

# A practical method for enumerating cysts of ciliates in natural marine sediments

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**ABSTRACT:** In order to determine the abundance of intact cysts of planktonic ciliates in natural sediments, we developed a new practical method using autofluorescence of the fixative glutaraldehyde. After treatment with glutaraldehyde, the cyst walls fluoresced yellowish-green under blue excitation. The fluorescence was always clear in the intact cysts, while only part of the empty cysts was weakly fluorescent. This fluorescence made detection of the intact cysts easy among detrital materials, even for the cysts covered with detritus. It was also easy to distinguish the ciliate cysts morphologically from other organisms that also fluoresced in the treated sediments. This method was compared with the density gradient centrifugation technique, and the advantage of the former in frequent and routine investigations is discussed.

**KEY WORDS:** Enumeration · Cysts · Glutaraldehyde · Natural sediments · Planktonic ciliates · Density gradient centrifugation

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## INTRODUCTION

It is widely accepted that oligotrich ciliates play an important role as heterotrophic and/or mixotrophic components of the planktonic assemblage in freshwater and marine environments. Some of them form a resting cyst to survive unfavorable seasons (Reid & John 1978, Paranjape 1980, Reid 1987, Kamiyama & Anzai 1990, Kim & Taniguchi 1995, 1997, Kamiyama 1996, Müller 1996, Müller & Wünsch 1999, Kim et al. 2002, Müller et al. 2002). However, quantitative information about ciliate cysts in natural sediments is still limited because of the absence of a quick method for cyst enumeration. To determine the abundance of ciliate cysts routinely, we need to develop a practical method similar to that developed for a particular group of dinoflagellate cysts (Yamaguchi et al. 1995).

In this study, we show that the cysts of marine planktonic ciliates always turn a fluorescent yellowish-green color after glutaraldehyde fixation and propose that glutaraldehyde treatment is a simple and quick method to quantitatively determine the cyst populations of ciliates in natural sediments.

## MATERIALS AND METHODS

A sediment sample was collected at a station in Onagawa Bay (38° 26.30' N, 141° 27.70' E; 22 m depth) near the transition zone between the subarctic and subtropical area of the western North Pacific in February 2004. The top 2 cm of sediment was sliced off and stored at 5°C in the dark. Two 1 g (wet weight) aliquots of the sample were suspended in ca. 20 ml of distilled water and sonicated for 30 s; 20 to 100 µm fractions were retained on a sieve. Of the sieved fractions, one was diluted with 10 ml of distilled water and used for direct enumeration of cysts without treatment (control). The other fraction was treated with glutaraldehyde before cyst enumeration as follows: 1 ml of 25% glutaraldehyde (Wako Pure Chemical) was diluted to 10 ml with distilled water to give a final glutaraldehyde concentration of 2.5% and this was added to the sieved fraction. The treated suspension was stored in a refrigerator for 1 d.

Each 1 ml aliquot of the treated and control suspensions was placed on a counting slide and examined under a regular light microscope to count the ciliate cysts. The ciliate cysts were categorized into 2 groups,

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i.e. *Strombidium conicum* and others, since the cysts of *S. conicum* have been well described by Kim (1995) in Onagawa Bay and are highly recognizable. The fluorescence capability of every ciliate cyst in the treated sample, regardless of state (intact or empty), was examined under blue-light excitation (450 to 480 nm). Both intact and empty cysts were counted up to 50 cells. The fluorescent ratio of each group of cysts was calculated as the percentage of fluoresced cells to the total cells examined. An epifluorescence microscope (Olympus BX50) was equipped with a blue-light excitation filter held in a DM500 dichroic prism, with a barrier filter BA515 and a 100 W mercury lamp (U-MWB cube). The statistical difference in the cyst number between the treated and the control suspensions was determined using Student's *t*-test.

To assess the stability of the fluorescence capability of the treated cysts, the same treated sample was stored in a refrigerator for 1 mo and then examined again in the same way. An additional sediment sample, which had been obtained at a station in Uranouchi Bay (32° 26.19' N, 130° 23.41' E; 20 m depth) open to the subtropical Pacific on March 2003, was also treated with glutaraldehyde. The sediment had been stored for about 1 yr in a refrigerator without any pretreatment.

Recovery of the ciliate cysts by the density gradient centrifugation technique, which is commonly used in studies on dinoflagellates (Blanco 1986), was also carried out for comparison with the detection efficiency of the present method. The sediment sample obtained in Onagawa Bay in February 2004 was used. As the gradient medium, Ludox TM TM-50 colloidal silica (Aldrich) with a density of 1.40 g cm<sup>-3</sup> was diluted with distilled water, after filtration through a paper filter to remove insoluble floccules (De Jonge 1979), into a series of 1.12, 1.20, 1.28, 1.36 and 1.40 g cm<sup>-3</sup> in specific weight. Five 1 g aliquots of wet sediment were suspended in distilled water and sonicated for 30 s. Their 20 to 100 µm fractions were resuspended individually in ca. 20 ml of distilled water and transferred to 50 ml centrifuge tubes containing 20 ml of each solution of the series of Ludox TM solutions. The tubes were centrifuged at 700 × *g* for 15 min. Detrital material between the layer of distilled water and the Ludox TM solution and on the bottom was withdrawn separately with a pipette onto a 20 µm mesh filter and washed with distilled water. Detrital material retained on the filter was transferred into a vessel to which 1 ml of glutaraldehyde was added and then made up to 10 ml with distilled water. Recovered intact cysts of *Strombidium conicum* were counted under an epifluorescence microscope. As a control, the intact cysts in the 20 to 100 µm fraction of 1 g of sediment treated with glutaraldehyde were counted without density gradient centrifugation.

In this investigation, only natural cyst assemblages were examined because no culture method to obtain a sufficient number of cysts of a single species in the laboratory is available. The ciliate cysts >20 µm in width were counted in the 20 to 100 µm fraction in this investigation. This size range was reasonable since the loss to <20 and >100 µm fractions was only 3.8% of total cysts. Cyst species were identified by referring to Reid & John (1978, 1983), Kim (1995) and Kim et al. (2002). Tintinnids could only be identified to genus due to breakage of the loricae, which are the key to species identification. All the examinations were replicated 5 times.

## RESULTS AND DISCUSSION

### Fluorescence capability of ciliate cysts

Fig. 1c,d shows microphotographs taken under regular lighting and epifluorescence lighting of a cyst of *Strombidium conicum* after glutaraldehyde treatment. The fluorescence of the cyst wall was always clear and yellowish-green under blue-light excitation and that of the papula or the plug of the extruding hole of an intact cyst was characteristically strong. This strong fluorescence made the intact cysts clearly visible even among detrital material in both sediments from Onagawa and Uranouchi bays (100%; Table 1). Their fluorescence capability was fairly stable; the intact cysts were still brightly fluorescent 1 mo after glutaraldehyde treatment. Furthermore, the fluorescence capability was unchanged for the intact cysts in the sediment from Uranouchi Bay that had been stored for 1 yr in a refrigerator, though their abundance decreased by ca. 20%. On the other hand, weak fluorescence of empty cysts due to the absence of a papula, which had been lost during the excystment, makes the empty cysts indistinct, especially among detrital material (Fig. 1o,p). Only a portion of the empty cysts fluoresced poorly for *S. conicum* and other ciliates, i.e. 31.6 and 35.6%, respectively. The rest of the empty cysts were not fluorescent. Therefore, glutaraldehyde treatment is useful for detecting and counting intact cysts in sediment suspensions.

It has previously been reported that many organisms and tissues fixed with fixatives such as formaldehyde and glutaraldehyde form covalent cross-links and become fluorescent (Weber et al. 1978, Epstein 1995, Haraguchi & Yokota 2002, Johnson et al. 2003). However, fluorescence of ciliate cysts after formaldehyde fixation was weak or they were totally non-fluorescent (data not shown). Within our samples, cysts of dinoflagellates, resting spores of diatoms, resting eggs of zooplankters and pollen of terrestrial plants were

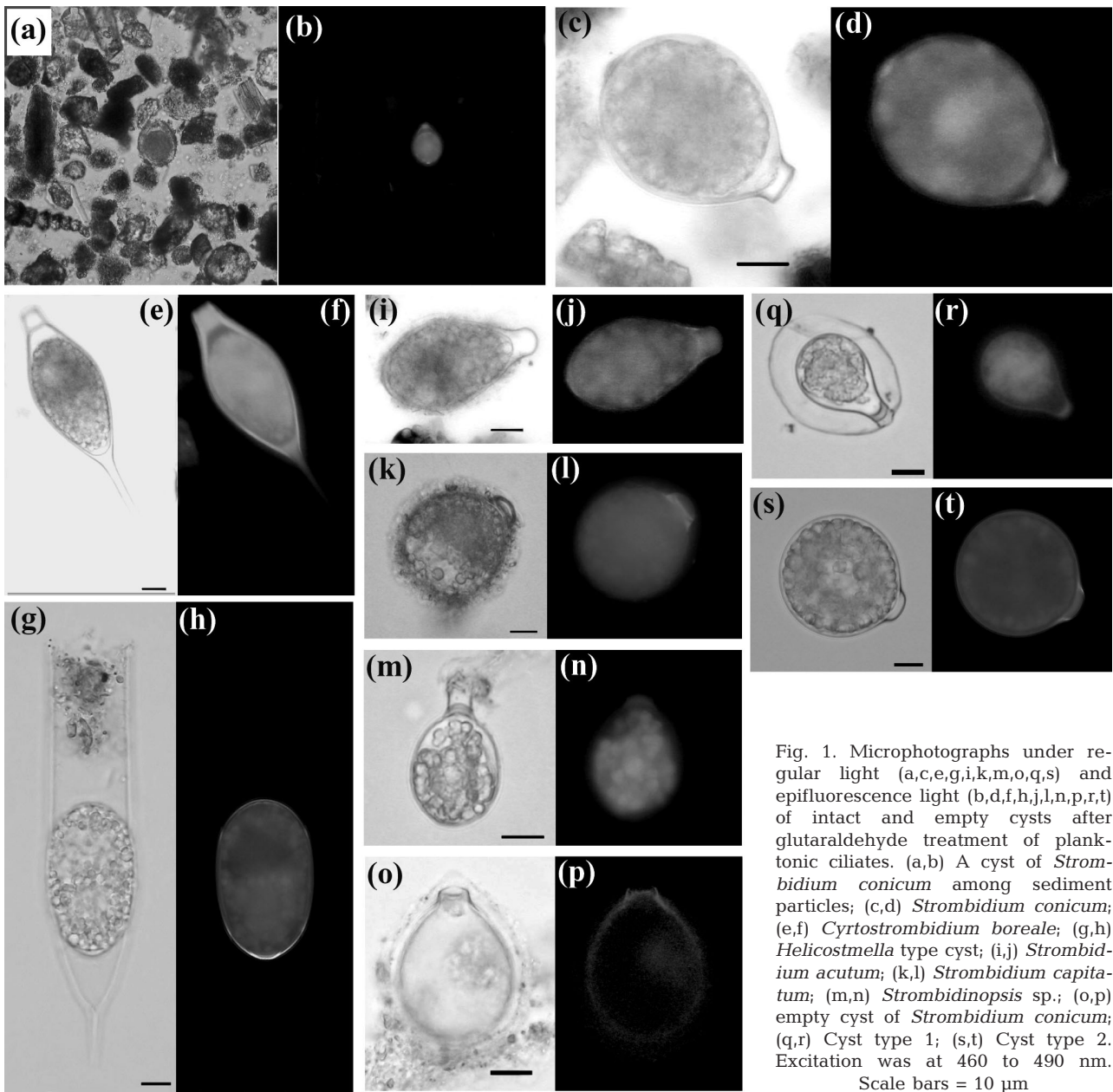


Fig. 1. Microphotographs under regular light (a,c,e,g,i,k,m,o,q,s) and epifluorescence light (b,d,f,h,j,l,n,p,r,t) of intact and empty cysts after glutaraldehyde treatment of planktonic ciliates. (a,b) A cyst of *Strombidium conicum* among sediment particles; (c,d) *Strombidium conicum*; (e,f) *Cyrtostrombidium boreale*; (g,h) *Helicostmella* type cyst; (i,j) *Strombidium acutum*; (k,l) *Strombidium capitatum*; (m,n) *Strombidinopsis* sp.; (o,p) empty cyst of *Strombidium conicum*; (q,r) Cyst type 1; (s,t) Cyst type 2. Excitation was at 460 to 490 nm. Scale bars = 10 μm

Table 1. Ratios(%) of fluorescent cysts to total intact and empty cysts for *Strombidium conicum* and other ciliates determined 1 d and 1 mo after glutaraldehyde treatment for Onagawa Bay sediment and the ratio for Uranouchi Bay sediment stored for 1 yr in a refrigerator. Number of tested cysts was 50 and every test was done in 5 replicates

		<i>Strombidium conicum</i>		Other ciliates	Total cysts
Onagawa Bay	1 d after glutaraldehyde treatment	Intact cysts	100 ± 0	100 ± 0	–
		Empty cysts	31.6 ± 4.6	35.6 ± 4.4	–
	1 mo after glutaraldehyde treatment	Intact cysts	100 ± 0	100 ± 0	–
Uranouchi Bay	1 yr after storage without glutaraldehyde treatment	Intact cysts	–	–	100 ± 0

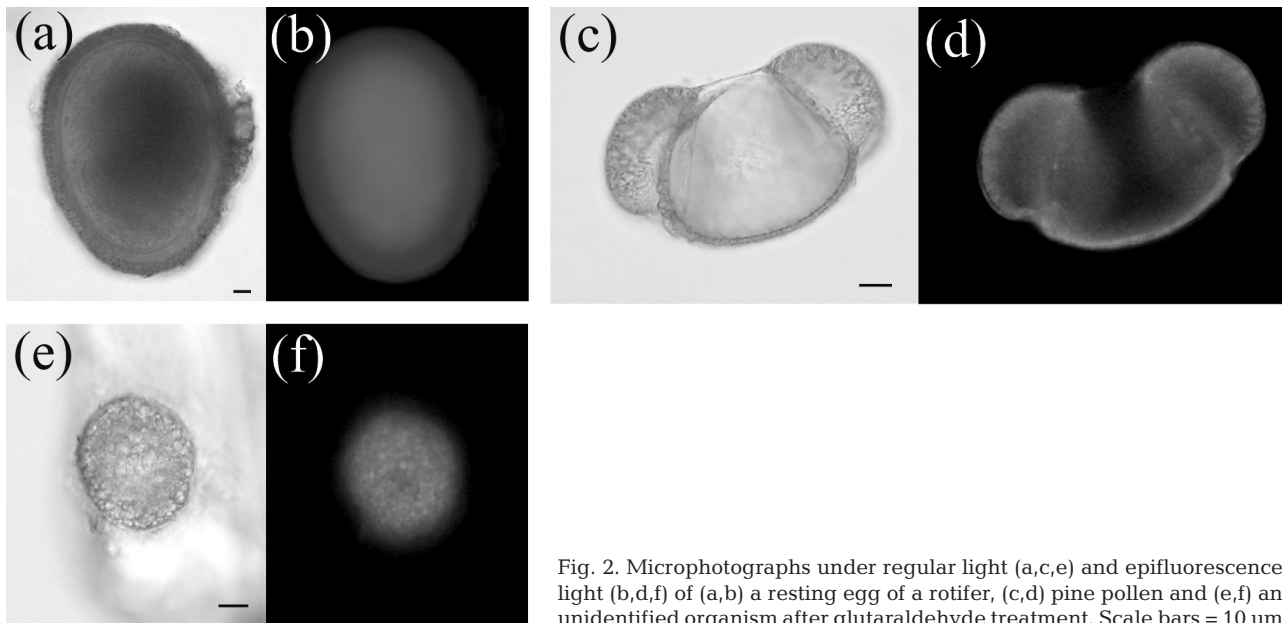


Fig. 2. Microphotographs under regular light (a,c,e) and epifluorescence light (b,d,f) of (a,b) a resting egg of a rotifer, (c,d) pine pollen and (e,f) an unidentified organism after glutaraldehyde treatment. Scale bars = 10  $\mu\text{m}$

also found. After glutaraldehyde treatment, some of them became fluorescent, such as pine pollen, resting eggs of zooplankters (probably rotifers) and other unidentified organisms (Fig. 2). However, cysts of dinoflagellates, resting spores of diatoms and resting eggs of copepods were very poorly fluorescent or not fluorescent at all. Among the fluorescent organisms, the ciliate cysts were clearly distinguishable due to their characteristically strong fluorescence, particularly of the papula (Fig. 1a,b).

#### Enumeration of cysts in natural sediments at different localities

In natural sediments collected from Onagawa Bay, *Strombidium conicum* cysts were the most abundant among the cyst assemblage, in both the glutaraldehyde treatment and the control (Fig. 3). However, the observed abundance was significantly higher in the glutaraldehyde treatment than in the control. The same was the case for *Strombidium acutum*, *Strombidium capitatum* and *Helicostomella* spp. It is noteworthy that the counts for the glutaraldehyde treatment were always higher than the control even for the spiniferous cysts of *S. acutum* and *S. capitatum* covered with detrital materials, while no difference was detected for species of lower abundance (Fig. 1i–l).

Among the cyst assemblage in Onagawa Bay, *Strombidium conicum* was the most dominant with 59.6 cysts  $\text{g}^{-1}$  wet sediment, followed by *Strombidium acutum* with 35.0 cysts  $\text{g}^{-1}$  wet sediment and *Helicostomella* spp. with 20.6 cysts  $\text{g}^{-1}$  wet sediment (Fig. 3). *S. conicum* has been reported to be the most dominant, with *S. acutum* and *Helicostomella* spp. as the secondary constituents, among planktonic ciliates in Onagawa Bay (Kim & Taniguchi 1997). In contrast, in Uranouchi Bay abundance of the cysts of these species was low

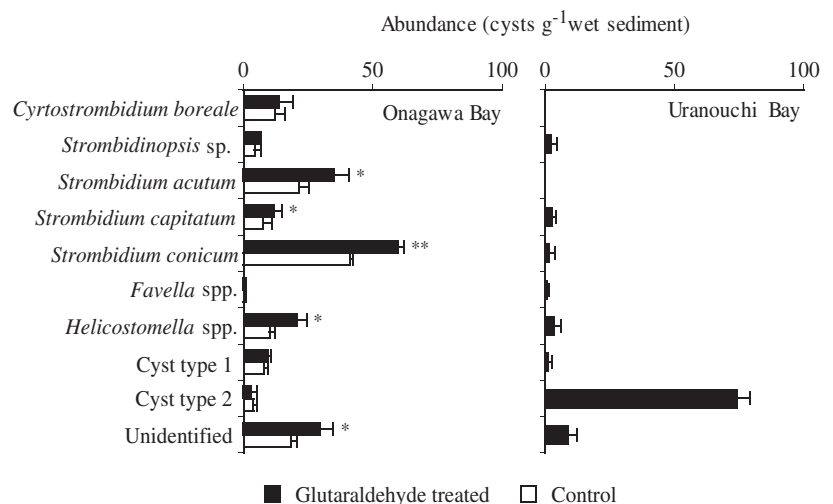


Fig. 3. Abundance of ciliate cysts (cysts  $\text{g}^{-1}$  wet sediment) counted for control and glutaraldehyde treated sediments collected in Onagawa Bay and for treated sediment in Uranouchi Bay. Unidentified: cysts with a papula, but their taxonomic affinity was not clear. Error bars indicate  $\pm$  SD,  $n = 5$ . \* $p < 0.05$ , \*\* $p < 0.01$  (Student's *t*-test)

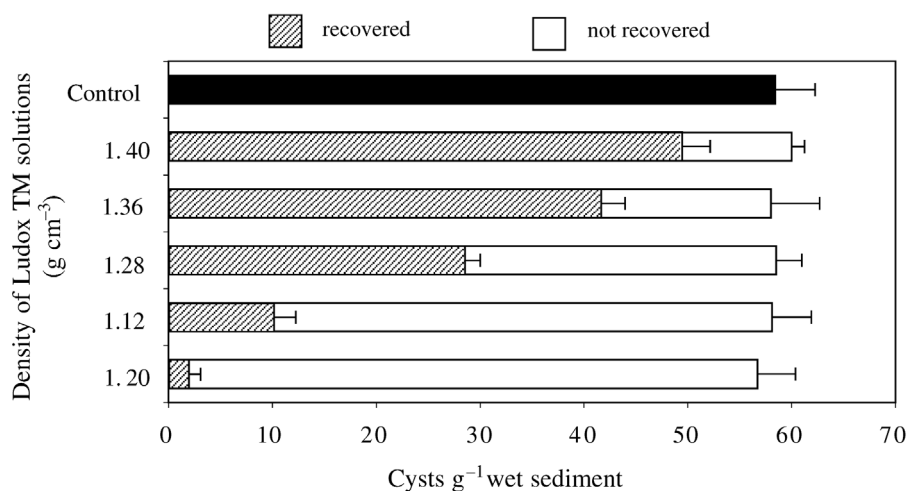


Fig. 4. *Strombidium conicum*. Number of recovered and not recovered intact cysts (cysts g<sup>-1</sup> wet sediment) from the sediment in Onagawa Bay by the density gradient centrifugation technique using Ludox TM solutions. The cysts recovered by each solution were counted by the glutaraldehyde treatment method. Error bars indicate  $\pm$ SD, n = 5

(0 to 3.6 cysts g<sup>-1</sup> wet sediment for the 3 species). However, the Cyst type 2 (a species independent of those previously mentioned and of unclear taxonomic affinity, Fig. 1s,t) was most abundant (74.0 cysts g<sup>-1</sup> wet sediment) (Fig. 3), though total abundance was at a similar level as in Onagawa Bay. These results suggest that the species composition of the planktonic ciliate community is essentially different between the 2 bays.

#### Comparison of glutaraldehyde treatment with the density gradient centrifugation method

The recovered cysts of *Strombidium conicum* gradually increased as the density of the Ludox TM solutions increased, being lowest (1.9 cysts g<sup>-1</sup> wet sediment) at the lowest density of 1.12 g cm<sup>-3</sup> or 3.3% of the cysts detected by glutaraldehyde treatment (58.4 cysts g<sup>-1</sup> wet sediment) in the control sediment (Fig. 4). On the other hand, at the highest density of 1.40 g cm<sup>-3</sup> the recovered cysts were 49.4 cysts g<sup>-1</sup> wet sediment or 85% of total cysts, which was similar to the value for dinoflagellate cysts in a previous study (Bolch 1997). However, 8.8 cysts g<sup>-1</sup> wet sediment were lost to the bottom because of excess weight due to attached detrital material (cf. Yamaguchi et al. 1995). Therefore, the present method is more practical for enumerating ciliate cysts in sediments than the density gradient centrifugation method and the panning technique (Matsuoka et al. 1989) based on the same principle. However, it must be noted that density gradient centrifugation is advantageous in collecting live cysts to be used for culture experiments when non-toxic gradient media such as Nalco 1060 (Schwingamer et

al. 1991) and sodium polytungstate (Bolch 1997) are employed.

Although the most probable number (MPN) method (cf. Imai et al. 1984) is also commonly employed in enumeration of resting stage cells in sediments, it is only adaptable for viable (ready-to-germinate) cells and requires *a priori* information about the culture conditions suitable for their excystment, such as optimal temperature and irradiance.

#### CONCLUSION

To understand the ecology of planktonic ciliates, a quantitative investigation of their cyst populations in the sediment and their vegetative populations in the water column is indispensable. Although the present method is not adaptable to empty cysts, which emit weak or no fluorescence (Table 1), it is valid for intact cysts and can be employed as a practical method in future routine analysis of cyst populations in natural sediments.

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