Observations of Algae and Their Floc in Water Using Low-Vacuum SEM and EDS

Hiroshi Konno†, Takeo Suzuki††, Masato Mizushiri† and Hideharu Kashiwakura††

Department of Civil Engineering, Tohoku Institute of Technology
"Electron Optics Division, JEOL Ltd.

Algae and their floc and the effects of chlorine treatment were observed using SEM and EDS. These observations revealed that (1) samples like algae and their floc can be held firmly by making the SEM pore filter surface adhesive using poly-L-ricin, (2) chlorine treatment causes cracks in the end and center of Nitzschia, allowing observation of how the entire surface is fractured, though morphological changes with the number of days of culturing algae are not known exactly, and (3) the floc of two types of coagulants-aluminum sulfate and ferric chloride-is taken into suspending hydroxide and turned into an aggregate of silica and phosphorus in water, combined with aluminum (or iron) in the coagulant and algae, in addition to carbon and oxygen.

Introduction

Humankind have been using water in their daily life and have gradually increased this consumption volume over many years. Now, this volume has reached a great extent, close to the limitation on water capacity. We can say that humankind have been taking advantage of the potential power of water. In that sense, they have enhanced the utility value of water, but the use of water by humankind is only momentary when viewed from the standpoint of the water circulating cycle that will last for an indefinite time. Humankind have been using water by borrowing it temporarily from nature. If they cannot stop discharging entropy into water [1] through their social activities, then they have to regenerate water, in particular, water quality. Here arises the need of wastewater treatment and water purifying techniques for utilization of water.

Activities of humankind in daily life have increased and discarded entropy, which has directly or indirectly given rise to a wide variety of substances with diversified types and concentrations, including suspended substances, melting substances, organic substances, organisms, toxic substances, viruses, and furthermore, those substances at molecular level. In recent years, the progress in the environment measurement technology has revealed new substances, which have been unknown to us and have to be eliminated from the earth. These include combined products that contaminate the environment and man-made substances that have not existed in nature. These environmental contaminants are increasing drawing our attention as serious obstacles to the earth.

Here lies an indispensable need for of conservation of water environment and research on water purification treatment. These tasks are part of risk assessment and risk management for the earth environment. We must continue monitoring, locating their sources and determining the amount, while conserving water environment and continuing use of water by using wastewater treatment and water purifying techniques.

However, more serious and important issues are the assessment and management of aquatic biota, which is more complex than aquatic inorganic substances and of which unknown field is expanding. Aquatic plankton had existed in water before humankind lived on the earth and should have been able to coexist with humankind that utilized water. However, it so happened that aquatic plankton stank and produced toxic substances. Whether or not such a cycle is attributed to the result of life struggle in nature will be clarified by biological research sooner or later, but such a process is deemed a sort of risk. The elucidation of the mechanism and development of techniques related to this mechanism are awaited.

Water purification treatment is greatly affected by the existence of algae when the water supply source is eutrophic. They include coagulation interference [2-7], abnormal smell/taste, filter clogging [8-15], toxic substances, residue of soluble metals [3, 9], and generation of trihalomethane (THM). Algae are often pretreated with chlorine for the reason that coagulation treatment is not easy, but its treatment is limited by generation of THM. There are several reports that chlorine treatment improves the sedimentation of algae, but this reported method is not regarded good, in terms of the improvement of coagulation. On the other hand, there is no report about how the algae treated with chlorine will change. According to comparative experiments of aluminum-based coagulant and iron-based coagulant about the generation of algae floc, it is reported that aluminum-based coagulant is better than iron-based coagulant.

In this paper, we discuss the coagulation mechanism of algae based on the observations of algae and their floc formed by two types of coagulant, using a scanning electron microscope (SEM) and an energy dispersive X-ray spectrometer (EDS). Also, the results of our examinations, which cover how the cells of algae are fractured and how the algae change morphologically in the multiplication period, are discussed.

Cultivation of Algae, Experimenting Conditions, and Method of Sample Preparation

Cultivation of algae and experimenting conditions

Algae were collected from Lake Kamafusa, Miyagi Prefecture, Japan, using plankton net. They were concentrated by a centrifugal separator and then were isolated in agar medium. The medium used for isolation was Nitzschia of algae, and BG-11 was used as medium, except for EDTA. Cultivation conditions were 18°C and 2000 Lx [7]. Coagulants used in examining the algae floc were aluminum sulfate as aluminum-based coagulant and ferric chloride as iron-based coagulant. The coagulation conditions were such that pH was 7.0, alkalinity was 50 mg/L, and the coagulant was 12 mg/L at each concentration of aluminum and iron. Incidentally, the chlorine demand of the raw water was 0.2 mg/L.

Method of preparing sample t-butyl alcohol freeze-drying method

While the critical point drying method is conducted at a pressure of about 100 atm using liquefied carbon dioxide, the conventional freeze-drying method has some disadvantages. That is, it takes time because it freezes a sample containing solution with liquid nitrogen and then is left in a high vacuum (10⁵ Pa or less) for several to several tens of hours, while being kept at a cryogenic temperature of 80K to 190K. As a result, the sample may be adversely affected by cryohydrate when frozen. However, the t-butyl alcohol freeze-drying method, in which the freezing temperature of t-butyl alcohol is high, has become popular as a method that permits drying at a temperature relatively close to...
room temperature within a short time.

A fixed, dehydrated sample is substituted in t-butyl alcohol and is placed on the sample stage of a freeze-drying unit, then the stage temperature is lowered to about 5°C (or may be left in a refrigerator for several tens of minutes). When t-butyl alcohol is frozen, water is discharged by using a vacuum pump to sublime frozen t-butyl alcohol. When sublimation is completed and pressure starts lowering abruptly, the stage temperature is restored to room temperature, thereby a dry sample being completed with restoration of atmospheric pressure.

**SEM pore**

The SEM pore is mounted on the sample stage to filter and trap particles floating in liquid. It is a polycarbonate milli-pore filter (10 µm thick, 0.6 µm pore diameter) supported with a hard conductive resin frame and is attached to a syringe when used for aspiration. It can trap particles in liquid effectively within a short time. Also the special-purpose adapter for the SEM pore allows the SEM pore to be easily installed on the sample holder. (Fig. 1)

**Preparation of sample for SEM**

Table 1 shows the procedure for preparing the sample for SEM. First, a syringe was set to the SEM pore filter, the sample was fixed, and poly-L-ricin (0.1 w/v%) was injected to the sample, and the sample was left for two minutes.

If the sample were not prepared in this way, the sample would have been scattered into air when the sample chamber was in vacuum after injecting coagulant to observe the algae floc.

Then, the sample was placed on the SEM pore filter to absorb moisture. Distilled water was dropped to clean the sample with the SEM pore filter, then t-butyl alcohol was introduced onto the SEM pore filter to immerse the sample in it. Upon completion of the process above, the SEM pore filter was removed from the syringe, and it was set on the special-purpose sample stage, then it was freeze-dried with liquid nitrogen. The sample was set in the SEM within 15 seconds after freeze-drying, and observation was made in the low vacuum (LV) mode.

**Algae Nitzschia and Changes in Shape with Number of Culturing Days**

In this study, we used a JEOL low-vacuum scanning electron microscope (LV-SEM). It permits observation of a sample freeze-dried with liquid nitrogen simply by sublimating moisture in low vacuum without complex pretreatment nor coating the sample with platinum.

**Fig. 2** shows the cultivation curve of *Nitzschia*. *Nitzschia* enters a logarithmic multiplication period, when the multiplication rate is high, about one week after starting cultivation, followed by a static period when concentration is constant, and reaches the extinction period when the number concentration decreases about four weeks later.

**Figures 3(a) to 3(g)** show the results of observations of algae *Nitzschia* using an LV-SEM with elapse of cultivation days. Although distinctive changes in the shape of *Nitzschia* are not observed, portions that look partially dense depending on the length of cultivation period can be observed sometimes distinctively and sometimes not distinctively. Changes depending on the length of cultivation period could not be identified on the micrographs.

**Figures 4(b) and 4(c)** show the results of EDS analysis of **Fig. 4(a)** of algae *Nitzschia* that was taken several tens of days later. It can be seen that the surface material of *Nitzschia* is covered with silica peculiar to algae. It seems that in this period, a large portion often looks white on the photograph as if the cell interior has been condensed.

Analysis of element distribution by EDS strongly suggests that it is the distribution of phosphorus. Assuming that the portion is an aggregate of phosphorus, it can be identified as a cell nucleus. In this portion, the activity of algae is high depending on the length of cultivation period and cell division takes place actively when it is frequently observed. But when the number of algae, in which cell nucleus is not clearly observed, the activity lowers. Also, the number concentration does not increase, and that of algae only with shell increases. However, the cell division of this type of algae takes place separately as if the upper half and lower half of a box come apart. It is said that the cell nucleus is divided before cell division takes place, so that the biological activity of algae cannot always be evaluated by whether cell nucleus can be observed well or not.

In addition, observations of algae using an LV-SEM revealed that the size of *Nitzschia* that could be separated in the observation of algae was 20 to 30 µm, but the conditions of *Nitzschia* in each cultivation period could not be characterized by shape or other information.

**Changes in Shape and Other Parameters of Algae by Chlorine Treatment**

In this study, we conducted chlorine treatment of normal *Nitzschia* and observed how the algae change morphologically. We used sodium hypochlorite solution as chlorine. To prepare sample before injecting chlorine, we placed a sample on the SEM pore filter according to the ordinary sample preparation process and then injected chlorine onto the filter with adequate care not to allow the surface to dry. The concentration of chlorine at this time was 10 mg/L. Then, we left the sample for 60 minutes while taking care not to allow it to dry up. In the meantime, the filter was kept covered with chlorine. Then the sample was cleaned in the same manner as in ordinary sample preparation, and freeze-dried, then observed in low vacuum. **Figure 3(a)** shows needle algae *Nitzschia* on the fifth day after start of cultivation. We observed how chlorine treatment changed it morphologically from this standard form.

**Figures 5(a) to 5(e)** show the results. It was made clear that injection of chlorine causes cracks in the end and center, and that it is differs widely from the ordinary *Nitzschia*. Thus, the cells of algae that had been treated with chlorine was fractured and the glassy portion was crushed later or simultaneously.
(a) This photo shows the shell annular surface. What element the *Nitzschia* and the white object represent on the photo is yet to be examined.

(b) Silica distribution. EDS detected silica and phosphorus as well as carbon and oxygen with exceedingly high concentration. This photo shows the elemental distribution of silica, which indicates that the surface of *Nitzschia* is covered with silica peculiar to algae.

(c) Phosphorus distribution. This photo shows the elemental distribution of phosphorus. Aggregation of phosphorus in the white, round portion suggests that the central portion like the cell shell of algae exists in this white portion.

Fig. 4. *Nitzschia* (LV-SEM observation and EDS analysis).

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(a) Cultivation period: 5 days. *Nitzschia* as viewed from the shell surface.

(b) Cultivation period: 5 days. Enlarged photo of *Nitzschia* in (a) On this photo can be seen white spots peculiar to *Nitzschia* in a line on one side, two rows of lines running in the transverse direction and lines that intersect the line at right angle.

(c) Cultivation period: 14 days. Cell nucleus cultivated for 14 days, in which cell nucleus-like objects can be clearly perceived through the shell surface.

(d) Cultivation period: 14 days. The top photo shows the shell annular surface that is the lateral face of *Nitzschia*. Careful observation of this photo will show a joining surface in longitudinal direction at the center that will be split into two sooner or later. The bottom photo shows the shell surface as with the *Nitzschia* shown above. The shell surface is contracted on both ends, but the shell ring surface is rectangular.

(e) Cultivation period: 14 days. This photo shows the shell surface, with cell division of another *Nitzschia* at the bottom.

(f) Cultivation period: 16 days. Observation of numerous *Nitzschia* at low magnification shows cell division of some *Nitzschia* under way.

(g) Cultivation period: 22 days. This photo also shows the shell surface, presumably immediately before cell division in vertical direction.

Fig. 3. *Nitzschia* (LV-SEM observation).
with it, losing its original shape. Even if shells remained after the cells had been fractured, they cannot be identified as algae after elapse of a certain period of time. Thus, we may say that chlorine treatment destroys shells like hyalin of algae considerably, leaving no form of algae.

**Observation of Algae Floc**

**Aluminum-based coagulant**

As mentioned earlier, coagulation conditions were such that pH was 7.0 and alkalinity was 50 mg/L. 12 mg/L of aluminum sulfate was injected as coagulant and the floc was prepared for observation after two minutes of quick agitation with a jar tester, followed by slow agitation for 15 minutes, as shown in Table 2.

Figures 6(a), 6(b) and 6(c) show the SEM micrographs of aluminum sulfate floc. These photos show how some of the algae *Nitzschia* are taken into whitish suspended matter and that 20 to 30µm *Nitzschia* aggregate to form a block. The field-of-view of the micrograph is about 200 × 300 µm, but the floc appears much larger than this.

This was analyzed by the energy dispersive X-ray spectrometer (EDS). It can measure X-rays generated from the sample surface by converting them into electrical signals, thus analyzing constituent elements of the sample from the area displayed on the entire screen of an SEM image. The elemental-distribution display function includes elemental distribution and elemental mapping.

Elemental distribution is to display the intensity counts (concentrations) of each element as peak. Element map, which is obtained by elemental mapping, displays the respective constituent elements separately and quantitatively, by selecting the peaks of the respective elements. Element map is color-displayed, where a portion that contains elements in high concentration can be seen by the comparison of the relative changes in colors presented in each element map. The color order is black, blue, green, yellow, red, and white. Black indicates the lowest concentration region and white is the highest in the map.

EDS analysis shows that elements that are shown distinctly on the SEM micrograph are aluminum, silica, phosphorus, etc. in addition to carbon and oxygen. This was analyzed by the energy dispersive X-ray spectrometer (EDS). It can measure X-rays generated from the sample surface by converting them into electrical signals, thus analyzing constituent elements of the sample from the area displayed on the entire screen of an SEM image. The elemental-distribution display function includes elemental distribution and elemental mapping.

**Iron-based coagulant**

Figures 9(a), 9(b), 9(c) and 9(d), which show the SEM micrographs of ferric chloride floc. It can be assumed that as seen in the case of aluminum sulfate, numerous algae *Nitzschia* acquired into suspended component constitute a floc. EDS analysis of this sample in these micrographs display distinctive elements, and high concentration of silica, phosphorus, iron and calcium are detected, in addition to carbon and oxygen.

**Conclusion**

Algae, floc and the effects of chlorine treatment were observed by means of SEM imaging and EDS analysis. The analysis results revealed the following:

1) In SEM observation, samples like algae floc can be supported firmly by making the SEM pore filter surface adhesive using poly-L-ricin.
2) Chlorine treatment causes cracks in the end and center of *Nitzschia* and destroys cells, good proof that indicates its coprecipitation.

![Fig. 5. *Nitzschia* subjected to chlorine treatment (LV-SEM observation).](image)
allowing easy observation of how the shell surface and shell annular surface are fractured.

3) The flocs of two types of coagulant - aluminum sulfate and ferric chloride - are acquired into suspended hydroxide, and are turned into an aggregate of silica and phosphorus in water that is combined with coagulant aluminum (or iron) and algae, as well as carbon and oxygen. This could be observed well.

These observations greatly contributed to the clarification of the mechanisms of coagulation treatment and chlorine treatment of algae. This means that such techniques can greatly contribute to the clarification of these phenomena. We will continue further study of these mechanisms.

References

6. Mitachi K: Effects of Aggregation

Fig. 6. *Nitzschia* floc by coagulant (aluminum sulfate). (LV-SEM observation)

Fig. 7. Aluminum sulfate floc (LV-SEM observation and EDS analysis)

**Fig. 8. Nitzschia floc by coagulation (ferric chloride).** (LV-SEM observation)

(a) Nitzschia floc by coagulation (ferric chloride). On this photo, the Nitzschia floc is about 100 x 200 μm, which is smaller than that by aluminum sulfate.
(b) Nitzschia floc by coagulation (ferric chloride).
(c) Nitzschia floc by coagulation (ferric chloride). This photo shows the bridging action between Nitzschias, assuming that the white portion represents hydroxide.

**Fig. 9. Floc (ferric chloride).** (LV-SEM observation and EDS analysis)

(a) Floc (ferric chloride). This is an SEM micrograph of ferric chloride floc. Analysis by EDS shows that distinctive elements are silica and phosphorus, in addition to carbon and oxygen.
(b) Silica distribution of floc (ferric chloride). This photo shows the silica distribution of ferric chloride floc. As with ferric chloride floc, Nitzschia contains much silica, as a matter of course. This photo shows how the silica contained in the culture medium is adsorbed to the floc portion, too.
(c) Iron distribution of floc (ferric chloride). This photo shows the iron distribution in ferric chloride. The white portion of the SEM micrograph can be considered hydroxide like ferric hydroxide as with aluminum floc.
(d) Phosphorus distribution of floc (ferric hydroxide). This photo shows phosphorus distribution of ferric hydroxide floc. Comparison of phosphorus and iron shows distribution in nearly the same position. It is well known that phosphorus is adsorbed to floc and undergoes coprecipitation. This photo shows a good example of coprecipitation.