NIES-512. After 120 hours, 2-MIB concentrations of cell suspension were 2.2 and 1.7ng•(L culture)$^{-1}$ under

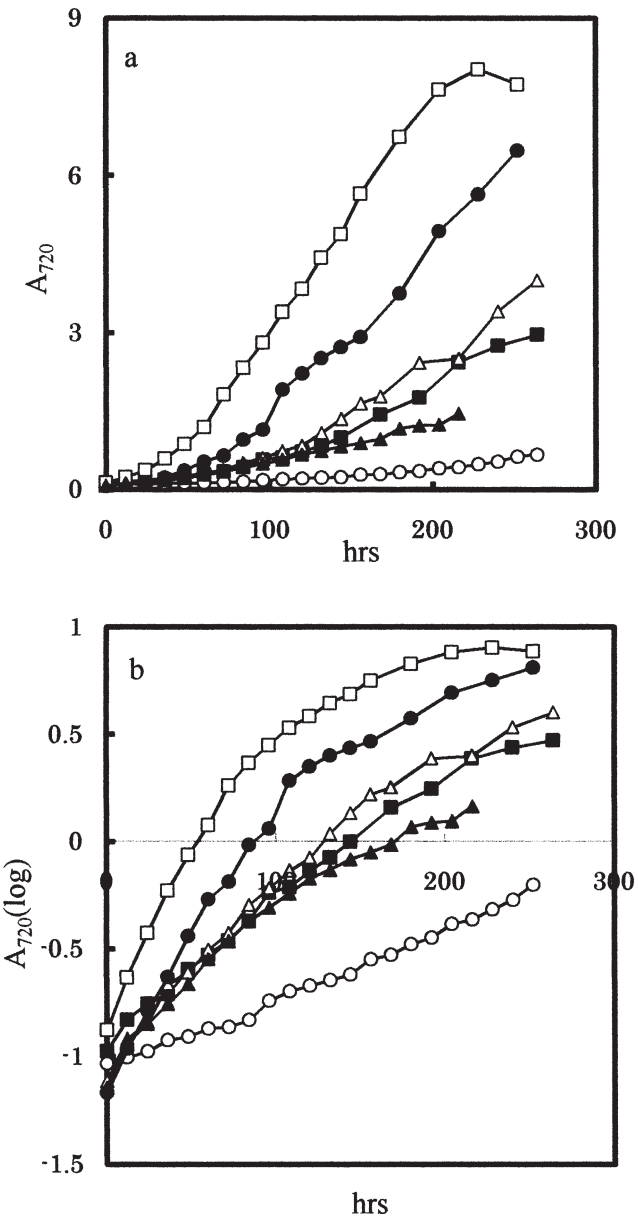


Fig. 5 Growth curves of *Phormidium tenue* NIES-512 at 10°C(○), 15°C(■), 20°C(△), 25°C(●), 30°C(□), and 35°C(▲)

The cells were grown under the same condition as in Fig. 2 except temperature.

phosphate- and light-limiting growth condition, respectively, where the concentrations were significantly higher than that of the cell suspension grown in BG11 medium , but were in decline after 200 hours (Fig. 8), suggesting that the cells under both growth-limiting conditions should decompose owing to the lack or shortage of phosphate and light energy.

The concentration of phosphate in lakes and reservoirs where musty-odorous compound producers are dominant is usually below 0.1μM (data not shown), and

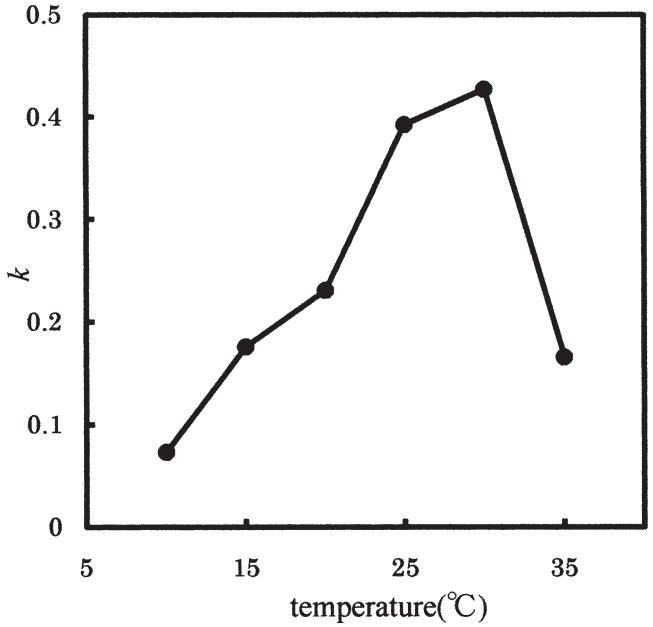


Fig. 6 Temperature dependent growth constant (k) in *Phormidium tenue* NIES-512.
k's were calculated from the data shown in Fig. 5.

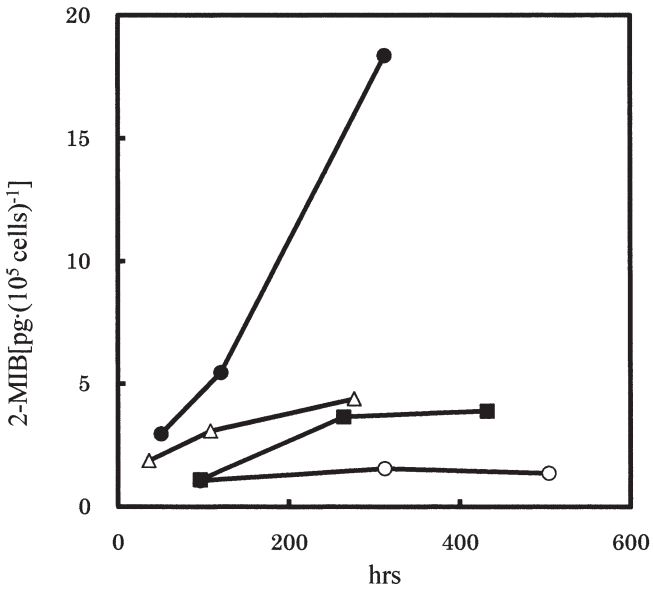


Fig. 7 Time dependency of 2-MIB concentration during growth of *Phormidium tenue* NIES-512 at 10°C(○), 15°C(■), 20°C(△), and 25°C(●).
The cells grown under the same condition as in Fig. 2 except temperature were withdrawn at intervals, and 2-MIB and cell number were measured.

hence their multiplication would be inhibited by the shortage of phosphate. Even under such an unfavorable growth condition, blue-green algae such as *P. tenue* NIES-512 still synthesize 2-MIB and bring about off-flavor episode.

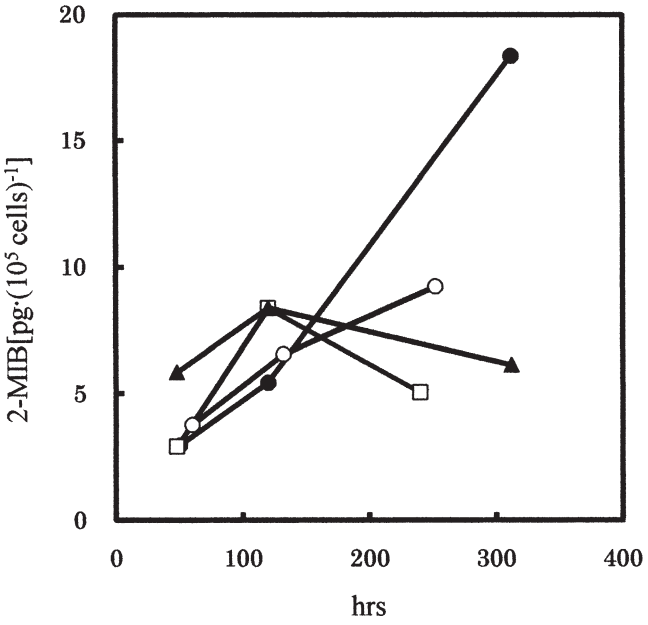


Fig. 8 Time dependent change in 2-MIB concentration during growth of *Phormidium tenue* NIES-512 under phosphate-limiting and photon-limiting conditions.
The cells grown under the same condition as in Fig. 2 except K_2HPO_4 concentration and PFD (Photon Flux Density) were withdrawn at intervals, and 2-MIB and cell number were measured.
The cells grown at 25°C under the conditions as follows:
●: 180μM K_2HPO_4+18μE•m^{-2}•s^{-1}
□: 1μM K_2HPO_4+18μE•m^{-2}•s^{-1}
▲: 180μM K_2HPO_4+3μE•m^{-2}•s^{-1}
○: 1μM K_2HPO_4+3μE•m^{-2}•s^{-1}

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要　旨

藍藻のカビ臭物質の同定と生育に伴う濃度変化に関する研究

岩瀬理子・安部俊彦

藍藻オシラトリア3種とフォルミディウム6種において，カビ臭物質の同定と生育に伴う濃度変化を検討した．パージトラップGC-MS分析により，M-71株からはカビ臭物質であるジェオスミンが，NIES-512株からは2-MIBが検出された．ジェオスミン濃度は，培養開始後24および288時間においてそれぞれ190，31 ng・(L culture)$^{-1}$・A_{720}^{-1}であった．一方，2-MIB濃度は，培養開始後50および312時間においてそれぞれ3.0，18pg・(10^5 cells)$^{-1}$であった．これらの結果から，定常期における2-MIB濃度はジェオスミン濃度の200倍以上であり，また，細胞あたりの2-MIB濃度は細胞増殖に伴って増大し，逆にジェオスミン濃度は低下することが示された．

生育温度と2-MIBの生産量の関係を調べたところ，NIES-512株は10-35℃で増殖し，25℃における2-MIB濃度がもっとも高かった．特筆すべきことに10℃においても2-MIBは生産された．

以上の結果から，冬季であっても貧栄養条件であっても，カビ臭の主な原因物質である2-MIBは発生することが示唆された．