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Application of a novel cleaning method using low-temperature plasma on tidal flat diatoms with heterovalvy or delicate frustule structure

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ABSTRACT.—For clear observation of diatom frustules, both inner- and extracellular impurities need to be eliminated from samples. This process is usually performed using oxidizing acids that can completely decompose organic matter. However, oxidizing acids often result in dissociation of frustules, and if oxidative byproducts are not washed out completely at the end of the process, they can act as impurities. Moreover, the treatment is time-consuming, and the necessity for subsequent rinsing may reduce the number of diatom frustules and/or valves because of loss during rinsing. Application of low-temperature plasma (LTP) was examined for diatom cleaning. LTP treatment is an extremely gentle cleaning method in which all the processes can be performed on a cover slip without rinsing with distilled water or the addition of chemicals. Application of this method to the tidal flat sample was found to be effective for the preservation of complete frustules, such as those observed in *Amphora*, *Cocconeis* and *Planothidium* species; with this treatment, there was no reduction in the diatom population during the cleaning process.

INTRODUCTION

In order to conduct morphological and taxonomical investigations on diatom frustules, it is necessary to eliminate impurities of both organic and inorganic origin (Round et al., 1990). Organic impurities can be removed by various methods. In many studies, diatom samples were washed with concentrated oxidizing acids, such as H_2SO_4, HNO_3, or HClO (Nagumo, 1995, Nagumo and Kobayasi, 1990, Patrick and Reimer, 1966, Round et al., 1990), which can completely remove organic impurities. However, they often lead to partial or complete dissociation of frustules (Round et al., 1990). Hydrogen peroxide can be used for gentle treatment; van Der Werff (1955) succeeded in maintaining frustules intact by using this chemical without heat treatment. However, the prominent drawback is that it takes several days for this cleaning. Its action is much more rigorous when used in combination with UV irradiation (Swift, 1967). Although this treatment hardly generates dissociated frustules, it is inefficient in the case of samples that include many impurities or a small amount of diatoms. And in every chemical cleaning method, if the reaction mixture is not rinsed properly, the oxidative byproducts themselves may act as impurities, impeding observation. Repeated rinsing may also decrease the number of diatom valves. Most important, regardless of which cleaning method is used, treatment of the diatom samples before preparation of the slide is time-consuming. The microwave digestion technique was introduced as one of the new diatom cleaning methods (Acker et al., 2002, Parr et al., 2004). It can be applied even for a small amount of sample and cleans impurities with the addition of few chemicals (nitric acid/hydrochloric acid) within a short time in comparison with the conventional methods (Acker et al., 2002, Parr et al., 2004). However, the microwave method also involves rinsing, which may cause loss of the diatom sample. In diatom taxonomy, when only a small amount of precious material, such as loaned classical exiccata and type material, can be examined, it is disadvantageous to reduce the number of specimens.

The biodiversity of tidal flats is usually very high, and a number of diatom species have been recorded in tidal flat areas (Ohtsuka, 2005, Sawai and Nagumo, 2003, Witkowski et al., 2000). We have also studied diatoms in similar environments (unpublished data) and observed diatom assemblages composed of various araphid, biraphid,

and monoraphid species. However, with conventional oxidizing acid treatment, frustules are often separated into single valves and many bands; therefore, it is often difficult to identify organisms even at the generic level. Rapheless valves belonging to several small monoraphid taxa such as *Achnanthidium*, *Cocconeis*, and *Planothidium* are quite similar to those of some other araphid taxa; this leads to questionable or misleading identification.

Low-temperature plasma (LTP), which is nowadays often employed in industries for cleaning or sterilizing the surface of electronics and precision instruments (for the detailed process see Lieberman and Lichtenberg, 2005), can be used to remove organic matter from diatom cells. In the process we used, excited-state oxygen ions, which were generated by collisions between oxygen molecules/atoms derived from the atmosphere and freely moving electrons, oxidized and sputtered organic matter; this was followed by ultimate production of CO_2. Since the entire treatment is performed on a cover slip without addition of chemicals or rinsing, it is easy to obtain cleaned frustules in their intact form. In this study, we examined the application of LTP treatment on diatom species with delicate frustule structure.

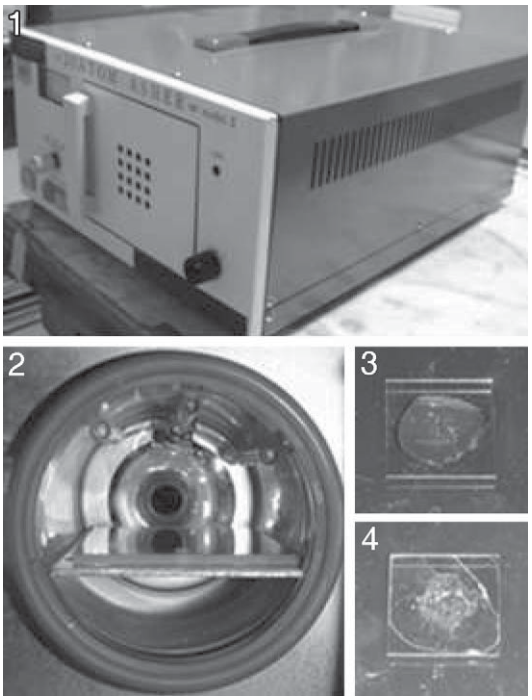
MATERIALS AND METHODS

The diatom samples were collected from several tidal flats in Japan (Table 1). A small amount of diatom suspension, in which marine water was replaced with distilled water, was dropped on a cover slip (Fig. 3). The cover slip with the wet slurry of diatoms was directly inserted in the chamber of an LTP-generating device (Diatom asher model II; Kyotodensikeisoku Co., Kyoto, Japan) (Fig. 1). Oxygen plasma is generated from atmospheric O_2 gas under a high-frequency wave (13.56 MHz) in the chamber for 10–60 minutes (Fig. 2). Because the cleaned diatoms (Fig. 4) were obtained in the dry state, they were mounted immediately in Mountmedia (Wako Pure Chemical Industries, Osaka, Japan), a commercial equivalent of Pleurax (Table 1). The cleaning process for freshwater samples is simpler than that for marine samples because it is not necessary to remove the salt from the freshwater sample.

Our LTP apparatus consists of 2 units—an LTP-generating device and a vacuum-pumping system. The cost of the LTP-generating device alone is under 300,000 yen. The LTP apparatus does not need ventilation because oxygen plasma utilizes atmospheric O_2, and the exhaust gas is discharged into the atmosphere as CO_2 and H_2O. For further information, contact YK, the second author.

The samples were also boiled in a solution of concentrated sulfuric acid with potassium permanganate for 20 minutes and rinsed in distilled water more than 5 times (Patrick and Reimer, 1966). The cleaned sample was dropped onto a cover slip, dried on a hot plate, and mounted on a slide. All the samples and slides are preserved in the lab of SM at Tokyo Gakugei University (Table 1).

Light microscopic analyses of these slides were performed using an Optiphot 2 (Nikon, Tokyo, Japan).



Figs 1-4. (above) LTP treatment. **1,** LTP-generating device. **2**, Chamber of the LTP-generating device. **3**, The tidal flat sample on a cover glass before the cleaning process. **4**, The tidal flat sample on a cover glass after the cleaning process.

Figs. 5**–**25. (right) Cleaned valves. Scale bar = 10 μm. A for Figs. 5–20, B for Figs 21-25. **5-14**, *Planothidium delicatulum* (Kütz.) Round and Bukht. sensu lato. **5–8**, Cells cleaned with oxidizing acids (H_2SO_4) showing separated frustules with either raphid (**5**, **6**) or pseudoraphid valves (**7**, **8**). **9–14**, Frustules cleaned with LTP treatment showing intact frustules with paired raphid (**9**, **11**, **13**) and pseudoraphid valves (**10**, **12**, **14**). **15**, **16**, Frustule of *Cocconeis hauniensis* Witk. cleaned with LTP treatment showing frustules with paired raphid (**15**) and pseudoraphid valves (**16**). **17**, **18**, *Amphora coffeaeformis* (C.Agardh) Kütz. Samples treated with H_2SO_4 (**17**) and LTP (**18**). **19**, **20**, *Amphora cymbamphora* Cholnoky. Samples treated with H_2SO_4 (**19**) and LTP (**20**). **21**, **22**, *Amphora* sp. Samples treated with H_2SO_4 (**21**) and LTP (**22**). **23**, **24**, *Amphora longa* Hustedt. Samples treated with H_2SO_4 (**23**) and LTP (**24**). **25**, A colony of *Melosira* sp. cleaned with LTP.

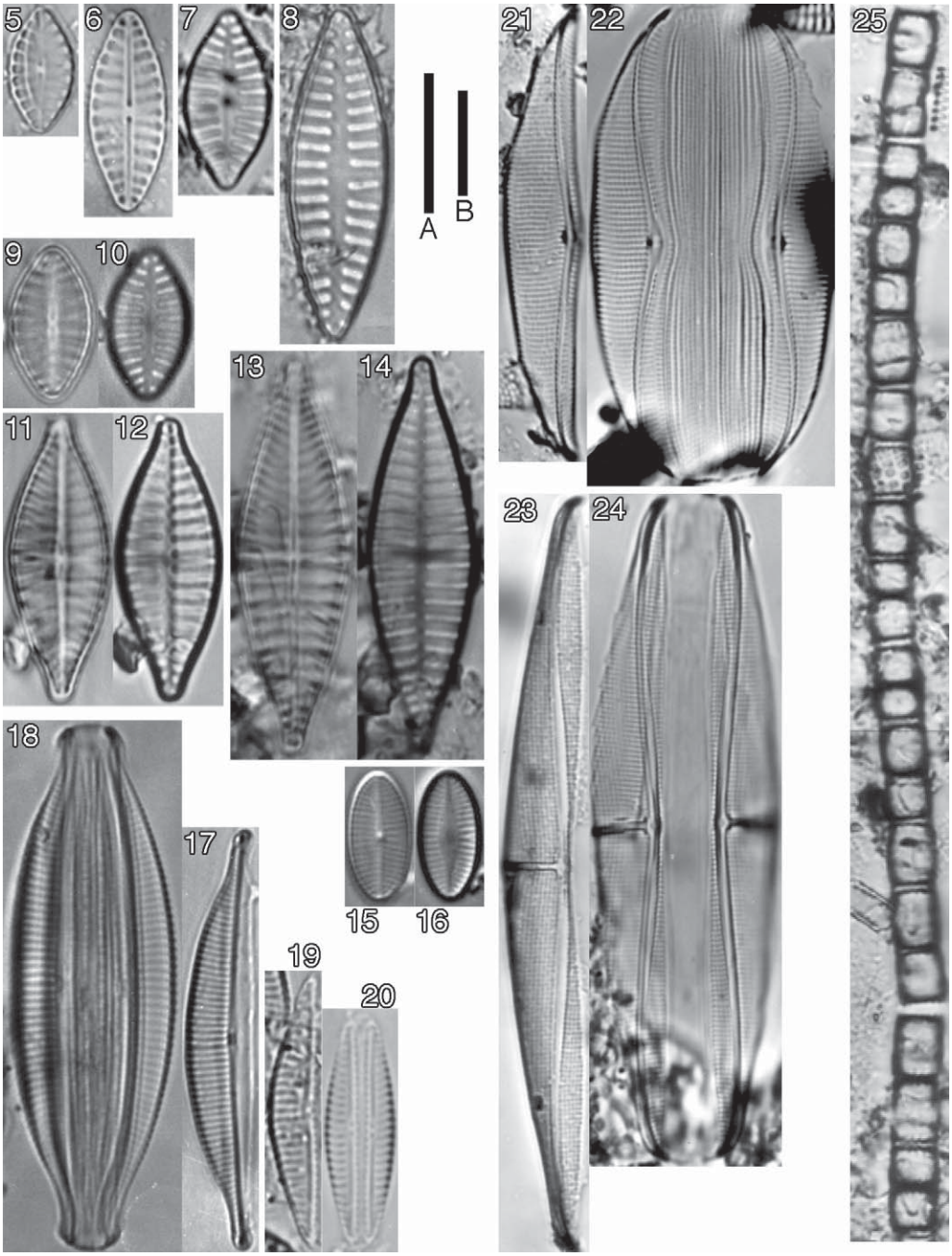

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Table 1. Sample and slides in this study.

Sample #	Slide #	Date	Location	Cleaning Method*
M075	H.K.3420	1999.7.31	Nakaura tidal flat in Saga Pref.	SA
M269	H.K.3400	2003.4.21	Sanbanse tidal flat in Tokyo bay, Chiba Pref.	SA
M269	H.K.3401	2003.4.21	Sanbanse tidal flat in Tokyo bay, Chiba Pref.	SA
M330	H.K.3402	2004.2.26	Sanbanse tidal flat in Tokyo bay, Chiba Pref.	SA
M330	H.K.3403	2004.2.26	Sanbanse tidal flat in Tokyo bay, Chiba Pref.	SA
M331	H.K.3405	2004.6.5	Futtsu tidal flat in Tokyo bay, Chiba Pref.	SA
M331	H.K.3406	2004.6.5	Futtsu tidal flat in Tokyo bay, Chiba Pref.	SA
M331	H.K.3415	2004.6.5	Futtsu tidal flat in Tokyo bay, Chiba Pref.	LTP
M332	H.K.3407	2004.6.5	Yatsu tidal flat in Tokyo bay, Chiba Pref.	SA
M332	H.K.3408	2004.6.5	Yatsu tidal flat in Tokyo bay, Chiba Pref.	SA
M332	H.K.3409	2004.6.5	Yatsu tidal flat in Tokyo bay, Chiba Pref.	SA
M333	H.K.3404	2004.6.5	Sanbanse tidal flat in Tokyo bay, Chiba Pref.	SA
M400	H.K.3416	2004.6.5	Futtsu tidal flat in Tokyo bay, Chiba Pref.	SA
M401	H.K.3417	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	SA
M401	H.K.3423	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	LTP
M401	H.K.3425	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	LTP
M401	H.K.3426	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	LTP
M401	H.K.3427	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	LTP
M401	H.K.3428	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	LTP
M401	H.K.3429	2004.8.13	Banzu tidal flat in Tokyo bay, Chiba Pref.	LTP

*LTP, Low-Temperature Plasma; SA, Sulphic Acid.

RESULTS AND DISCUSSIONS

Various diatom valves were observed in the sample cleaned by oxidizing acids. Because the samples often contained separated valves (Figs. 5–8), it was sometimes difficult to identify them. For example, this treatment frequently resulted in the dissociation of frustules of *Amphora*, in which the cingula are thin and arranged delicately (Figs. 17, 19, 21, 23).

Although we could not rigorously compare the LTP method with the acid-boiling methods, the sample treated with LTP contained much more intact frustules than the sample treated with acid; thus, the information necessary for their identification was easily available. For example, in *Planothidium delicatulum* (Kütz.) Round et Bukht. sensu lato and *Cocconeis hauniensis* Witk., both raphid valves and those without raphe could be easily distinguished in single frustules (Figs. 9–16). The frustule structure of members of *Amphora* showed characteristic amphoroid symmetry with complete sets of girdle bands (Figs. 18, 20, 22, 24). Moreover, long chains of diatoms were observed as in the case of *Melosira* sp. (Fig. 25); such a long chain of diatoms is rarely found after acid oxidizing treatment. Thus, with LTP treatment, diatom colonies and growth forms can be observed.

Special care should be taken when identifying species of *Planothidium*, *Cocconeis, Amphora*, etc., which sometimes occur dominantly in tidal flats. LTP treatment is advantageous for floristic studies in these habitats because it is useful for preparing intact frustules on a cover slip. In addition, LTP can save processing time: the time required for LTP treatment is only 10–60 minutes. LTP treatment is easier to perform than the other cleaning methods, because for this treatment, it is not necessary to add/defuse any of the chemicals in/from the diatom sample. Although, for a long time, diatom taxonomists have paid attention mainly to valves, it was reported that frustules as well as girdle views provide valuable taxonomic information (Idei and Mayama, 2001, Mayama, 2001). LTP cleaning would promise good results in these observations and implies that LTP would be useful in taxonomic, floristic, and ecological studies.

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Around 27 years ago, I (SM) met Dr. C.W. Reimer for the first time in the herbarium of Philadelphia. He inspired many of my thoughts on diatom taxonomy, and this paper is dedicated to Dr. C.W. Reimer, diatomist par excellence.

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