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The New Practical Method For Screening Musty-odor / Non-odor Species In Oscillatoriales (Cyanophyta)

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ABSTRACT:

A musty-odor problem, caused by cyanobacteria belonged to Oscillatoriales, is not understood enough in reservoirs. One of causes, Oscillatoriales has morphological similar species including musty-odor / non-odor species. Musty-odor species cannot be identified by present morphological identification technique or a separation method of green / brown strains using a fluorescence microscope. Recently, Komárek and Anagnostidis have proposed a new classification system of Oscillatoriales. It is revealed that the detailed classification research based on this classification system can determine a separation of musty-odor / non-odor species. As a result, cases of successful separation of musty-odor / non-odor species in Oscillatoriales have increased. However, classification and identification of Oscillatoriales based on a new classification system is too hard to be directly applied in a regular phytoplankton monitoring, since it is performed by focusing on motility of trichome and detailed morphological characteristics at high magnification with microscope. In order to implement the daily water quality management, an elucidation and measures of musty-odor phenomenon in dam reservoirs, it is necessary to develop simple method for identification of musty-odor species, which is based to a new classification systems proposed by Komárek, et al. This paper introduces the consideration on a development of new morphological identification method to discriminate musty odor-species from Oscillatoriales. This method will be possible for reservoirs to predict risk of a musty-odor outbreak. This identification has been characterized by having adopted four new standards in a conventional standard. We assumed four conditions as classification standards.

Keywords: Oscillatoriales (Cyanophyta), morphological identification, 2-Methylisoborneol

1. INTRODUCTION

To Japanese tap water, we expect to be hygienic and good taste water. Under such circumstances, 2-Methylisoborneol (hereinafter 2-MIB) and geosmin, which are responsible for musty odor, were established as quality demand standards of a tap water based on the Water Supply Act of March 2003. Generally, a musty-odor producing materials are produced from some Cyanophyta and Actinomycetes. The abnormal bloom of Cyanophyta associated with the eutrophication of lakes, swamps, dams and reservoirs is often the cause of the production of such materials. Water suppliers and dam managers have implemented various measures to preserve water quality as a measure to solve musty odor problems. Although such measures have produced definite effects, they have not provided fundamental solutions. One of the reasons that musty odor problems have not been eliminated is the problems concerning the morphological identification of algae, including, *Phormidium tenue*, belonging in *Oscillatoriales*, Order Nestocales and Class Cyanophyceae (hereinafter *Phormidium tenue*), which is considered to be the algae that cause abnormal odor and taste. In many cases, the problems of 2-MIB induced abnormal odor and taste in dams and reservoirs are thought to be originated in *Phormidium tenue*. Yet, some have pointed out that some organisms in this species produce 2-MIB, while some do not. Studies have verified that they belong in genetically different groups. As a method to distinguish them at fields, methods such as focusing on the differences in antenna pigment have been suggested, but exceptions have also been reported. Therefore, it has been concluded that accurate screening of these two is difficult. Thus, as in many cases, a clear relationship between variations in the cell density of *Phormidium tenue* and the 2-MIB concentration is not recognized. As a result, studies have neither fully revealed the mechanisms of the onset of abnormal odor nor the taste and the effects of reducing them. This situation is forcing people at fields where *Phormidium tenue* has been found, but no 2-MIB has been detected, to conduct management while being anxious about the onset of problems which could occur at any time. In 2005, Komárek and Anagnostidis suggested a new classification system for Cyanophyceae based on unique viewpoints which differ from conventional perspectives. Studies in Japan are also starting to report cases suggesting the possibility of screening musty-odor / non-producing species based on detailed algae classification research conducted using the method of Komárek and Anagnostidis. Yet, it is difficult to use this morphological identification of Cyanophyceae directly based on the new classification system at actual fields because it is conducted by focusing on detailed morphological characteristics of algae and the mobility of trichome. Therefore, it is necessary to develop simplified identification methods so that people at actual fields can identify musty-odor producing species with their identification techniques and in their identification environment using the classification method suggested by Komárek et al. in order to use the method for daily water quality management and finding causes of the musty odor phenomenon. This paper is introducing the simple identification manual for *Phormidium tenue* (hereinafter manual) that the authors have been working to develop since 2011 and reporting the results of verifications conducted using the data obtained from 2011 to 2012.

2. IDENTIFICATION MANUAL

2.1. Overview of the simple identification manual

The authors prepared the simple identification manual which is intended to separate *Phormidium tenue* into musty-odor and non-producing species based on comparisons between conventional morphological identification methods and morphological

identification based on "Komárek and Anagnostidis, 2005", research of published literature on musty-odor causing algae, and results of morphological identification at Kasumigaura. This manual adopted the following four conditions as classification standards which were prepared by organizing the findings of past research in addition to conventional morphological identification standards for *Phormidium tenue* which is based on "cell diameter: 5 μm or less and the L/W ratio of a cell (the ratio between the length (L) and the width (W) of a cell): 6 or less." This enables the selection of four types of musty-odor producing species from 14 species of *Phormidium tenue*, which used to be identified as one species without conducting detailed morphological identification, and distinguishing ten remaining species as non-producing species. (See Fig 1 and Table 1.)

(i) Cell diameter: 0.5 to 2.5μm
(ii) L/W ratio of a cell: 2 to 5
(iii) Deformation at the tip of a cell: None
(iv) Cleavage on cell wall: Available

Based on the above, 2-MIB producing algae can be distinguished among *Phormidium tenue* only by simple observation under a microscope without depending on the detailed identification of species by Cyanophyceae classification experts who are very limited in number.

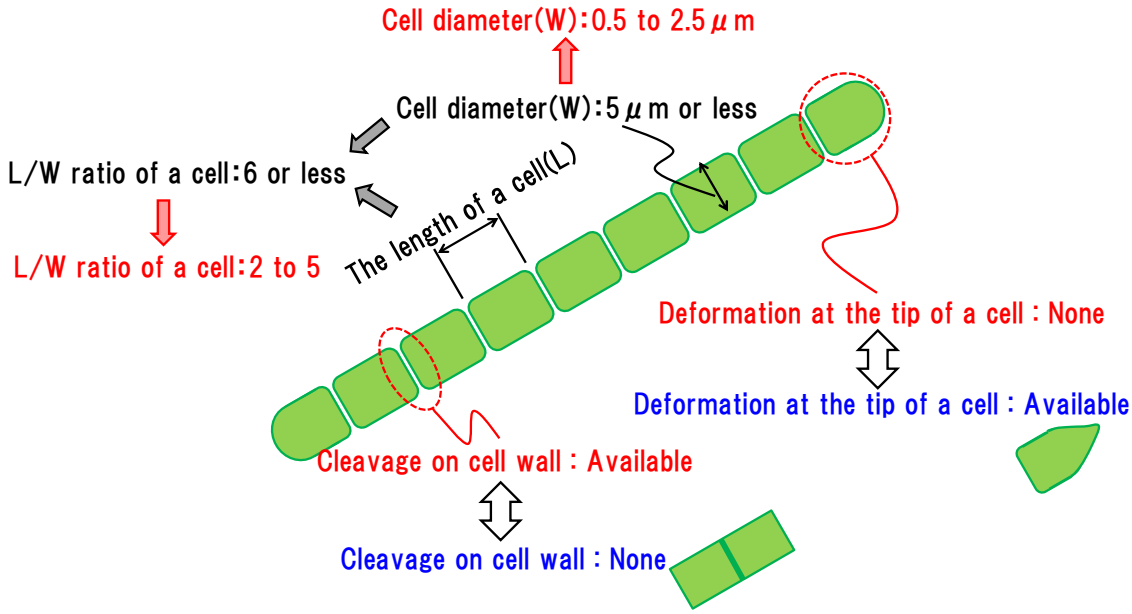

Figure 1. Point of interest on the simple identification manual

2.2. Expected effects of using the simple identification manual

This simple identification manual cannot reveal names of existing algae species. Its feature is to enable the screening of species which produce musty odor and ones which do not produce the odor. There are mainly two effects expected from using this manual. One is that it enables the identification and forecast of the risks of musty odor generating in higher accuracy than conventional methods in regular water quality monitoring. This means that incorporating this manual into daily water quality monitoring (e.g. periodical water quality investigation) in dams and reservoirs where *Phormidium tenue* is found but no musty odor is detected, enables identification of whether the detected *Phormidium tenue* belongs in the group of musty-odor producing species.

Table 1. Correspondence between the species name and point of interest on the manual

		Deformation at the tip of a cell					
		Available			**None**		
		The species name	Cell diameter 0.5～2.5μm	L/W ratio 2～5	The species name	Cell diameter 0.5～2.5μm	L/W ratio 2～5
Cleavage on cell wall	**Available**	*Komvophoron cf. minutum*	×	×	***Pseudanabaena catenata***	○	○
		Komvophoron schmidlei	×	×	***Pseudanabaena limnetica***	○	○
		Leptolyngbya tenuis	○	○	***Pseudanabaena galeata***	○	○
		Geitlerinema sp.	○	○	***Pseudanabaena biceps***	○	○
					Planktolyngbya limnetica	△	△
					Leptolyngbya sp.	△	△
	None	*Geitlerinema nematodes*	○	△	*Geitlerinema amphibium*	○	○
		Geitlerinema splendidum	○	○	*Limnothrix redekei*	○	△
					Limnothrix planktonica	○	○
					Jaaginema gracile	×	×

The species name : The species name that is based on the identification by experts.
(Gray Hatching is 2-MIB producing algae)

Thick line box : Grope of 2-MIB producing algae that is based on the simple identification manual

○ : In range、△ : Both、× : Out of range

In addition, this manual enables the forecasting the increasing risks of the onset of musty odor if a group of musty-odor producing species starts to be detected although no such species were found in the same dams and reservoirs before. Such forecasts can be used for the implementation of detailed investigations and the provision of smooth communication and warnings to relevant organizations. This is an extremely beneficial in water quality management. The other point is the possibility of progress in revealing mechanisms at the onset of musty odor which used to have many unknown aspects. As mentioned earlier, in many cases, no clear relationship is recognized between variations in the cell density of *Phormidium tenue* and the concentration of 2-MIB which are identified using the currently available morphological identification methods. Yet, if the onset of odor can be identified by specifically extracting the group of musty-odor producing species, analyzing its responses to environmental factors of the same time enables one to specify the preferred environment for the bloom of musty-odor producing species, which can then be used for effective measures to prevent the bloom. A case in which morphological identification was actually conducted and examined based on this manual is introduced below. *Phormidium tenue* is being detected almost throughout the year in dam K in Japan. Yet, the onset of its bloom is not necessarily synchronized with the bloom of 2-MIB. Similarly, no clear relationship is found between the peak value of the cell density of *Phormidium tenue* and the peak value of the 2-MIB concentration. Therefore, the mechanism of the onset of the musty odor had not been revealed. Therefore, *Phormidium tenue* at the surface was identified and counted at the center of the lake (see Fig 2) based on this manual. As a result, 2-MIB was found in two timeframes, from early April to early July (period (i)) and from mid-August to late September (period (ii)). A clear difference found here was that in the period (i), a group of musty-odor producing species was found, whereas no musty-odor producing species were found in the period (ii). This suggests that the bloom of the group of musty-odor producing species in the reservoir water became the cause of the production of 2-MIB in the period (i), while 2-MIB was detected in the water of the reservoir although the group of musty-odor producing species was not present in the water in the period (ii). Also, algae investigation found no musty-odor producing algae besides *Phormidium tenue*.

This indicates that the musty odor in period (ii) occurred because there was a source of odor outside of the water of the reservoir, and 2-MIB was supplied to the water in the reservoir in a dissolved state from the source. Given this finding, people at dam K are now looking for sources of the odor outside of the reservoir and investigating and examining the mechanisms of the supply of musty odor from the source to the lake water. As discussed above, the development of the simple identification manual is necessary for improving the level of water quality management in dams and reservoirs (early detection of problems and the establishment of better methods to counteract problems).

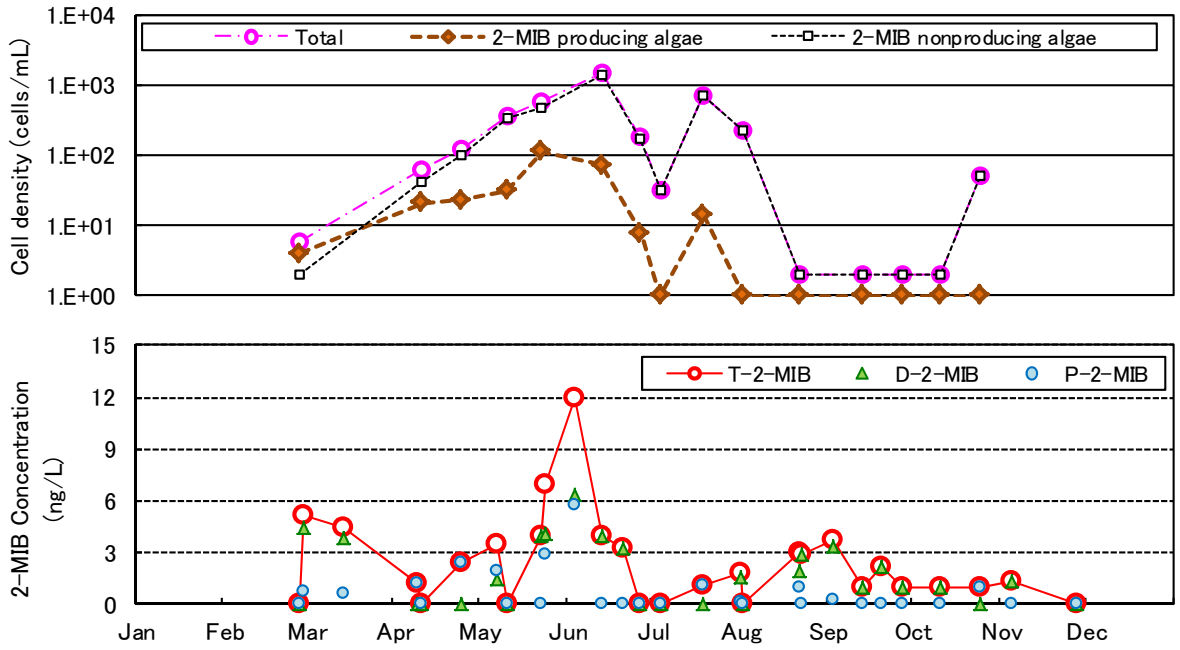

Figure 2. Correspondence relationship between identification and count that is based on the simple identification manual and 2-MIB concentration

T-2-MIB：It means total 2-MIB. We extract the 2-MIB algal cells by sonication method, to analyze the 2-MIB of all of the water.

D-2-MIB：It means dissolved 2-MIB. The obtained value by analyzing the 2-MIB, which are dissolved in water

P-2-MIB：It means particulate 2-MIB. The difference between the D-2MIB and T-2-MIB.

3. VERIFICATION OF THE SIMPLE IDENTIFICATION MANUAL

3.1. Method of verification

Verifications are conducted based on the method below to check whether musty-odor producing species can be accurately identified by people at fields based on this manual. “People at fields” as used here means engineers in private companies that are identifying, counting, and analyzing algae as a part of periodical water quality examinations in reservoirs. They are expected to be capable of identifying and counting algae using classification books and pictorial references which are commonly used today.

- Eighteen samples, including the *Phormidium tenue* collected from August to October, 2011 and from July to August, 2012 in dam K in Japan (ten samples from 2011 and eight samples from 2012) were sent to the people at fields, who examined them under microscopes based on this manual.

- In the microscope examination, they identified *Phormidium tenue* and then examined (i) cell diameter, (ii) the L/W ratio of cells, (iii) deformation at the tip of the cell, and (iv) the presence of cleavage on cell walls; and took photographs of targeted algae.
- Results obtained in the microscope examination were plotted in the recording format shown in Fig 3.
- Photographs taken by the people at fields were examined, and *Phormidium tenue* in the sample was identified based on "Komárek and Anagnostidis, 2005."
- The result of the identification by "Komárek and Anagnostidis, 2005" was compared with the results of the microscope examination conducted by people at fields to check their ability to accurately identify algae.

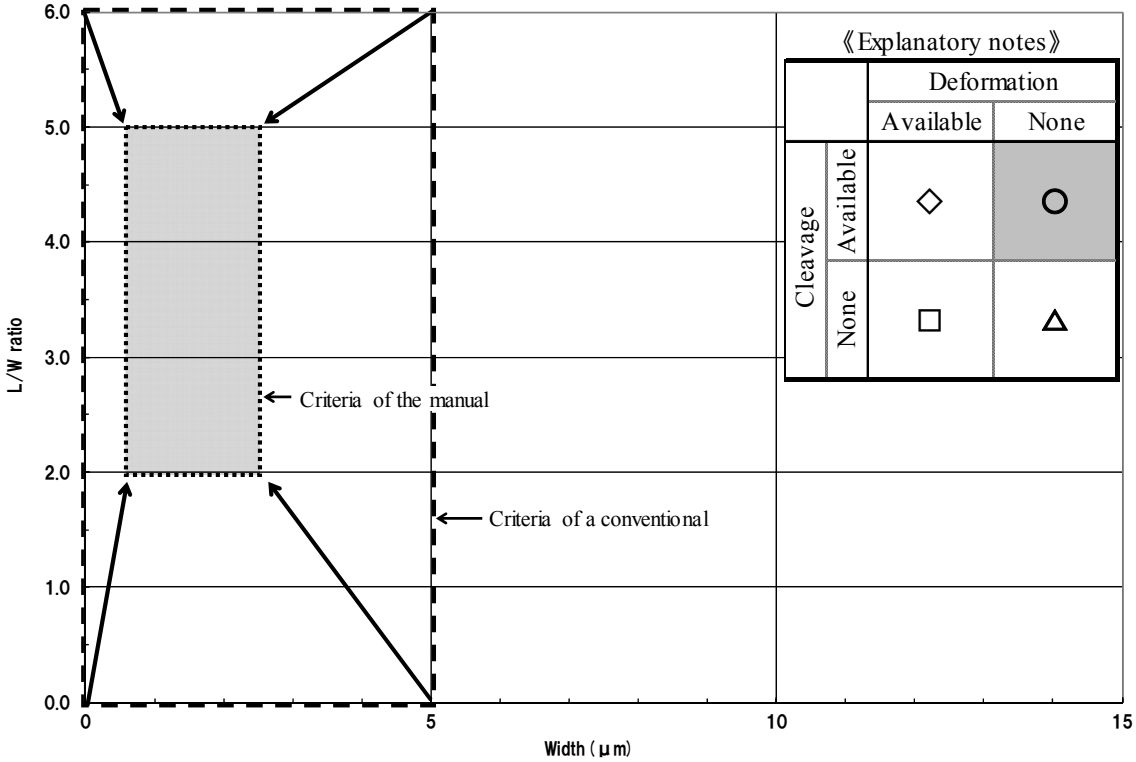

Figure 3. The recording format for verification

3.2. Result of verification

The result of the verification of the samples is described below. Fig 4 shows entries of the results in a recording format, and Fig 5 shows species that were identified in this verification.

- A hundred and twenty five filamentous algae were found within a size category which would be classified as *Phormidium tenue* in conventional classification systems.
- The morphological identification of these 125 filamentous algae by the authors based on the photographs taken by the people at fields found nine species shown in Table 2. Among them, *Pseudanabaena catenata* was the only 2-MIB producing species.
- Sixteen filamentous algae were recognized as 2-MIB producing species, and 109 filamentous algae were recognized as non-2-MIB producing species based on the manual.
- The result of the comparison between the 16 filamentous algae recognized as 2-MIB producing species and the result of additional morphological identification conducted

by the authors is as follows: Nine filamentous algae belonged in the genus *Pseudanabaena* (*Pseudanabaena catenata*: 2-MIB producing species), and remaining six filamentous algae belonged in the genus *Geitlerinema* (*Geitlerinema nematodes*: non-2-MIB producing species), the genus *Jaaginema* (*Jaaginema gracile*: non-2-MIB producing species), and the genus *Leptlyngbya* (*Leptlyngbya* sp.: non-2-MIB producing species).

- Among the non 2-MIB producing species listed above, *Geitlerinema nematodes* is supposed to be classified as "Deformation at the tip of a cell: Available, Cleavage on a cell wall: None," and *Jaaginema gracile* as "Deformation at the tip of a cell: None, Cleavage on a cell wall: None." However, the people at fields identified both of them as "Deformation at the tip of a cell: None, Cleavage on a cell wall: Available."
- The comparison of the 109 filamentous algae identified by the people at fields identified as non-2-MIB producing species with the result of the morphological identification by the authors found that all of them were non-2-MIB producing species.

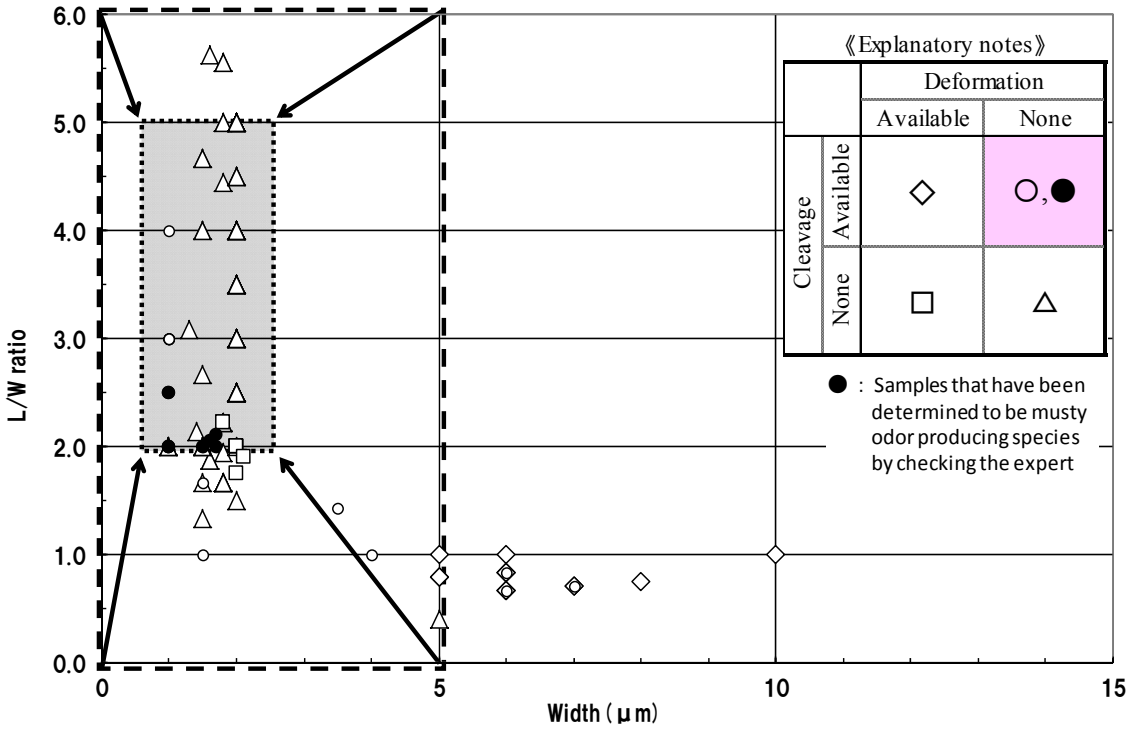

Figure 4. The result of the entry into the recording format

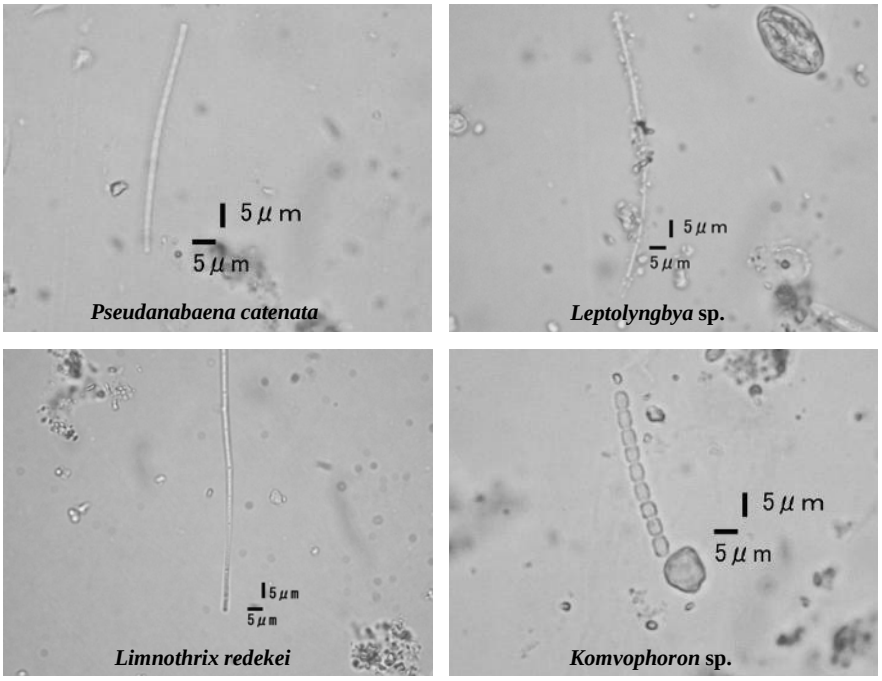

Figure 5. The main appearance species has been confirmed this research

Table 2. The verification of classification results between expert and people at fields

		Deformation at the tip of a cell					
		Available			**None**		
		The species name	Cell diameter 0.5～2.5μm	L/W ratio 2～5	The species name	Cell diameter 0.5～2.5μm	L/W ratio 2～5
Cleavage on cell wall	**Available**	*Geitlerinema sp.*	○	○	***Pseudanabaena catenata***	○	○
		Komvophoron sp.	×	×	*Leptlyngbya sp.*	△	△
	None	*Geitlerinema nematodes*	○	△	*Geitlerinema amphibium*	○	○
		Geitlerinema splendidum	○	○	*Limnothrix redekei*	○	△
					Jaaginema gracile	×	×

3.3. Adequacy of the simple identification manual

In this verification, the people at fields could not specifically isolate 2-MIB producing species using the manual. Yet, they were able to eliminate 109 out of 125 filamentous algae (six species) as non-2-MIB producing species and narrowed down the candidate of odor-causing algae into 16 filamentous algae (three species). The same person at fields identified two species belonging in the genus of *Geitlerinema* and *Jaaginema* based on this manual during the verification conducted over two years. The identification by the person in 2011 did not match with the identification based on this manual, but this mistake was accurately corrected in the verification in the following year. These two species have microscopic granular structures which scatter light in low-magnitude observation (x100 to 200), and make a cell appear as if it has a cleavage on the cell wall. Therefore, the magnitude of a microscope needs to be x500 to x1,000 in some cases to accurately identify whether a cell really has a cleavage on the cell wall. Providing proper descriptions and

explanations for classification in the manual is necessary for species which require special attention like this. At the same time, people at fields need to have some experience with these classifications. In addition, *Planktolyngbya limnetica*, which belongs in the same category as musty-odor causing algae (the genus *Pseudanabaena*) constantly form clearly visible mucilaginous sheaths; thus, it can be easily distinguished from species in the genus *Pseudanabaena*, which do not form mucilaginous sheaths. Other species in the genus *Leptolyngbya* (*Leptlyngbya* sp.) do not necessarily form mucilaginous sheath, and it is difficult to clearly distinguish them from musty-odor causing algae. These points are the issues which need to be improved in this manual. The verification above indicated a possibility that people at fields can screen musty-odor / non-producing species among *Phormidium tenue* using this manual. There is great value in improving the accuracy of screening 2-MIB producing species at fields in terms of water quality management at dams and reservoirs.

4. FUTURE STUDIES AND PERSPECTIVES

This paper discussed the development of a new morphological identification manual which allows people at fields to identify *Phormidium tenue* that used to be identifiable only when musty-odor and non-producing species are present in the same water, and examined its accuracy and workability at fields. As a result, the screening of musty-odor / non-producing species using this manual is found to be possible to a level that can be effectively used in actual operations, although there is a necessity to add descriptions for species which require special attention in identification and some experiences of people at fields. This verification indicated the adequacy of this manual.

There are still issues to be solved, however, concerning the adequacy of this manual, certainty of implementing this process by people at fields, and usability at fields because the result obtained this time is based on the verification targeting samples from one dam, and that verification only examined one person working at the fields. Therefore, the manual needs to be properly improved by continuing to accumulate and examine more data and adding descriptions with sketches and examples with photographs. The authors are now collecting samples from multiple reservoirs and conducting examinations targeting multiple people at fields. While this manual was only targeted to *Phormidium tenue*, there are reports from actual fields on musty odors caused by the genus *Oscillatoria* and by geosmin. Also, both musty-odor and non-producing species are present in large quantities in algae which used to be considered as a part of the genus *Oscillatoria*.Based on the above, these algae need to be organized in similar fashion and added in manuals in the future. In addition, if the use of this manual improves confusion at fields caused by the mixed presence of musty-odor / non-producing species which originate in problems in classifications, the analysis of morphological characteristics of these taxonomical groups is expected to progress, which results in the implementation of more appropriate measures to protect water quality.

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