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Chloroplast Retention by *Elphidium excavatum* (Terquem). Is it a Selective Process?

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Abstract

Some foraminifera retain chloroplasts from algae they partly digest. *Elphidium excavatum* (Terquem) was kept under laboratory cultures for a month and fed monoalgal cultures. The number of chloroplasts retained by this species of foraminifera depended on the algal diet. Higher numbers of chloroplasts were observed when the foraminifera were fed diatom species; the number of chloroplasts retained by each individual was approximately 3.7×10^4 chloroplasts. Significantly fewer green algal, and dinoflagellate chloroplasts were retained by each individual; the numbers were lower than the starved controls. These results seem to indicate that diatoms are the chloroplast donors for husbandry by *E. excavatum*.

Keywords: Foraminifera, *Elphidium*, chloroplast retention

1. Introduction

Retention of plastids first described in opistobranch mollusks (Green, 1970) was later found to occur in many protists including planktonic ciliates (Blackbourn et al., 1973; review in Dolan, 1992) dinoflagellates (reviewed by Stoecker, 1999), Heliozoa (Paterson and Durschmidt, 1987) and foraminifera

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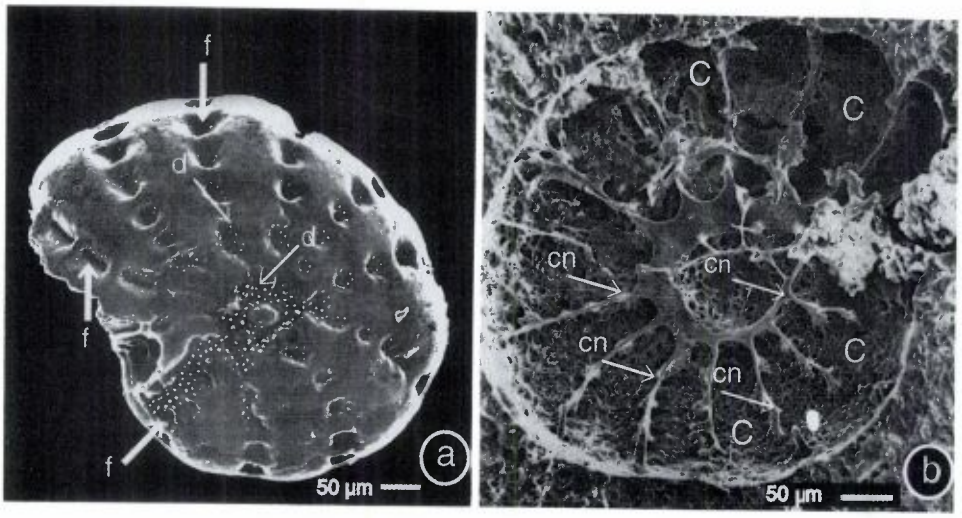


Figure 1. Morphology of a test of *Elphidium excavatum* (Terquem). a: Whole test showing denticles (d) and fossae (f). b: Cast of test (prepared by Hottinguer technique) showing areas where canals (cn) and connections between chambers (C) are located.

(reviewed by Berhard and Bowser, 1999). Chloroplast retention has been described in 5 families of foraminifera, almost all of which have sieve-like test ornaments (Fig. 1a) and in the case of the family Elphididae a complex canal system (Fig. 1b). These features have been thought to play a part in the phenomenon even though this has not been tested experimentally (Lee et al., 1988; Berhard and Bowser, 1999).

Most of the published accounts on chloroplast retention in foraminifera describe specimens obtained from the field. Lopez (1979) in one of the first experimental studies on the phenomenon in foraminifera tested the effects of different light/dark regimens on the number of chloroplasts retained. Experiments by Lee and Lee (1989) suggested that some algal groups or species were more suitable as chloroplast donors than others. They reported difficulties in counting chloroplasts in the chambers of foraminifera because of the thickness of the specimens. Even when the specimens were crushed they had difficulty distinguishing and enumerating individual overlapping fluorescing chloroplasts in an epifluorescent microscope. The development of confocal microscopy, which allows for thin optical slicing of specimens, gave us a new opportunity to continue studies on the phenomenon. It was the aim of this study a make a more comprehensive analysis of the question of whether chloroplast retention in *Elphidium excavatum* is a selective phenomenon.

2. Material and Methods

Sample collection

Samples were collected at Lake Tashmoo (Martha's Vineyard N41°32' W 70°40') in the first week of August 1997 and 1998. In this season it is easiest to collect large numbers of individuals of *Elphidium* spp. and *Hanesina germanica* in this habitat. Macroalgae (especially *Enteromorpha*) present on the sediment, were collected and washed in a bucket of local seawater to release the epiphytic forams and microalgae. Particles >0.5 cm were sieved out. The foraminifera were washed in plastic buckets in order to eliminate most of the organic material. After they settled to the bottom of the bucket the overlaying water was decanted. The remaining sediment was stored in clear plastic bottles in a 1:3 ratio of sediment/water and transported to the laboratory in New York City in a picnic cooler. Individual specimens of *Elphidium excavatum* were picked from the sediment with sable paint brushes and divided into groups of 20–25 foraminifera. Each aliquot contained individuals of approximately the same size selected at random.

Algae

All of the algae used in these experiments were isolated from littoral benthic marine communities. They were cloned on agar as described in Lee et al. (1975). *Nitzchia frustulum, Amphora coffaeformis, Cylindrotheca closterium, Dunaliella salina,* unidentified green alga clone 5 and clone 8, were isolated from the sublittoral epiphytic community of Lake Tasmoo or the nearby Greater Sippewissitt Salt Marsh. *Navicula salinicola, Amphidinium* sp. were isolated from the benthos of sedimentation ponds at the National Center for Mariculture in Eilat Israel. *Chlorella* sp. was isolated as an endosymbiont of the foraminifer *Amphistegina lobifera,* harvested from the Gulf of Eilat. It was partially characterized by Kessler et al. (1982).

Experimental setup

Each group was fed one of 9 monoclonal algal cultures, or a mixture of these and incubated at 25°C with a 12 hour light:12 hour dark cycle, or in complete darkness. The algae used were diatoms (*Amphora coffaeformis, Cylindrotheca closterium, Navicula salinicola,* and *Nitzchia laevis*); chlorophytes (*Chlorella* sp., *Dunaliella salina,* unidentified green alga #5 and unidentified green alga #8) and a dinoflagellate (*Amphidinium* sp.). These algae were chosen because they are abundant in the natural habitat of *E. excavatum* and therefore likely to be ingested by this foraminifer. The amount of algae added

to each culture in a 250 ml tissue culture flask brought the final concentration of algae to 10^6 cells /ml. Starved controls were maintained under the same conditions as experimental cultures. The reason for these controls is that freshly captured organisms already have a complement of chloroplasts. Even if starved for several months they still retain some chloroplasts. The number of chloroplasts retained by the starved controls in our experiment served as a baseline which was subtracted from the experimental data. By subtracting the average number of chloroplast retained by the starved controls from each of the experimental groups, we calculated the change in number of chloroplasts due to feeding by the foraminifera in the laboratory. The controls were collected at the exact same time as the experimental groups and were subject to the same treatment. The only difference was that the controls were incubated in sterile seawater instead of seawater with algae. Half of the controls were incubated in the dark, the other half were in a 12 hour light/12 hour dark cycle. Every week, 10 individuals were selected from each flask, placed on a slide with a drop of glycerol and kept at –20°C. The experiment lasted for 4 weeks and the starved controls still looked healthy under the dissecting microscope. The number of chloroplasts per individual, of the frozen foraminifera, was estimated using a Confocal Laser Scanning Microscope (CLSM, Molecular Dynamics Multiprobe 2001 with an Argon/Krypton laser). Scans were done using a 568 nm exciter filter and a 590 nm barrier filter. Optical sections, 1 micron thick, were made at 5 micron intervals. Serial sections were made throughout each individual foraminifer. In most cases, the whole cytoplasm was scanned in 20 serial sections. At random at least three sections were chosen to represent each organism. The number of chloroplasts in each section was counted and multiplied by an appropriate factor to calculate the number of chloroplasts in the entire volume of the organism. In order to calculate the total number of chloroplasts per individual, the ratios described above, were multiplied by the volume of an average specimen of *E. excavatum*. That volume was calculated assuming each individual has the approximate shape of a disk and the average diameter and thickness described by Buzas (1966) for specimens isolated from the same salt-marsh.

Statistical analysis

All ANOVA calculations were done using the SAS®. statistical package version 6.12 The values obtained in the ANOVA analysis were compared pair-wise and the comparisons were corrected using a sequential BonFerroni analysis to decrease the inflation of the error associated with multiple pair wise comparisons (Rice, 1989). The alpha value used for the comparisons was 0.05.

Figure 2. Specimen of *Elphidium excavatum* (Terquem) observed on a CSLM. Chloroplasts can be observed as fluorescent bodies (Cp) scattered throughout the cytoplasm. Note opaque areas (W) which correspond to test walls separating the several chambers (C). Test was sometimes interrupted by cytoplasm filled canals (Cn) connecting the chambers.

3. Results

Chloroplast distribution within Elphidium excavatum

Chloroplasts within *E. excavatum* specimens were easily and individually visualized as fluorescent bodies under the CSLM (Fig. 2). Chloroplasts seem to be distributed evenly throughout the cytoplasm. Those in the youngest chambers were more intensely fluorescent and more compact than those found in older chambers. Non-fluorescing (opaque areas) in Fig. 2 correspond to the test wall separating the chambers. We observed chloroplasts in the canal system as well as in the interior cytoplasm of the chambers. The volume occupied by the chloroplasts was approximately between 10.9% and 25.4% of the total cytoplasmic volume of the specimen.

Feeding experiments

Significantly more chloroplasts of *Amphora coffeformis* were retained by *E. excavatum* than the chloroplasts of any other species of algae tested (Fig. 3 and Table 1). There were no significant differences between the numbers of chloroplasts of *Cylindrotheca closterium*, *Navicula salinicola*, and *Nitzchia laevis* retained by the forams.

In only one case, when the foraminifera were fed a diet of *Amphora coffeformis*, did we find that significantly more chloroplasts were retained when the forams were incubated in the light than when they were incubated in the dark. On diets of all the other species of diatoms tested there were no significant differences between the number of chloroplasts retained by foraminifera incubated in either the light or the dark. When compared to the diatom species, fewer chloroplasts from green algal species tested were retained. The number of chloroplasts retained by foraminifera fed *Amphidinium* and the two unidentified chlorophytes were less than the starved controls (Fig. 3).

When the data were analyzed by grouping the algae by types (Fig. 4), there were significantly more chloroplasts from diatoms retained by *E. excavatum* than by those of other groups. When incubated in the light, there were significant statistical differences between the number of chloroplasts retained by foraminifera fed each of the diets. After diatom diets, foraminifera fed a diet of a mixture of several diatoms and green algae retained the next highest number of chloroplasts. When *Elphidium* were fed dinoflagellates or green algae, chloroplast retention was zero or negative (when compared to the control). In the light, significantly more diatom chloroplasts were retained than on all the other diets. The number of chloroplasts retained in the dark when the diets consisted of green algae and the dinoflagellate were not

Table 1. Average number of chloroplasts retained per individual (Chl/ind). Diatoms refers to the average of all diatom species used. The mixture is composed of diatoms and green algae.

Diet	Diatoms	Mixture	Ah	Na	Ni	Ci
Chl/ind	3.7E+4	2.9E+4	3.9E+4	3.6E+4	3.8E+4	3.6E+4

Ah = *Amphora Halamphora coffeformis;* Na = *Navicula;* Ni = *Nitzchia laevis;* Ci = *Cylindrotheca closterium;* Field = specimens observed right after collection.

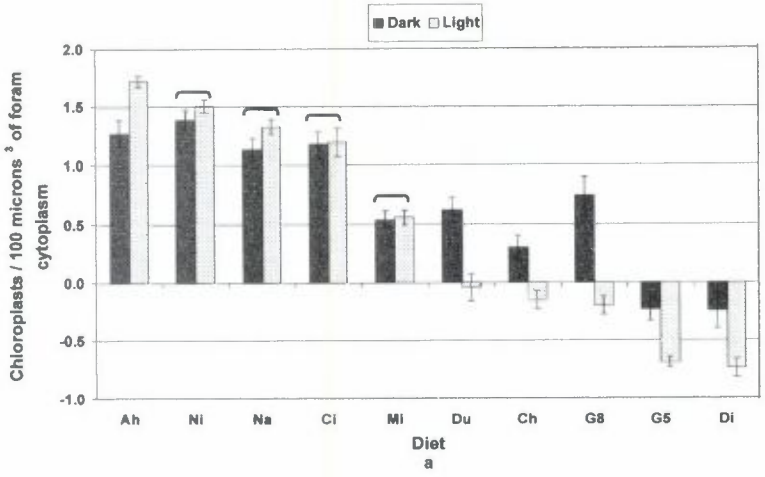
statistically different from each other and did not differ from the starved control. There were no significant differences between the number of chloroplasts retained after incubations in the light or the dark for two of the diets (diatoms and mixture) (Fig. 4). There were significantly more green algal chloroplasts retained in forams incubated in the dark than those incubated in the light.

4. Discussion

Food quality

Of the nine algal species tested, *Amphora coffaeformis* seems to be the best chloroplast donor for *Elphidium excavatum*. This is in agreement with the results of Lee and Lee (1989) who found that *A. coffaeformis* is among the preferred algae when *E. williamsonii* and *Haynesina germanica*, are the hosts, even though the differences between the values for different diatoms were not treated statistically. However, Lopez (unpublished) did not find significant differences between the retention number of diatom species she tested. She suggested that the forams seemed to react to fluctuations in the abundance of food particles and not the particular algal species available as food. In fact, in our results, no significant differences were found between the diets of *Nitzchia*, *Navicula* and *Cylindrotheca* tested. Lopez (unpublished) tested closely related species of *Amphora* whereas this study and Lee and Lee (1989) dealt with four different genera. This may explain the apparent disparity of results.

Lee and Lee (1989) found that five times more diatom chloroplasts were sequestered than were those from green algae. This is not a surprising finding since fine structural studies (Lopez, 1979; Leutenegger, 1984; Lee et al., 1988; Cedhagen, 1991) and pigment analysis (Lopez, 1979; Knight and Mantoura, 1985) have suggested that diatoms were the chloroplast donors.


Dark
Light
Chloroplasts / 100 microns ³ of foram cytoplasm
2.0
1.5
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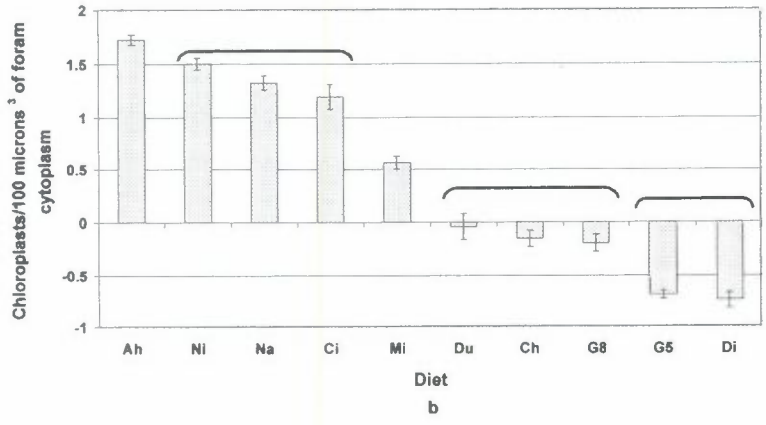
Light
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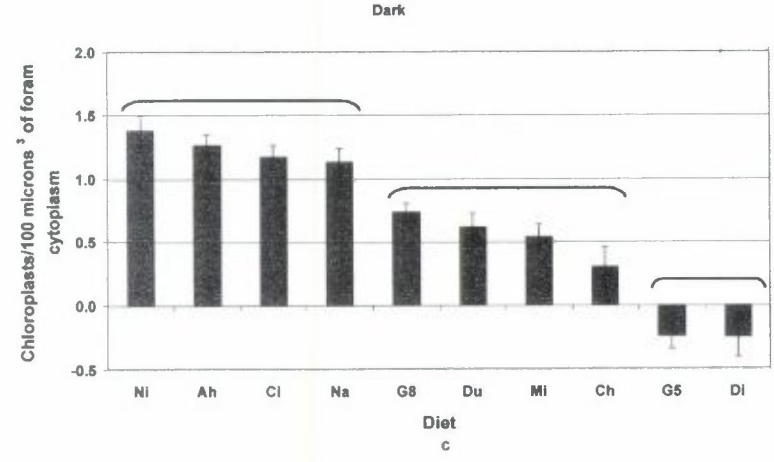
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Lee and Lee (1989) found that green algae were retained in lower numbers than diatoms. It was interesting to find that the chloroplasts from the endosymbiotic *Chlorella* were not retained at a significantly higher rate than any of the other chlorophphyte chloroplast donors fed to the foraminifera. It was significant that chloroplast retention was even lower than in the starved controls. This seems to indicate that feeding on green algae in the light somehow increases the digestion of the chloroplasts already present. A benthic dinoflagellate (*Amphidinium*) was also a poor chloroplast donor for potential plastid retention by the foraminifera. When the foraminifera were fed a diet of *Amphidinium* they seemed to digest the chloroplasts they had sequestered before the experiment started.

When the values obtained from the counts are converted to absolute numbers of chloroplasts/individual foram (Table 1), they are larger than the numbers obtained previously by other authors who studied this phenomenon. In the case of Lee and Lee (1989), the results obtained in this study differ by 2 orders of magnitude when *Amphora* and *Nitzchia* are considered and by four orders of magnitude when *Navicula* is considered. In the studies published by Lopez (1979), only values for field samples are presented. These values, are also two orders of magnitude lower than the ones obtained in this study. The explanations for these huge differences in the results can be attributed to two sets of factors. On the one hand, the extrapolation methods were different. In fact, only Lee and Lee (1989) determined, through microscopy the numbers of chloroplasts per individual directly. In this study and in Lopez (1979) estimations were made. Estimates are always subject to errors and if these error are in two different directions (under and over estimation) the differences between the values could be magnified. It can be assumed that the chloroplasts present in the foraminifer's cytoplasm have a random distribution and that they all have an equal probably of being sectioned through. This is true because regardless of what algae the chloroplasts originated from, once they are inside

Figure 3. Number of chloroplasts retained by foraminifera being fed diets of different algae. Analysis done considering the specific algae used to feed the forams. Ah = *Amphora* (*Halamphora*) sp., Ni = *Nitzchia frustulum*, Na = *Navicula* sp., Ci = *Cylindrotheca closterium*, Mi = mixture of *Amphora* (*Halamphora*) sp., *Nitzchia frustulum*, *Navicula* sp., *Cylindrotheca closterium*, *Chlorella* sp. and *Dunaliella salina*, Du = *Dunaliella salina*, Ch = *Chlorella* sp., G8 = green alga clone 8, G5 = green alga clone 5, Di = *Amphidinium* sp. Values graphed were obtained by substracting retention values for starved controls from the mean for each diet. a: Comparison between dark and light incubations; b: comparison between light incubations; and c: comparison between dark incubations. The square brackets indicate values which are not statistically different from each other with an $\alpha = 0.05$.

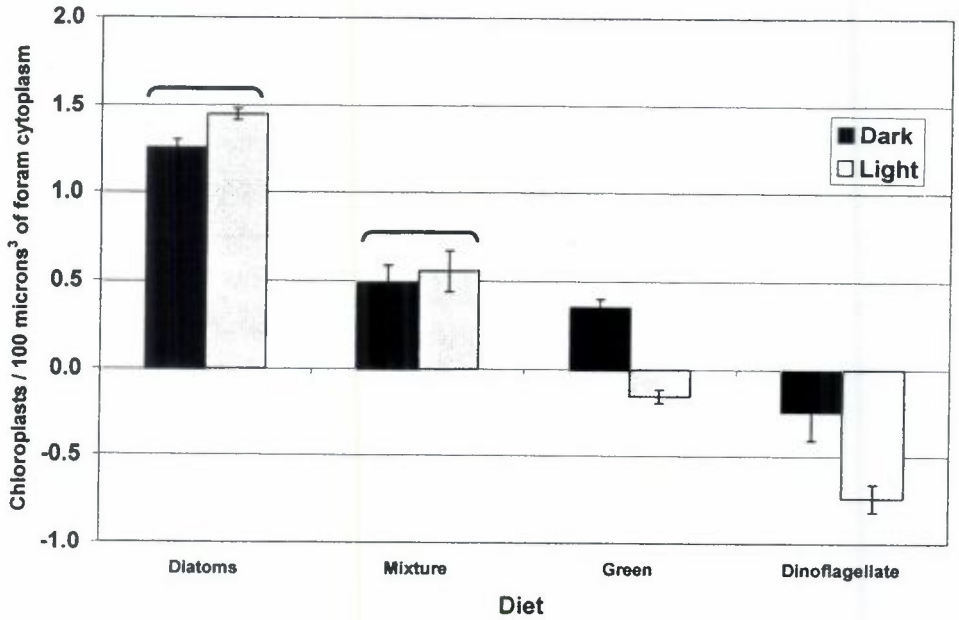

Figure 4. Number of chloroplasts retained by foraminifera being fed diets of different algae. Analysis done considering the specific algae used to feed the forams. Analysis done considering the algal groups to which the diets belonged to. The diatoms include *Amphora* (*Halamphora*) sp., *Cylindrotheca closterium, Navicula* sp., and *Nitzchia frustulum*. The dinoflagellate is *Amphidinium*. The green algae include *Chlorella, Dunaliella salina,* the green alga clone 5 and clone 8. The mixture is composed of *Amphora* (*Halamophora*) sp., *Cylindrotheca closterium, Navicula* sp., *Nitzchia frustulum, Chlorella* sp., and *Dunaliella salina*. Values graphed were obtained by substracting retention values for starved controls from the mean for each diet. Square brackets indicate values that are not statistically different from each other with an α value of 0.05.

the foraminifer's cytoplasm they assume a more or less round to oval shape due to the absence of the frustule's physical constraint. On the other hand, the studies done by Lopez (1979) and Lee and Lee (1989) used regular epifluorescence microscopy for their counts. This microscope presents two major problems. First, the thickness of the specimens in concert with characteristically bright background fluorescence, make accurate counts of the individual chloroplasts very difficult and probably led to the under estimation of the chloroplasts. Lopez (1979), solved this problem by making "extracts" of 100 crushed forams. This however, increases the chances of loosing chloroplasts by lysis and by bleaching of the fluorescence. When regular epifluorescence microscopy is used, the chloroplasts are continuously flooded with intense light from a mercury

lamp which causes the fluorescence to quench extremely fast. These two reasons, prompted us to use the Confocal Scanning Laser Microscope (CSLM). This instrument decreases the bleaching because the UV light from the laser is only briefly focused on the point being scanned. Furthermore, it is possible to do optical sectioning of the specimens. In this way, the thickness problem is solved without the need to crush the individuals. This, together with the fact that images are digitized, makes the counts more accurate. These new technologies, not available two decades ago, greatly improve the accuracy of the counts and explain the increase in numbers which was obtained in this study.

Effect of light on chloroplast husbandry

The results of the present experiments gave a mixed message. There were no statistical differences between the chloroplasts retained by foraminifera incubated in a light/dark cycle or in the dark when the foraminifera were fed most of the diatoms tested, the mixture (Fig. 3a) or starved controls. This suggests that gradients, energy captured by photosynthesis, or photosynthetates, did not affect the retention process. However, on diets of *A. coffaeformis*, all the green algal diets and the *Amphidinium* diet (Fig. 3a) values of chloroplast retention by foraminifera incubated in light/dark cycles were statistically different than those incubated in the dark. This might be interpreted as a slow down of digestion by the foraminifera in the dark, or to a higher rate of degradation of the retained chloroplasts in the light. Lopez (1979) found that *Nonion* (*Haynesina*) germanicum survived for a longer time when individuals were adapted to continuous darkness, than when they were kept in alternating light/dark cycles. She suggested that the loss of chloroplasts might be accelerated by degradation and/or loss of light sensitive components of active chloroplasts. The fact that foraminifera from below the photic zone retain chloroplasts (see review in Bernhard and Bowser, 1999), seems to support the hypothesis that photosynthesis (or at least the light phase of this process) is not absolutely necessary for the retention of the plastids.

Why diatoms?

It is known that the plastids eventually get digested or undergo autolysis (Lee et al., 1988) and therefore need to be replaced. Therefore the abundance of donor species is important for the foraminifera. Diatoms are the most abundant microalgal group in the salt marshes where many species of *Elphidium* abound (Lee et al., 1975). It comes as no surprise that they would be an important

component of the diet of *E. excavatum*, the organism we chose for our experiments. Diatoms are also found as endosymbionts in quite a number of families of larger foraminifera. Paradoxically endosymbiotic diatoms are extremely rare (less that 0.5% occurrence) in the habitat where larger foraminifera feed (Lee et al., 1989). This rarity in abundance does not seem to be a problem since the host foraminifera mainly reproduce asexually and transmit the symbionts from one generation to the next. Recent studies by Chai and Lee (1999a, 2000) have shown that diatoms which are endosymbiotic have a surface antigen not found on the surfaces of diatoms which are digested. They also demonstrated that there is a receptor for this antigen on the surfaces of pseudopodia and that the antigen was necessary for the maintenance of symbiosis. The fact that plastid retention in *E. excavatum* seems to be a selective process suggests that there may be characteristics of either the host, the chloroplasts, or both in combination, which makes diatom plastids more suited for retention. We could imagine that the foraminiferan cytoplasm contains factors most conducive to diatom survival. We can wonder about the factors that underlay the partial digestion of the diatoms. Why are the chloroplasts not digested at the time that the rest of the algal cell is degraded? Does the canal system of *Elphidium* play any role in the chloroplast husbandry? These questions, and many more, challenge us to design experiments aimed at better understanding of the factors which underlie the chloroplast retention phenomenon.

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