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A

CHLOROPLAST RETENTION IN *Elphidium excavatum* (Terquem)

By

Maria Jose Serol de Brito Correia

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

Chloroplast retention in *Elphidium excavatum* (Terquem). Selectivity, persistence and fine structure of the chloroplasts.

By
Maria Correia

Adviser: Professor John J. Lee

Chloroplast retention by mollusks and several groups of protozoa has sparked the interest of several researchers in the last 3 decades. One of the protozoa groups found to retain chloroplasts from algae they partially digest are the foraminifera. *Elphidium excavatum* (Terquem) an abundant species of foraminifer in salt marsh areas was studied. Feeding experiments were done in the laboratory using several algae present in the foraminifer's natural habitat in order to ascertain the selectivity of the retaining process. Foraminifera were fed algae belonging to the diatoms, green algae and dinoflagellate groups. Results show a preferential retention of chloroplasts originating from diatoms (especially *Amphora coffaeformis*). Further feeding experiments were done with the aim of testing whether "survival" time of the isolated plastids within the foraminifera was dependent on the type of chloroplast ingested. Foraminifera were fed, in the laboratory, monoalgal diets of *Amphora coffaeformis* (a diatom), *Dunaliella salina* (a green alga) and *Amphidinium* sp. (a dinoflagellate). Feeding was allowed to continue for a weeks after which the foraminifera were cleaned and kept in medium without any algal food particles. Weekly samples of the foraminifera were observed using a confocal laser scanning microscope (CSLM) and the number of chloroplasts retained by individual counted. Isolated chloroplasts were retained inside the foraminifer for at least 8 weeks and possibly up to 20 weeks. When compared to green algae (6 weeks) or dinoflagellate

(4 weeks), diatom chloroplasts (9 weeks) were retained for longer periods of time under starvation of the foraminifera. Fine structure analysis of individuals fed different algal groups and of individuals fixed immediately after collection from the field were also performed. Transmission Electron Microscopy shows that the only chloroplasts retained belong probably to diatoms and that chloroplasts of green algae and dinoflagellates are not retained. The results from these three sets of experiments suggest that, just as has been shown for the ascoglossan mollusk *Elysia chlorotica*, chloroplasts from chromophitic algae are better suited for survival in a symbiotic host away from the algal nucleus.

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I would like to express my thanks to Dr. John Lee who has helped me throughout the almost ten years we have been working together. I specially thank him for having sparked my interest in these awesome creatures the Foraminifera. It all started with a set of reprints he sent me around 1991. Without his help none of this would have been possible and I would never had the possibility of working with forams. I would also like to thank everyone in my committee especially Dr. Bowser and Dr. Rockwell who were always ready to answer my questions and discuss details of the work even when they were extremely busy. Everyone who has passed through the Marine Microbial Ecology laboratory since the Fall of 1994 has helped me at some point or another and I am thankful to them. Particularly helpful in discussing projects and ideas were: Dr. John Chai, Megan Harrison, Dr. Eva Hawkins, Jorge Morales, Dr. Brina Nathanson, Dr. Fernando Nieto, and Andrew Symons. I would also like to thank Patricia Brito, Claudia Ribeiro, Robert Wallace and Marcelo Weskler for reviewing the manuscript and always trying to help whether through discussing ideas, solving software problems or just listening to my complaints. Several students helped with the collection and maintenance of the specimens: Taisha Benjamin, Sandy Dorcelus, Vanessa Marcano, Sophia McLeod, Michael Pearson and Yessenia. The sampling was also made possible with the help of Commodore Steven Moore of the Woods Hole Yacht Club and Mr. Paul Fucile from the Woods Hole Oceanographic Institution who provided the vessels necessary for sampling. The hospitality of the Lee family both in Woods Hole and in South Hampton was also much appreciated during the sampling periods.

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Finally, but most important of all, this thesis is dedicated to my parents to whom I owe everything. Very special thanks go also to Sergio Martins who has encouraged me through the whole process and didn't let me give up.

Table of Contents

List of Tables

List of Figures

Chapter I

General introduction

INTRODUCTION

As a group, foraminifera are one of the most abundant protists in modern seas. They occupy a wide range of habitats from the plankton to the sediments of the deep sea (Buzas and Culver, 1991). A few species have also been described in brackish and fresh water bodies (Arnak, 1958; Boltovsky and Lena, 1971 and Reisig, 1974 cited by Lipps, 1983). Their size varies widely from the larger foraminifera, which can reach several cm, to the tiny forms like *Metarotalia* and other microforaminifera which are 100 micrometers or less (Pawlowski and Lee, 1991). Foraminifera have a wide variety of survival strategies and exhibit almost all forms of nutrition from the uptake of dissolved nutrients, herbivory, carnivory, omivory, suspension feeding, detritivory, symbiosis, parasitism and cannibalism (reviewed by Murray, 1991).

In the last few decades, foraminifera have been studied as models of protozoan symbiosis. Algal symbiosis seems to be established, almost exclusively in the larger forms that inhabit the shallow tropical and semitropical seas, where the nutrient levels are low, and in planktonic foraminifera of oligotrophic seas. The phenomenon is thought to be an adaptation to these oligotrophic environments (Lee and Anderson, 1991). Foraminifera have characteristics that make them specially suited for algal symbiosis. These characters include:

1. General morpho-physiological characteristics such as the multi-cameral structure (Lee and Hallock, 1987) which subdivides cellular activities. Digestion, a cellular process

particularly important when discussing symbiosis, is initiated in the external granuloreticulopodal net and continues in the younger chambers (Lee *et al.*, 1991). This pattern of external formation of phagolysomal vesicles keeps the symbiotic algae apart from the algae being digested.

2. Vertical transmission of the symbionts facilitated by: a) asexual reproduction (agamogony and schizogony), and b) the potential for the zygotes to capture their parent's symbionts if their sexual phase is gamontogamic.

3. The diversity of algal types found in the foraminifera suggests some general mechanisms which facilitate symbiotic relationships (Lee, 1992).

4. The looseness of some associations (e.g. diatom-bearing hosts) in natural populations (Lee *et al.*, 1995) and also in experimental conditions (Lee *et al.* 1983, 1986) also suggests general adaptive mechanisms to support the phenomenon.

5. The existence of some algae that are rare or minor symbionts suggests that the host/symbiont relationships are less than finical.

6. The survivability of symbiotic algae in non symbiont-bearing foraminifera (Lee and Zucker, 1969) also support the hypothesis that even these foraminifera have some characteristics of a system that may allow some internalized algae to escape digestion (Lee and Anderson, 1991).

In addition to symbiotic relationships between algae and foraminifera, some groups of foraminifera partially digest algae they capture and retain their chloroplasts (Lopez, 1979, Lanners, 1983; Leutenegger 1984; Lee *et al.*, 1988; Cedhagen, 1991; reviewed by Bernhard and Bowser, 1999). These chloroplast retaining species inhabit salt marshes (Lee and Lee, 1989), other shallow benthic habitats (Lopez, 1979) and benthic habitats below the photic zone (Leutenegger, 1984; Cedhagen, 1991; Bernhard and Bowser, 1999).

I.1-Chloroplast retention

Retention of chloroplasts by metazoan cells had been reported in opistobranch mollusks (Green, 1970) and under experimental conditions from mouse fibroblast cells in culture (Nass, 1969 and Giles & Sarafis, 1971, cited in Blackbourn *et al.*, 1973). Blackbourn and others (1973) that some planktonic ciliates kept chloroplasts of the algae they ingested. This was the first description of the phenomenon in a protozoan. Since then, evidence of the phenomenon occurring in other groups of protists has been accumulating. The *Oligotrichs* and *Tinitinnids* in particular have been studied as chloroplast-retaining ciliates in marine environments (reviewed by Dolan, 1992). There is one description of a freshwater plastidic ciliate by Perriss and others (1994). Other protists retaining chloroplasts include some dinoflagellates (reviewed by Stoecker, 1999), Heliozoa (Patterson and Dürschmidt, 1987) and Foraminifera (reviewed by Berhnard and Bowser, 1999).

I.1.1-Groups of organisms which practice kleptoplastidy

I.1.1.1-Mollusks

The retention of chloroplasts (kleptochloroplasty) by sacoglossan mollusks was first noted by Brüel in 1904 in *Caliphylla mediterranea* (Clark *et al* 1990). But the first attempts at studying the phenomenon were done much later (Trench, 1969 and Green, 1970). Most of the studies following the re-discovery of plastid retention (reviewed by Hinde, 1983) were concentrated on the photosynthetic activity of the retained chloroplasts (Trench, 1969) and the longevity of the sacoglossan mollusks with the chloroplasts as their sole energy source (Green, 1970). Chloroplast retention in this group seems to occur in a gradient between the extremes of long-term retention in which chloroplasts are retained and photosynthesize for several weeks and total digestion without any retention (Clark *et al.*, 1990). It is a wide spread phenomenon among the non-shelled sacoglossan mollusks (Clark *et al.*, 1990).

The plastids may be surrounded by host membranes or free in the cytoplasm (Marín and Ros, 1993). In species or ontogenetic stages in which the photosynthetic activity is more intense, the chloroplasts are free in the cytoplasm (Clark, 1992). The chloroplast retaining mollusk, seem to have several anatomical, physiological and behavioral adaptations to optimize the benefits from the kleptochloroplasts. Species that are more adapted to kleptochloroplasty have a higher degree of branching of the digestive diverticula (cladohepatic digestive glands). In the case of elysiids and baseliids the entire dorsal surface of the animal is permeated with diverticula. Elysioid ascoslossan mollusks have a pericardial vascular complex, in which veins facilitate the excretion of excess O_2 and uptake of CO_2. This seems to be an important physiological adaptation to plastid retention by this group. Body shape maximizes surface area for light absorption and gas exchange. Phototaxic behavior has also been observed (Clark, 1992).

Recently focus has shifted to understanding how the chloroplasts keep their protein synthetic mechanisms working without algal nuclear gene products. Pierce and others (1996) found that the chloroplasts within the mollusk could synthetize the large subunit of RuBisCO and the PSI protein D1. Using protein synthesis inhibitors, they also found evidence that some chloroplast-associated proteins were synthesized in the host cytoplasm and then translocated into the plastids. In addition, Mujer and others (1996) found that rbcL, rcbS (RuBisCO subunit genes), psaB (photosystem I gene), psbA (photosystem II gene) and 16S rRNA were all found in DNA extracts of the slug. Using RNA synthesis inhibitors these authors found that active transcription of at least the 16S rRNA gene is taking place during the time the slugs are starved. Using a radionuclide tracer ([^{35}S] methionine) *de novo* synthesis of several thylakoid membrane proteins was also observed (Mujer *et al.*, 1996).

Green and others (2000) have shown that the symbiont chloroplasts maintain photosynthetic oxygen evolution and electron transport activity through photosystems I and II for several months in the absence of algal food. In addition, these authors have shown that the slugs maintain functional levels of chloroplast-encoded photosystem proteins and confirmed *de novo* protein synthesis of chloroplast encoded proteins such as D and RuBisCO large subunit. The absence of algal genome in the slug's tissue implies that the photosynthetic activity in the retained chloroplasts is regulated solely by the algal chloroplast and the animal nuclear genome (Green *et al.*, 2000). Pierce and others (1999) suggest that a virus, which infected the *Elysia* population for 9 years, could be a vector for the transfer of genetic information from the nucleus of the alga to the nucleus of the slug. Algal chloroplasts fix $^{14}CO_2$ in slug species which retain chloroplasts longer. The radioactive carbon appears in a variety of compounds in the animal tissues depending on the species. In *Placobranchus ianthobapsus* ^{14}C-hexose appears in mucus produced by the pedal gland (Trench *et al.*, 1970 cited by Green and Muscatine, 1972). In extracts of both *Elysia hedgpethi*, and *Placobrachus ianthobapsus* the radiomuclide tracer (^{14}C) was found in succinic acid and other TCA cycle intermediate compounds (Green and Muscatine, 1972).

I.1.1.2-Protozoa

I.1.1.2.1-*Ciliates*

The *Oligotrich* and *Tintinid* ciliate, are among the protists found to share the ability to retain chloroplasts. According to Stoecker (1991) there are 15 species of ciliates that retain chloroplasts. These species have been found in the Pacific, Atlantic and Mediterranean, but their absence from other waters may just be due to lack of appropriate sampling. Johnson and co-workers (1995) reported plastid and mitochondrial retention by *Perispira ovum* (family Spathidiidae) collected at a river estuary. Mixotrophy in ciliates is not restricted to a particular environment. There are freshwater chloroplast-retaining species (*Strombidium viridae*) that inhabit a broad range of lakes (oligo- meso- and eutrophic) (Perris *et al.*, 1994). Though most chloroplast-retaining species studied are from eutrophic coastal waters (Stoecker *et al.*, 1988; Dolan, 1992), there are also reports of chloroplast-retaining ciliate species in oligotrophic and mesotrophic waters (Perriss *et al.*, 1994). Mixotrophy is not a widespread phenomenon even in the oligotrichs, most species are holozoic and a few species have endosymbiotic algae. Most chloroplasts retained by ciliates seem to originate from a variety of chromophytic and chlorophytic algae (Blackbourn *et al* 1973; Laval-Peuto *et al.*, 1986;

Stoecker *et al.*, 1988). Eight different types of chloroplasts were found in a single species of the ciliate *Tontonia apendiculariformis* (Laval Peuto and Febvre, 1986). The results of some experiments (Stoecker *et al.*, 1988) suggest that there is no preference in the ingestion of the algae from which the chloroplasts are kept. In the same ciliate species there is variability in the treatment of ingested algae. Some specimens of the same algal species may be either completely or partially digested, with the chloroplasts remaining in the cytoplasm of the ciliate (Stoecker and Silver, 1990). However, when specimens of *Strombidium capitatum* were starved, they survived longer if the chloroplasts retained belonged to a cryptophyte (Stoecker and Silver 1990), which indicates that some associations of ciliates and plastids may be more successful than others. Even though theoretically the ciliates are capable of meeting their carbon requirements through the photosynthetic activity of their kleptochloroplasts, they need to keep feeding actively (Stoecker *et al.*, 1988). This may indicate that: 1) the energy generated by the photosynthetic chloroplasts is not enough for the metabolic needs of the ciliates (Putt 1990) and energy must be obtained by feeding; 2) the ciliates have some nutritional requirements not satisfied by the plastid activity; 3) the plastids have a short half-life inside the ciliate's cytoplasm compared to the host's life cycle and because they don't reproduce, the number of plastids can be maintained only by ingestion (Laval–Peuto and Febvre, 1986, Stoecker, 1988; Stoecker and Silver, 1990); 4) even if the plastids in the algal cells have a long half-life, the ciliate is not able to provide them with the metabolites necessary to maintain them for a longer time.

The relative integration of the plastid in the ciliate's cytoplasm varies. In *Laboea strobila* (Stoecker *et al.*, 1988) and *Strombidium capitatum* (Stoecker and Silver, 1990) the chloroplasts were reported to be free in the cytoplasm without any membranes surrounding them. On the other hand, Laval-Peuto and Febvre (1986) showed that in *Tontonia apendiculariformis* the chloroplasts are separated from the area where digestive vacuoles are found by a continous and dense endoplasmic reticulum. In this way, the chloroplasts are kept in the periphery of the cell (Laval-Peuto *et al.*, 1986). Other descriptions of the peripheral location of the chloroplasts have been made by Stoecker and others (1988) and Stoecker and Silver (1990). Radionuclide tracer labeling (^{14}C) studies of photosynthetic rates have yielded variable results. In general, the rates of photosynthesis in plastidic ciliates were comparable to those of microphytoplankton, but considerably lower than those of the donating algae (Dolan, 1992). Photosynthetic rates were dependent on the intensity and spectral quality of the light used as well as other variables such as the ciliate species studied (Putt, 1990) and algae fed (Stoecker *et al.*, 1988). Furthermore, Stoecker and Michaels (1991) found that the ^{14}C estimates reported probably underestimate the actual amount of fixed carbon. Several aspects of the association make the measurement of fixed ^{14}C difficult, and internal recycling of the carbon within the symbiotic system may underestimate the values obtained and mask the actual importance photosynthesis may have for the host.

Division of the chloroplasts inside the ciliates has not been observed and it has been suggested that these organelles probably degenerate (Laval-Peuto and Febvre, 1986).

It is not known why some chloroplasts are retained and others digested, or even exactly what the relative proportion of these processes is in ciliates.

I,1.1.2.2-*Dinoflagellates*

Some dinoflagellates had been described as having inclusions which were called "Chromatophores" or possessing plastids with pigments and membrane characteristics not typical of dinoflagellates (Stoecker, 1999). Recently some of these have been shown to be either symbionts or kleptochloroplast obtained from ingested algae.

Kleptochloroplasty has been found in at least two orders: *Gymnodiales* and *Dinamoebales*. In the former, *Amphidinium latum*, *A. poecilochroum*, *A. wigrense*, *Gymnodinium aeruginosum* and *G. acidotum* (Horiguchi and Pienaar, 1992; Larsen, 1988; Wilcox and Wedemayer, 1985; Schnepf *et al.*, 1989; Skvogaard, 1998). In the latter, chloroplast-retention has been described in the toxic dinoflagellate *Pfiesteria piscicida* (Lewitus, 1999) and seems possible in *Paulsenella* (Schnepft *et al.*, 1989). Except for *Gymnodinium aeruginosum* and *G. acidotum*, all the species mentioned are marine. Two of the species belong to a benthonic genus (*Amphidinium*), but the majority of the dinoflagellates involved in this process are found in the water column.

In all but one of the cases studied, ultrastructural observations indicate that the most likely source of the plastids is a cryptophyte (Horiguchi and Pienaar, 1992; Larsen, 1988; Wilcox and Wedemayer, 1985; Schnepf *et al.*, 1989; Skvogaard, 1998, Lewitus *et al.*, 1999). The cryptophyte origin of the chloroplasts is suggested by observation of paired thylakoids with an electron opaque lumen, sometimes described as inflated thylakoids (Horiguchi and Pienaar, 1992; Larsen, 1988; Wilcox and Wedemayer, 1984; Schnepf *et al.*, 1989) and the presence of nucleomorphs in most cases (Horiguchi and Pienar, 1992, Larsen, 1988). The nucleomorph is absent in 90% of *G. aeruginosum* studied (Schnepf *et al.*, 1989). None of the researchers cited studied the pigments present in the kleptochloroplast retaining dinoflagellates. This analysis would be an important way to confirm that the algal remnants found in the dinoflagellate's cytoplasm are crptophyte in origin. Fucoxanthin and phycobilins should be found and peridinin should be absent. The source of plastids kept by *Paulsenella* is probably a diatom since these algae are the prey of this dinoflagellate.

Electron microscopy has revealed that only the cryptophycean plastids remain in the cell (*Amphidinium latum*, and *Amphidinium wigrense*) (Horiguchi and Pienar, 1992; Wilcox and Wedemyer, 1985). In all other species studied some cryptophycean cytoplasm and other organelles such as mitochondria, ribosomes, Golgi apparatus and the nucleomorphs can also be found (Wilcox and Wedemyer, 1984; Larsen, 1988; Schnepf *et al.*, 1989). The presence of mitochondria in remnants of the prey's cytoplasm can be especially useful markers because cryptophycean mitochondria have flattened cristae (Wilcox and Wedemayer, 1984 and 1985) in contrast to the tubular cristae present in the dinoflagelates (Larsen, 1988; Schnepf *et al.*, 1989). In this way, the presence of these particular mitochondria further supports the cryptophyte origin of the retained chloroplasts. In every case, the "endosymbiont" is separated from the dinoflagelate cytoplasm by a membrane (Wilcox and Wedemayer, 1984; Wilcox and Wedemayer, 1985; Schnepf *et al.*, 1989; Larsen, 1988; Horiguchi and Pienaar, 1992).

In the studies described above, the integrity of the chloroplast ultrastucture has prompted researchers to consider that the chloroplasts remain active after digestion of other parts of the cryptophyte cell. Only recently, radiotracer methods were used to determine photosynthetic activity of the plastids. Skovgaard (1998) studied photosynthetic rates of *Gymnodinium "gracilentum"* feeding on a cryptophyte. In the light, *G. gracilentum* fixed a significantly higher amount of carbon than it did in the dark, which indicated that the sequestered chloroplasts are photosynthetically active. With light microscopic-autoradiography, Lewitus and others (1999) showed that *Pfiesteria piscicida* containing kleptochloroplast is also capable of photosynthesis, although it is at rates lower than the free-living cryptophytes from which the chloroplasts originated.

In most of the cases, ultrastructural observations of were made from samples collected from the field and not from laboratory experiments. It is therefore presently not possible to determine the permanence of the association between the dinoflagellates and the plastids. Evidence of digestion of cryptophytes in some sections (Larsen, 1988), coupled with the inability to culture the dinoflagellates without food (Larsen, 1988 and Horiguchi and Pienaar, 1992) indicates that the plastids need to be replaced by feeding. In *Gymnodinium "gracilentum"*, the kleptochloroplast seem to be active and essential for survival. After approximately 2 days, they are digested and/or diluted by division (Skovgaard, 1998). These observations were based on growth and survivability rates of the dinoflagellates and not on ultrastructural evidence. In *Pfiesteria piscicida* ingested chloroplasts were retained and kept for at least 9 days, but the authors suggest that higher retention times are possible (Lewitus *et al.*, 1999).

I.1.1.2.3-*Heliozoa*

There has only been one observation of kleptochloroplasts in specimens of *Acanthocystis serrata*, *Rhaphidocystis tubifera* and *Chlamydaster fimbriatus* collected in British lakes Heliozoa (Patterson and Durchmidt, 1987). The fine structure of the retained plastids seems to indicate that they originated from Chlorophytes. The chloroplasts had thylakoids composed of three lamellae. Girdle lamellae, pyrenoids and storage products were sometimes present. In one case the storage product was starch and in the other, lipids. The chloroplasts in all three species were separated from the heliozoan cytoplasm by a single membrane possibly remaining from the phagosomal membrane formed when ingestion took place.

In these plastidic Heliozoa, photosynthesis was inferred from the structural integrity of the chloroplast, but not demonstrated experimentally. The ability to retain chloroplasts in the Heliozoa is not universal. *A. serrata* occurred with *A. erinaceoides* and *Raphidiophyris ambigua*, but the latter did not retain chloroplasts. *Rhaphidocystis tubifera* occasionally contain chloroplasts but *R. lemani* does not (Patterson and Durchmidt, 1987). The chloroplasts retained by the three different species, and even within each species, had different ultrastructure, which indicated that they originated from different ingested algae. This association may be classified as neither obligatory nor stable.

I.1.1.2.4-*Foraminifera*

I.1.1.2.4.1-*Distribution of the chloroplast retaining species*

The foraminiferal species shown to retain chloroplasts belong mainly to the families *Elphididae* and *Nonionidae* (Table I). They are distributed through temperate and tropical seas, most in shallow waters (Lopez, 1979, Lee *et al.*, 1988 and Lee and Lee 1989) but some in deeper regions (Lee *et al.*, 1988, Leutenegger, 1984, Cedhagen, 1991 and Bernhard and Bowser, 1999). Chloroplast retention has also been reported in the tiny rotaliellids *Metarotaliella parva* and *M. simplex*.

TABLE I.1 - Documented occurrences of plastids found in the cytoplasm of foraminifera. *These occurrences were not found in samples from the field but were induced in laboratory cultures by feeding the forams with certain diatoms.

Family	**Species**	**Region**	**Depth**	**Reference**
Elphididae	*Elphidium* sp.	Red Sea	5m	Leutenegger, 1984
	E. incertum	Lake Tashmoo, US	≤ 0.5 m	Bernhard and Bowser, 1999
	E. williamsoni.	Denmark	≤ 6m	Lopez, 1979
	E. crispum	Mediterranean	1-5m	Leutenegger, 1984
	E. macellum aculeatum	Mediterranean	1-5m	Leutenegger, 1984
	E. excavatum	Denmark	≤ 6m	Lopez, 1979
	E. cradiculatum	New Caledonia	18m	Leutenegger, 1984
	E. umbiculatum	Sweden	115m	Cedhagen, 1991
	E.incertum	Drake's Island, UK	?	Lee and Lee 1989
	E. incertum	Long Island and Sipewisset Marsh, US	?	Lee and Lee 1989
	E. translucens			Lee and Lee, 1989
	E. crispum	Eilat Mombassa	38m ≤2m	Lee et al., 1988
Nonionidae	*Haynesina germanica*	Denmark Drake's Island, UK	6m	Lopez, 1979 Lee and Lee 1990
	Nonium depressulum			Cedhagen, 1991
	Nonionella stella	California, US	29m	Leutenegger, 1984
	N. stella	California, US	551-591m	Bernhard and Bowser, 1999
	N. labradorica	Sweden	20-300m	Cedhagen, 1991
Buliminidae	*Bulimina elegantissima*	California, US	551-591	Bernhard and Bowser, 1999
Stainforthiidae	*Stainforthia fusiformis*	Sweden	20-300	Cedhagen, 1991
***Rotaliellidae**	*Metarotaliella parva*	in culture		Lanners, 1983
	M. simplex	in culture		Lanners, 1983

Estimates of the number of chloroplasts retained from recently collected forams were made by Lopez (1979) in Denmark. She reported that *E. williamsoni* had the greatest number of chloroplasts ($9.7 \pm 4.9 \times 10^3$) per individual, *Nonium* (now *Haynesina*) *germanicum* ($1.3 \pm 0.4 \times 10^3$)per individual and *E. excavatum* ($1.2 \pm 0.7 \times 10^3$) per individual. Lee and Lee (1989) found almost an order of magnitude fewer chloroplasts than those reported by Lopez (1979) in the foraminifera they collected in salt marshes of the Northeast coast of the United States. They found 150 chloroplasts per individual in freshly collected *Elphidium incertum* and 175 chloroplasts per individual in *Haynesina germanica*. These huge differences in number may hsve resulted because the authors studied different species from different habitats, but may have also arose from the methods of counting the chloroplasts. While Lopez (1979) made estimates observing 100 crushed individuals and then assuming an average size for the forams, Lee and Lee (1989) estimated the chloroplast numbers by counting the number of chloroplasts in each individual foraminifer. In both studies, an epifluorescence microscope was used to count the chloroplasts, presenting two main problems. The fluorescence of the chloroplasts bleaches within 1-5 minutes, which may not be enough time to count the numerous chloroplasts. On the other hand, because the number of chloroplasts is usually very large there is a high amount of background fluorescence, especially if the foraminifer is observed whole. Use of the laser confocal scanning microscope solves these problems and allows a more accurate estimate of the number of chloroplasts retained by each individual foraminifera.

I.1.1.2.4.2-*Origin of the chloroplasts*

Determining the origin of the kleptochloroplasts is a difficult task because in most cases the plastids, in foraminifera, are the only structures retained. Therefore, one way to determine which algae the chloroplasts belonged to is by fine structural and pigment analysis. Even when these of data are available, it is possible to assign the chloroplasts only to an algal group and not a particular species. Data from these types of study seem to indicate that the most probable donors belong to the Bacillariophyceae (Lopez, 1979, Leutnegger; 1984; Knight and Mantoura, 1985), Chrysophyceae (Lopez, 1979; Leutnegger, 1984) and Dinophyceae (Cedhagen, 1991).

The chloroplasts retained by foraminifera generally have lamellae with three thylakoids (Lopez, 1979; Leutenegger, 1984; Lee *et al.*, 1988). Pyrenoids were often present in the plastids and they had either a simple internal matrix with no thylakoids entering the pyrenoid (Cedhagen 1991) or they were crossed by a lamella (Lopez, 1979). Leutenegger (1984) observed that the chloroplasts she found in *Elphidium craticulatum* had a girdle, lamella and an internal pyrenoid separated by a membrane and a thylakoid sheath. Lopez (1979) found membrane-like structures in the distal part of the pyrenoid in plastids within *E. williamsoni.* She frequently found thylakoids crossing from one lamella to another but she did not observe grana (stacks of thylakoids). Each chloroplast was surrounded by a girdle lamella, consisting of 3 thylakoids (Lopez, 1979; Leutenegger, 1984).

In order to determine which algae were preferred by the foraminifera as plastid donors, Lopez (unpublished) and Lee and Lee (1989) studied the association under different experimental conditions. Lopez (unpublished) used four different species of *Amphora* and two of *Nitzchia* and *Melosira*, labeled with a radionuclide tracer to feed *E. williamsoni*. Analysis of the crushed forams, revealed that they didn't select a particular algal species but that they were sensitive to differences in the amount of food offered, eating in proportion to the quantity of diatoms available (Lopez unpublished). Lee and Lee (1989) experimented with other diatom species and two green algae. Their results depended on the particular species of foraminifer observed. For example, *E. crispum* retained an average of 160-175 chloroplasts after being fed monoalgal diets of *Cocconeis placentula* or *Amphora sp.*; around 140 of *Nitzchia rhomboica*; 80 of *Navicula sp.*; 60 of *Entomoneis densistriata* and 30 of *Chlorella sp. Haynesina germanica* kept 60 chloroplasts per individual after being fed a mixture of *Navicula menisculus* and *Amphora tenerima*. This seemed to indicate that there is a pecking order in which chloroplasts of particular species are preferentially retained over others.

The retention of the chloroplasts seems to be different in the light and in the dark. Again, depending on the foraminiferal species studied and the procedures used, the results varied. Lopez (1979) found that under starvation chloroplasts in *E. williamsoni* and *Nonium germanicum* survived for a longer time in individuals adapted to continuous darkness than those kept in alternating light and dark. This was interpreted as being due to use of essential substances by the active chloroplasts thereby decreasing the longevity of these structures. However, Lee and Lee (1989) had dark controls in their feeding experiments, and, in general, the foraminifera kept in the light survived longer under starvation than those kept in the dark. For example *Haynesina germanica* survived for 13.5 weeks when kept in the light and only 9 when kept in the dark. Furthermore, they found that the number of chloroplasts kept per individual was greater when the foraminifera were kept in an alternating light/dark cycle than when they were kept in the dark. *Haynesina germanica,* when fed the same diet, kept 60 chloroplasts/individual in the dark and only 30 in the light/dark cycle.

I.1.1.2.4.3-*Fine structure of the chloroplasts*

Some authors have reported that the chloroplasts found inside the foraminiferal cytoplasm were of one type only (Lopez, 1979; Cedhagen, 1991). However, others have found that there were different types of chloroplasts in the same foraminiferal species and sometimes in the same individual (Leutenegger, 1984). When the chloroplasts are inside the host, three membranes are usually found (Lee *et al.*, 1988; Cedhagen, 1991). Lee and others (1988) interpreted the outer one as the phagosomal membrane; the middle, which is not always complete, is thought to be the algal cell membrane; and inside this one, the chloroplast's own membrane. Cedhagen (1991) suggested that all three membranes he observed belonged to the alga ingested and therefore hypothesized that the algae ingested belonged to the dinophyceae. This is the only description, so far, of dinoflagellate chloroplasts retained by foraminifera. The micrographs published by Cedhagen (1991) do not reveal the number of thylakoids present in the retained plastids of *Nonionella labradorica,* therefore the only distinctive structure is a pyrenoid similar to those in diatoms. Lopez (1979) found that the chloroplasts she observed were bound by a double membrane and sometimes situated towards the vacuolar lumen. She also observed fragments of a second double membrane. These different observations probably have more to do with the fixation and staining methods used than with real differences in the fine structure of the organelles.

Although the retained chloroplasts seem to keep photosynthesizing, they also appear to be slowly but actively digested by the host. Small vesicles (that could be microbodies or lysosomes) and Golgi bodies were found around the enveloping membranes (Lee *et al.* , 1988). However, there seems to be a consensus that most chloroplasts are intact: only a small fraction showing signs of disaggregation (Leutenegger, 1984, Lee *et al.*, 1988 and Cedhagen 1991). The signs of aging include a loss of thylakoid lamellar structure, the fusing of the thylakoids and the loss of the membranes (Lopez, 1979; Leutenegger, 1984; Lee *et al.*, 1988).

In most ultrastructural descriptions, only chloroplasts were found in the vacuoles of the foraminifer's cytoplasm. Exceptions were reported by Lee and others (1988) in specimens of *Elphidium* collected in tropical seas (Eilat and Mombassa). They found algal nuclei and mitochondria in some sections. Lopez (1979) found that the chloroplasts studied in *Elphidium williamsonii*, *Haynesina germanica* and *Elphidium excavatum* were distributed throughout the cytoplasm, but tended to be most concentrated in the outer most parts.

In one study, 10% of the chloroplasts were reported to be dividing (Cedhagen, 1991). However the micrographs presented as evidence for the division of plastids may have been misinterpreted. It seems reasonable to interpret two juxtaposed chloroplasts as either lobes of the same plastid or as two chloroplasts originating from the same alga.

I.1.1.2.4.4 - ^{14}C *studies*

Primary production studies of the husbanded chloroplasts showed carbon fixation by the retained plastids (Lopez, 1979, Lee *et al.*, 1988; Cedhagen, 1991). But again, due to the different methods used in these estimates, comparisons are difficult. At light saturation, *E. williamsoni* fixed 9.4 ± 3.4 x 10^{-5} mg C mg^{-1} ash-free dry weight per hour, which is almost five times more than *Haynesina germanica* (1.6 ± 0.6 x 10^{-4} mg C mg^{-1} ash-free dry weight per hour) (Lopez, 1979). For these two species, the carbon fixation rate was significantly different in the light and in the dark. In *E. excavatum* there were no significant differences between light and dark incubation which suggests that the chloroplasts in this association were not active (Lopez, 1979). *E. crispum* from the Red Sea at Eilat fixed 1.5 µg carbon/mg foram dry weight in 48 hours (Lee *et al.*, 1988). *Nonionella labradorica* in Swedish water fixed 2.12 x 10^{-10} mg of carbon/individual/hr which was considered by the author to be low even though the basis for this comparison is unclear (Cedhagen, 1991).

Data from TEM observations and the radioisotope studies support the hypothesis that the chloroplasts retained by most of the species discussed are photosynthetically active. Another indication that the chloroplasts are active inside the foraminifera was provided by Knight and Mantoura (1985) who used High Performance

Liquid Chromatography (HPLC) to show that the concentration of phaeophytin (degradation product of chlorophyll a) was low in the foraminifer's cytoplasm.

I.1.1.2.4.5-*Stability of the relationship*

It seems that at least in the species that inhabit deeper waters the retention of chloroplasts is not an obligatory phenomenon. In fact, Cedhagen (1991) reported that in the winter, samples of *Nonionella labradorica* were collected without chloroplasts Furthermore, Leuteneger (1984) reported that half of the specimens of *Nonionella stella* observed did not have chloroplasts. These findings corroborate the earlier observations of Lopez (1979) who also found chloroplast-free *Nonionella stella* in Danish waters. In this case, the specimens that did not have chloroplasts were collected in deeper water.

The main hypothesis proposed to explain retention of the plastids is that the foraminifera benefit from the association by being able to use the photosyntates produced by the plastids. Bernhard and Bowser (1999) noted that because some of the foraminifera retaining chloroplasts were collected below the photic zone, the production of photosynthates may not underlay chloroplast retention. Since the foraminifera they observed were living in dysoxic sediments, the authors speculate that some unknown biochemical pathway used by the chloroplasts benefited foraminifera.

Chloroplast retention in some species of foraminifera may be a seasonal phenomenon (*Nonionella labradorica*) (Cedhagen, 1991), but in others the same number of chloroplasts (in *Elphidium williamsoni* and *Haynesina germanicum*) were found in winter and summer (Lopez, 1979).

I.1.1.2.4.6-*Elphidium*

The genus *Elphidium* comprises a number of species that inhabit littoral and coastal sediments. The identification of the species and varieties within this genus is a hard task due to the fact that test morphology changes in response to various environmental variables (ecophenotypes) (Painter and Spencer, 1984). *Elphidium* is especially abundant in salt marsh sediments and in some areas can make up to 80% of the live foraminiferal community (Correia, personal observations). The separation of species of *Elphidium* from salt marsh and estuarine areas has been quite controversial. One of the most abundant forms was described initially by Cushman (1930) in the New England coastal waters as a translucent variety, yellowish to brownish in color. Cushman considered this species to be *Elphidium incertum* (var.) *clavatum*. Buzas (1966), working in the Long Island Sound, found a diversity of morphologies including the form *Elphidium incertum* var. *clavatum* described by Cushman (1930) and other forms closer to *E. incertum* (Williamson) and *E. subarticum* (Cushman). He used canonical analysis to test the hypothesis that what had been described as different species were really part of the same species complex. He tried to establish groups which could be separated in statistically based on measurements and invariant characters such as the presence or absence of organic linning and wall structure. Buzas declared that the species he studied should be named *Elphidium clavatum* and, based on the canonical analysis he felt that no further distinction within the species could be made. Feyling-Hansen (1972) and Miller and others (1982), nmed this group *Elphidium excavatum* (Terquem) and included 5 variants as part of this complex. They considered that the assemblages of *Elphidium clavatum* consists of morphotypes linked to one another by subtle gradations in characters. Painter and Spencer (1984) analyzed variants of *Elphidium clavatum* from the Chesapeake Bay area and, using discriminant analysis, tried to link ecological variables such as temperature and trace metals within the sediment to the morphological variation in those assemblages. They concluded that even though six forms could be distinguished visually, only two groups at best could be established by canonical analysis as *formae*. Furthermore, they concluded that the environmental factors they studied did not correlate with the distribution of the two forms. In the present study the individuals are considered as belonging to the *Elphidium clavatum* species with no further subdivision.

Elphidium species have denticles surrounding the apertures and the fossae, and a complex canal system, extending into each chamber. Because digestion in elphidids starts in the pseudopodial net (Lee *et al.*, 1991), the denticles found in the aperture and fossae may function as sieves, preventing diatom frustules or whole undigested cells from entering the foraminifer's test. In fact, unlike most other genera of foraminifera, diatom frustules have not been observed within the tests of elphidids (Lee *et al.*, 1988). No direct evidence has connected the particular morphology of this group and the fact that they are capable of retaining chloroplasts. A recent review by Berhard and Bowser (1999) highlights the fact that some of the chloroplast-retaining species such as *Elphidium*, have test ornamentations, but some others (specially the ones living in dysoxic environments) do not. However, the authors point out that in every case, where plastid retention was observed in Foraminifera, the species involved had teeth and tubercules, which could play a role in separating the plastids from the frustules. The tiny *Metarotaliella* is an exception; it is also known to retain kleptochloroplasts and has no test ornamentations (Lanners, cited by Lee 1992). Alexander and Banner (1984) showed that extrathalamous digestion probably happens within the umbilical cytoplasm where large amounts of chloroplasts are present in *Haynesina germanica* and *Elphidium williamsoni*. They also observed that diatoms are engulfed by the cytoplasm extracellularly and the frustules remained segregated outside as their content was sieved through the tubercules of the umbilical region. In *Elphidium williamsoni*, the umbilical granules are less dense and less concentrated than the ones in the main chambers. The septal-canal system was characterized by very long and narrow vacuoles strongly elongated along the length of the canal (Alexander and Banner, 1984).

The functional aspects of the canal system of *Elphidium* have never been extensively studied. A preliminary description of the canal system of *Elphidium* using the Hottinger technique (1979) was done by Lee (1992), but only gross external morphological observations were made and the function of the canal system remains a mystery.

Certain *Elphidium* species also have abundant pores in their tests. These pores are also abundant in the larger foraminifera *Amphistegina lobifera* where they were shown to allow $^{14}CO_2$ to be taken up through the test (Leutenegger and Hansen, 1979). Dissolved Organic Matter (DOM) (^{14}C labeled glucose) however, did not pass through these pores which are lined by an internal organic membrane.

Under experimental conditions individuals of *E. crispum* were positively phototaxic (Manley and Shaw, 1997). This behavior may aid the search for food or it may be a response linked to plastid retention. *Amphistegina lobifera* and *Amphisorus hemrichii*, two algal-bearing species, are phototaxic (Zmiri *et al.*, 1970 and Lee *et al.*, 1980).

I.1.2-Fine structure of algal groups involved in chloroplast retention

In chloroplast retention, most of the prey cell is digested, making it difficult to determine the origin of the plastids once they are inside the host tissue. Chloroplasts from different algal groups have some structural differences: shape of the plastid; type of membrane surrounding the plastid; pyrenoid presence, shape and location within the plastid; number of membranes enveloping the pyrenoid; number of thylakoids per lamella; arrangement of the lamella. It is possible to infer which algae were ingested and became chloroplast donors by studying the fine structure of the chloroplasts retained by a particular host. However, it has to be considered that: 1) some chloroplasts, for instance, those from diatoms, are very malleable, and can change their shape once the physical constraint of the frustule is removed; 2) fixation and other processing protocols may not be ideal and some structures may be artifacts and others not visible; 3) depending on the sections, it is possible that certain structures such as the membranes bounding the pyrenoid never appear in the plane of section if few individuals or sections are analyzed (as is often the case); and 4) the physiological state of the cells at the time of fixation as well as the environmental conditions they were grown in may change the fine structure

(Gibbs, 1962) . For these reasons ultrastructural studies should be accompanied by pigment analysis. A description of the typical plastids of the algal groups involved in chloroplast retention as plastid donors is provided in the third part of this section.

I.1.2.1-Chromophytes

Chromophyte algae have a plastid enclosed inside a rough endoplasmic reticulum envelope (Round, 1992). They include, among others, the Bacillariophyceae (diatoms) and the Chrysophyceae.

Diatoms plastids are enclosed by a double membrane which is, in turn, inside a vesicle made by endoplasmic reticulum (Drum, 1963; Crawford, 1973). The algae in this group seem to have lamellae comprising three thylakoids (Drum 1963; Drum and Pankrata, 1964; Stoemer *et al.*, 1964 and 1965; Crawford, 1973; Anderson, 1975; Rothchild and Heywood, 1987). The thylakoids rarely branch and are sometimes interrupted (Stoemer *et al.*, 1965; Crawford, 1973). Thylakoids continue through the pyrenoid in all species. Either one or two thylakoids cross the pyrenoid (Gibbs, 1962; Drum 1963; Drum and Pankrata, 1964; Stoemer *et al.*, 1964 and 1965; Crawford, 1973; Anderson, 1975). The pyrenoid is embedded in the chloroplast, but separated from it by a thin membrane (Drum 1963; Stoemer *et al.*, 1964; Crawford, 1973 and Anderson, 1975). Pyrenoid size is variable but it is always centrally located within the plastid (Gibbs, 1962; Drum 1963; Drum and Pankrata, 1964; Stoemer *et al.*, 1964 and 1965; Crawford, 1973; Anderson, 1975). Girdle lamellae usually extend completely around the periphery of the chloroplast (Crawford, 1973; Rothscild and Heywood, 1987). In the Crysophytes, however, some chloroplasts do not have a girdle band (Rothchild and Heywood, 1987). Another structure that characterizes diatom chloroplasts are osmophilic granules which may be associated with the pyrenoid and are thought to be lipids (Drum, 1963, Drum and Pankratz, 1964; Stoemer *et al.*. 1964; Anderson, 1975). Most species have only one or few chloroplasts per cell.

I.1.2.2 Cryptophytes

Cryptophyte chloroplasts are enclosed in a plastid complex delimited by endoplasmic reticulum and containing a nucleomorph, starch grains and ribosomes (Larsen, 1992; Santore, 1992). Cells have one or two plastids connected by a pyrenoidal bridge (Santore, 1992). The plastids have lamellae consisting of two thylakoids (Santore, 1992) which are often compressed but look different from grana (Gant *et al.*. 1971). Other authors report thylakoids arranged singly or in stacks greater than two in number (Rothchild and Heywood, 1987). The thylakoids never fuse (Gant *et al.*. 1971) or penetrate the pyrenoid (Santore, 1992). The pyrenoid may have various shapes and is sometimes seen joining lobes of the plastid (Santore, 1992).

I.1.2.3-Dinozoa

Plastids originating from dinoflagellates are enveloped by three membranes which are not connected to other algal structures (Dodge, 1973; Rothscild and Heywood, 1987). The lamellae are composed of three thylakoids, which can be diversely arranged. Some species have girdle lamellae (Dodge, 1973). The pyrenoids present in this group are diversified. There have been reports of simple internal pyrenoids with no thylakoids going through it (Dodge, 1973), but some authors have also described some species with stalked pyrenoids and thylakoids running through them (Gibbs, 1962). It has been suggested that there may have been multiple acquisitions of chloroplasts by dinoflagellates, a factor that makes their chloroplasts difficult to separate from other algal groups.

I.1.2.4-Chlorophytes

In this group the chloroplasts appear as cup-shaped structures enveloping the nucleus. The thylakoids usually occur in stacks of two to six (Rothchild and Heywood, 1987). The typical chlorophyte pyrenoid is partially enclosed in the chloroplast, surrounded by reserve materials, such as starch, and penetrated by thylakoids (Dodge, 1973).

I.1.3-Goals of the thesis

This dissertation was aimed at contributing to the knowledge of chloroplast retention phenomenon in foraminifera. Most of the studies done to date rely, on the observation of filed collected specimens. There are few published accounts of experimental treatment of specimens in culture. This thesis work will focus on *Elphidium excavatum* (Terquem). This species will be kept in laboratory and studied regarding: 1) It's ability to choose a particular type of chloroplast from several algal diets. 2) It's ability to maintain chloroplasts from different algae in it's cytoplasm for different periods of time. To study these problems two sets of techniques will be used: Confocal Scanning Laser Microscopy (CSLM) and Transmission Electron Microscopy (TEM). Insight on which, if any, algae are preferred by *Elphidium excavatum* (Terquem) will be helpful in understanding what are the requirements for chloroplast-retention to work. If a certain algal group is preferred as the chloroplast donor, it is likely that those chloroplasts have characteristics that make them best suited for retention.

Chapter II

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

Co-author:
John J. Lee

Symbiosis (submitted)
2000

Abstract:

Retention of isolated chloroplasts of partially digested algae has been described for several groups of organisms including the Foraminifera. Forams of the genus *Elphidium* are capable of chloroplast-retention and can be found in abundance in the saltmarsh areas in the Northeast US. Previous studies had suggested that the chloroplasts retained by these organisms originated from diatoms and possibly green algae. In order to ascertain if *Elphidium excavatum* (Terquem) preferably retained chloroplasts of particular algae species or groups, feeding experiments were conducted in the laboratory. Foraminifera were fed monoalgal cultures of diatoms, green algae and a dinoflagellate. Some of the foraminifera were also fed a mixture of diatoms and green algae and controls were kept in sterilized seawater and not fed. All incubations were done in replicates half of which were kept in a light:dark cycle and the other half in complete darkness. The number of chloroplasts retained per volume unit of cytoplasm was estimated using a Confocal Laser Scanning Microscope (CSLM). Results indicate that a greater number of chloroplasts are retained when the foraminifera are fed diatom diets and specially *Amphora coffaeformis*. The use of CSLM greatly improves the accuracy of the counts of the number of retained plastids Results are discussed by comparison with previous descriptions of the number of chloroplasts retained by other species of *Elphidium*.

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, reviewed by Dolan, 1992), dinoflagellates (reviewed by Stoecker, 1999), Heliozoa (Paterson and Durschmidt, 1987) and Foraminifera (reviewed by Bernhard and Bowser, 1999). Chloroplast retention has been described in five families of foraminifera, almost all of which have sieve-like test ornaments (Figure II.1A). In the case of the family Elphididae a complex canal system (Figure II.1B) is also present. These features have been thought to play a part in the phenomenon even though this has not been tested experimentally (Lee *et al.*, 1988; Bernhard and Bowser, 1999).

Most of the published accounts of chloroplast retention in foraminifera describe specimens obtained from the field. Lopez (1979), in the first experimental study of 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. Counting chloroplasts in the chambers of foraminifera is difficult because of the thickness of the specimens. Even when the specimens are crushed, it is difficult distinguishing and enumerating individual overlapping fluorescing chloroplasts under 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 to make a more comprehensive analysis of the question of whether chloroplast retention in *Elphidium excavatum* is a selective phenomenon. In other words, does *Elphidium excavatum* (Terquem) retain chloroplasts of certain algal species or groups more frequently than others? If it is found that *E. excavatum* prefers a particular group or species of algae, as chloroplast donor, the study of the characteristics of those algae might provide a better insight into the chloroplast-retaining phenomenon.

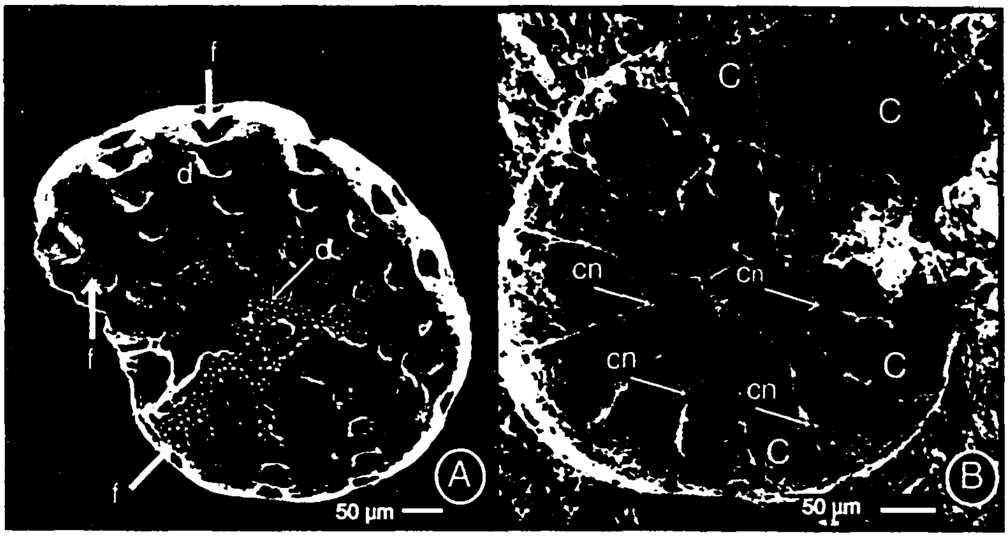

Figure II.1 - Morphology of a test of *Elphidium excavatum* (Terquem). A-Whole test showing denticles (d) and fossae (f). B-Cast of test (prepared by Hottinguer technique) showing areas where canals (cn) and connections between chambers (C) are located. Scale bars as indicated in the figures.

Material and Methods:

Sample collection

Samples were collected at Lake Tashmoo (Martha's Vineyard N 41°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 *Haynesina 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 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 by one of the authors from littoral benthic marine communities. They were cloned on agar as described in Lee and others. (1975). *Nitzchia frustulum*, *Amphora coffaeoformis*, *Cylindrotheca closterium*, *Dunaliella salina*, unidentified green alga #5 and #8, were isolated from the sublittoral epiphytic community of Lake Tashmoo or the nearby Greater Sippewissett Salt Marsh. *Navicula salinicola* and *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 and characterized by Lee and others (1982).

Experimental setup

Each group was fed one of nine monoclonal algal cultures, or a mixture of these and incubated at 25°C with a 12 hours light : 12 hours dark cycle, or in complete darkness. The algae used were diatoms (*Amphora coffaeoformis*. *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.*). The amount of algae added to each culture in a 250 ml tissue culture flask brought the final concentration of algae to $\approx 10^6$ cells /ml. Unfed controls were maintained under the same conditions as experimental cultures. Half of the controls were incubated in the dark, the other half were in a 12 hours light /12 hours dark cycle. Every week, ten individuals were selected from each flask, placed on a slide with a drop of glycerol and kept at -20°C. 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 568nm λ exciter filter and a 590nm λ 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 per micron^3. 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 *Elphidium excavatum*. That volume was calculated assuming each individual has the approximate shape of a disc and the average diameter and thickness described by Buzas (1966) for specimens isolated from the same saltmarsh.

Statistical analysis

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

Results:

Chloroplast distribution within Elphidium excavatum:

Chloroplasts within *Elphidium excavatum* specimens were easily and individually visualized as fluorescent bodies under the CSLM (Figure II.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 figure II.2 correspond to the test wall separating the chambers. The volume occupied by the chloroplasts was approximately between 10.9 and 25.4% of the total volume of the specimen.

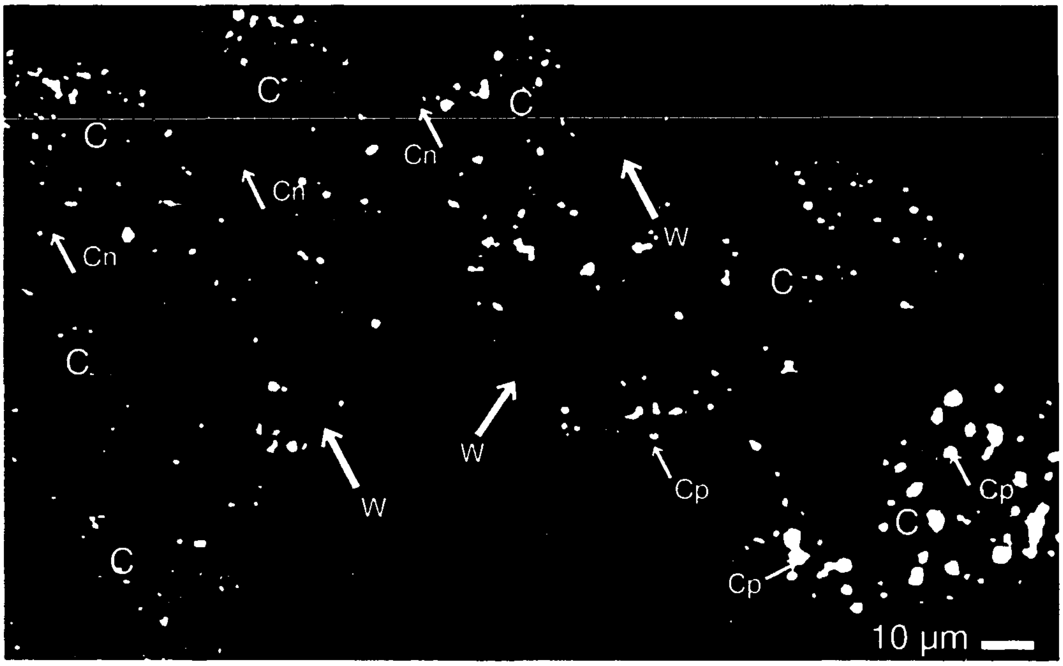

Figure II.2 - Specimen of *Elphidium excavatum* (Terquem) observed on a CSLM. Chloroplasts can be observed as fluorescent bodies (Cp) scattered throughout the cytoplasm. Note opaque areas (W) which correspond to test walls separating the several chambers (C). Test was sometimes interrupted by cytoplasm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

Feeding experiments:

Significantly more chloroplasts of *Amphora coffeformis* were retained by *Elphidium excavatum* than the chloroplasts of any other species of algae tested (Figure II.3). 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 numbers of chloroplasts retained by foraminifera incubated in either the light or the dark. Fewer chloroplasts from green algal species tested were retained. However, the numbers of chloroplasts retained by foraminifera fed *Amphidinium* and the two unidentified chlorophytes were less than the starved controls (Figure II.3).

When the data were analyzed by grouping the algae by types (Figure II.6), there were significantly more chloroplasts from diatoms retained by *Elphidium excavatum* than by those of other groups. When the experiments were incubated in the light, there were statistically significant differences between the numbers of chloroplasts retained by foraminifera fed each of the diets. After monoalgal diatom diets, foraminifera fed a diet of a mixture of several diatoms and green algae retained the next highest numbers of chloroplasts. When *Elphidium* were fed dinoflagellates or green algae, chloroplast retentions were zero or negative (when compared to the control). In the light, significantly more diatom chloroplasts were retained than on all the other diets. The numbers of chloroplasts retained in the dark when the diets consisted of green algae and the dinoflagellate are not statistically different from each other and do not differ from the starved control. There were no significant differences between the numbers of chloroplasts retained after incubations in the light or the dark for two of the diets (diatoms and mixture) (Figure II.6). There were significantly more green algal chloroplasts retained in forams incubated in the dark than those incubated in the light.

Since these experiments took place in successive years, we compared the numbers of chloroplasts retained by starved controls incubated in either the light or the dark (Figure II.7). There were no statistical differences between the values obtained for controls in successive years.

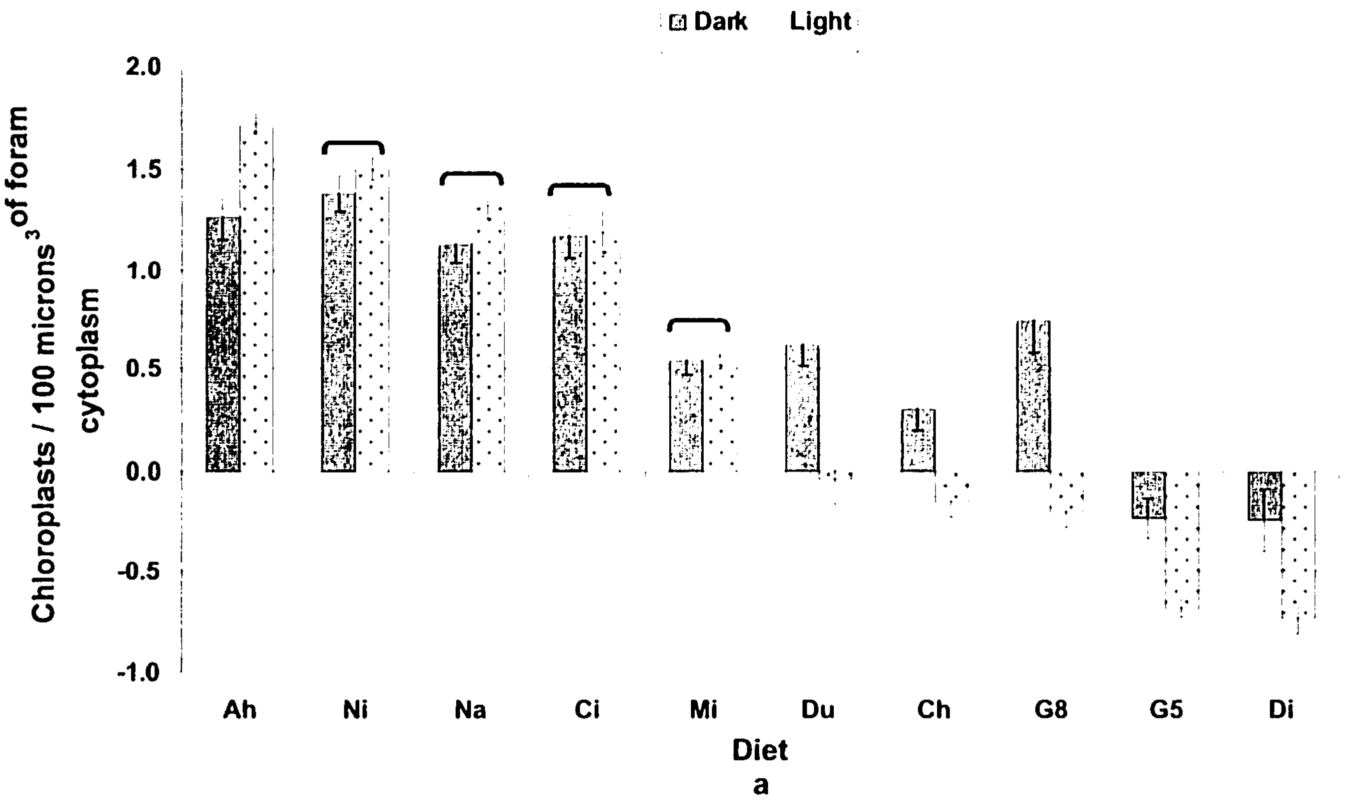

Figure II.3 –Number of chloroplasts retained by foraminifera being fed diets of different algae. Chloroplasts retained in a light:dark cyle (dotted bars) and in complete darkness (dark bars) Ah=*Amphora (Halamphora)* sp., Ni=*Nitzchia frustulum*, Na=*Navicula* sp., Ci=*Cylindrotheca closterium*, MI=Mixture 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 and Di=*Amphidinium* sp. Values graphed were obtained by subtracting retention values for starved controls from the mean for each diet. The brackets indicate values that are not statistically different from each other with an α= 0.05.

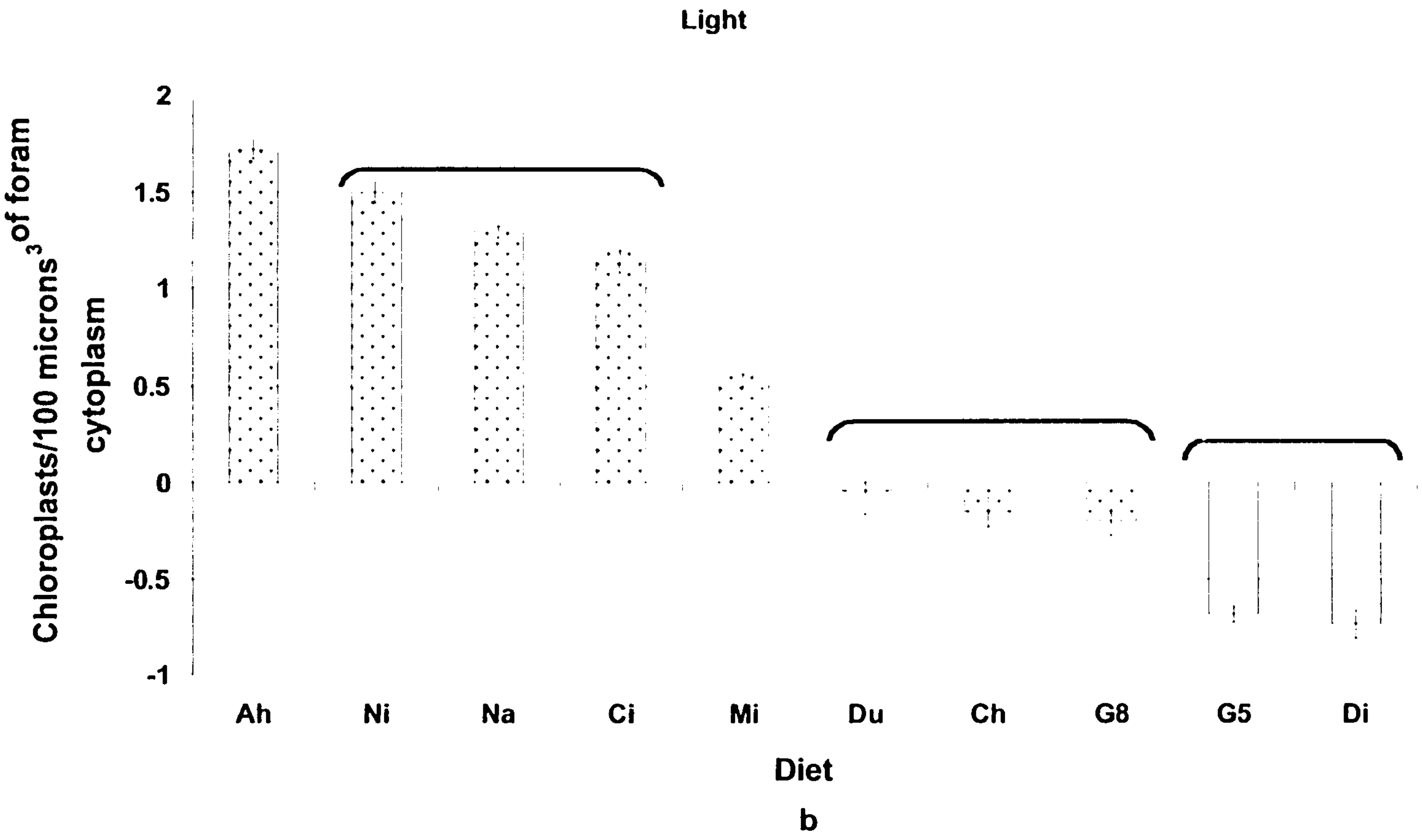

Figure II.4 –Number of chloroplasts retained by foraminifera being fed diets of different algae. Incubations in a light:dark cycle. Ah=*Amphora (Halamphora)* sp., Ni=*Nitzchia frustulum*, Na=*Navicula* sp., Ci=*Cylindrotheca closterium*, MI=Mixture of *Amphora (Halamphora)* sp., *Nitzchia frustulum*, *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 and Di=*Amphidinium* sp. Values graphed were obtained by subtracting retention values for starved controls from the mean for each diet. The brackets indicate values that are not statistically different from each other with an α= 0.05.

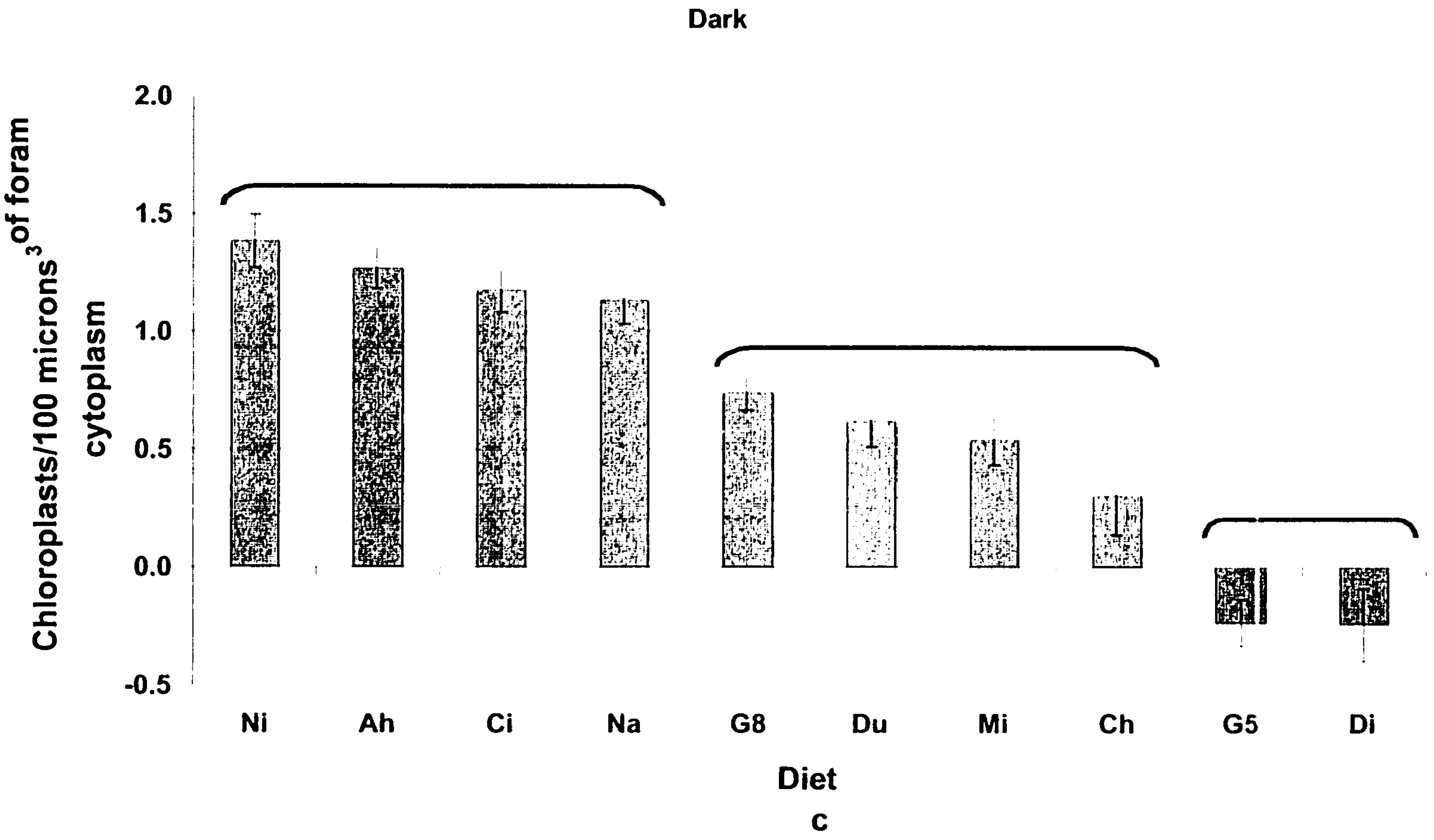

Figure II.5 –Number of chloroplasts retained by foraminifera being fed diets of different algae. Incubations in complete darkness Ah=*Amphora (Halamphora)* sp., Ni=*Nitzchia frustulum*, Na=*Navicula* sp., Ci=*Cylindrotheca closterium*. MI=Mixture of *Amphora (Halamphora)* sp., *Nitzchia frustulum*, *Navicula* sp., *Cylindrotheca closterium*, *Chlorella* sp. and *Dunaliella salina*, Du= *Dunaliella salina*, Ch= *Chlorella* sp., G8=green alga clone 8, G5=green alga clone 5 and Di=*Amphidinium* sp. Values graphed were obtained by subtracting retention values for starved controls from the mean for each diet. The brackets indicate values which are not statistically different from each other with an $\alpha = 0.05$.

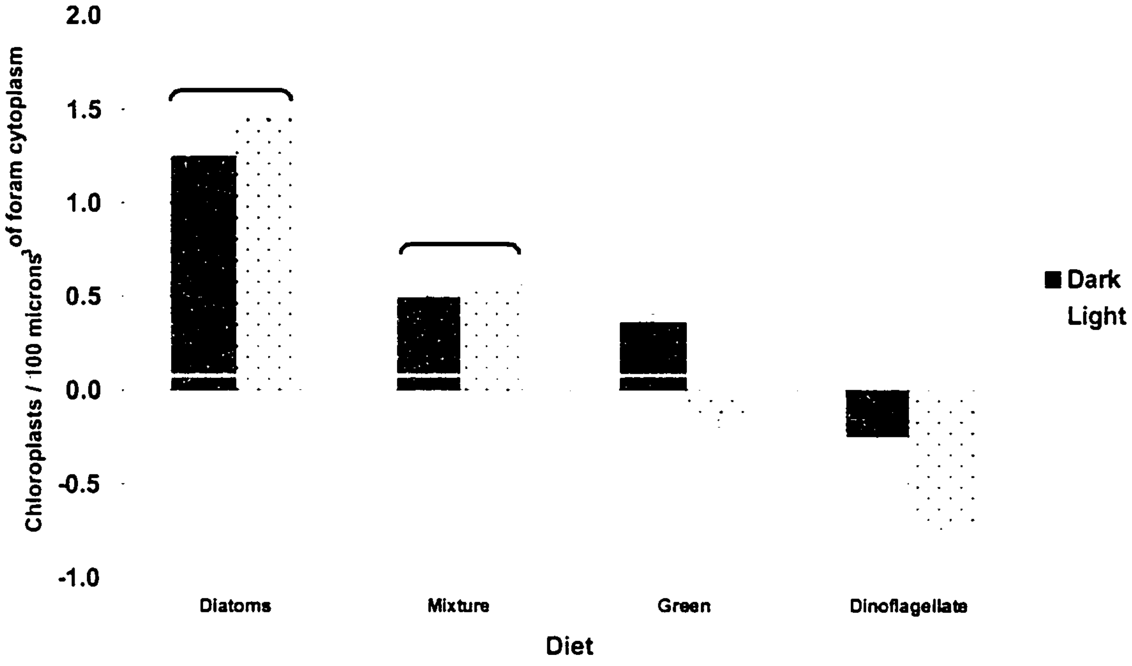

Figure I.6 - Number of chloroplasts retained by foraminifera fed diets of different algae. Analysis done considering the algal groups to which the diets belonged. The Diatoms include *Amphora (Halamphora)* sp., *Cylindrotheca closterium*, *Navicula* sp. and *Nitzchia frustulum*. The Dinoflagellate is *Amphidinium*. The Green include *Chlorella*, *Dunaliella salina*, the green alga clone 5 and clone 8. The Mixture is composed of *Amphora (Halamphora)* sp., *Cylindrotheca closterium*, *Navicula* sp., *Nitzchia frustulum*, *Chlorella* and *Dunaliella salina*. Values graphed were obtained by subtracting retention values for starved controls from the mean for each diet. Brackets indicate values that are not statistically different from each other with an α value of 0.05.

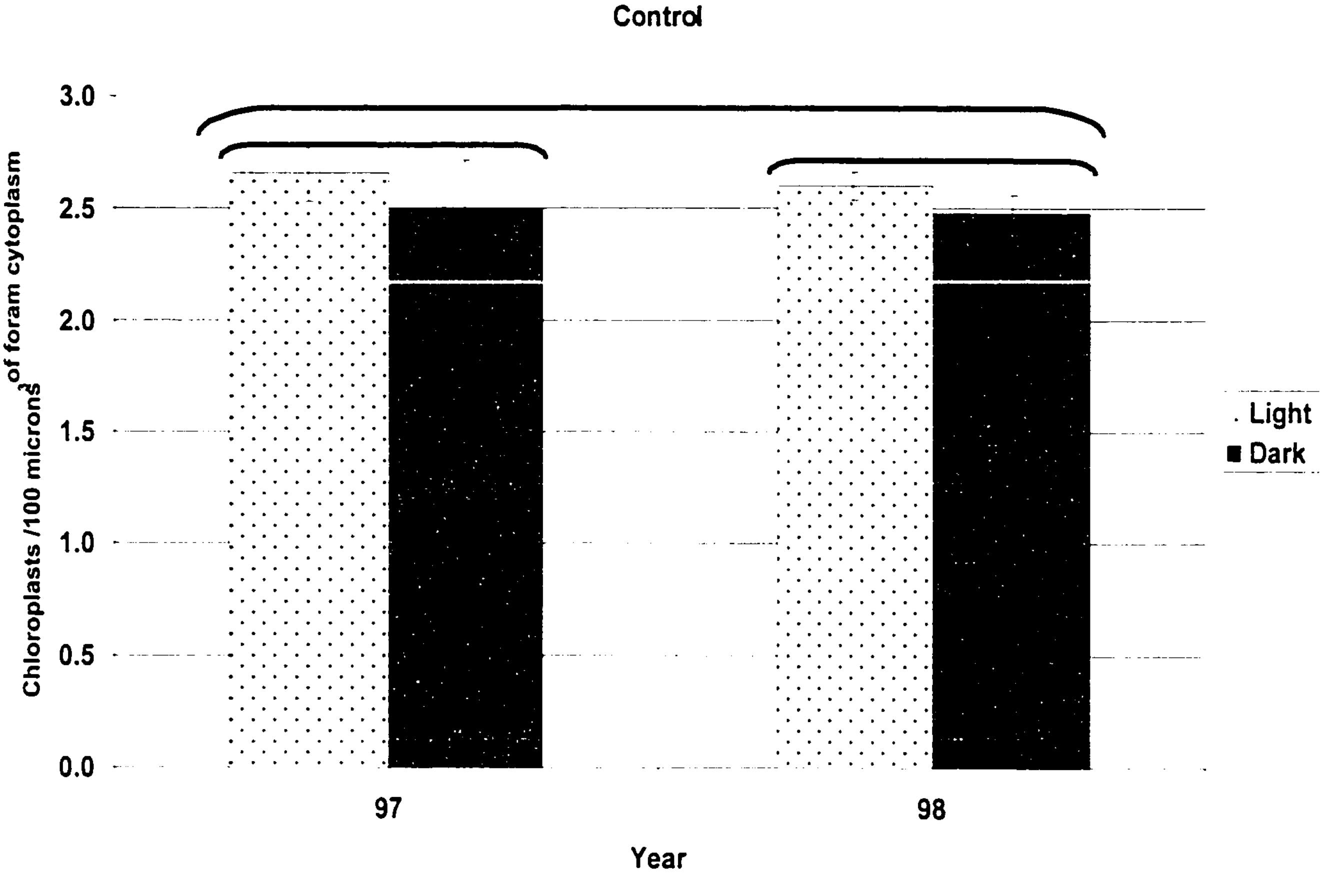

Figure I.7 - Comparison of the values obtained for starved controls incubated in the dark and in the light two consecutive years of experiments. Brackets indicate values which are not statistically different from each other with an α value of 0.05.

Discussion:

Food Quality.

Of the nine algal species tested, *Amphora coffaeformis* seems to be the best chloroplast donor for *Elphidium excavatum*. This agrees with Lee and Lee (1989) who found that *Amphora* (*Halamphora*) *coffaeformis* is among the preferred algae when *Elphidium 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 numbers diatom species she tested. She suggested that the forams were more sensitive to fluctuations in the abundance of food particles. 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 in 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. Green algae chloroplasts were also described as being retained in lower numbers than diatom chloroplasts (Lee and Lee, 1989). 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 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 (*Amphidiunium*) was also a poor chloroplast donor for potential plastid retention by the foraminifera. When the foraminifera were fed a diet of *Amphidinium*, they digested even more of the chloroplasts they had sequestered when the experiment started than did the starved controls.

TABLE II.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; Ah=*Amphora Halamphora coffeformis;* Na= *Navicula*; Ni= *Nitzchia laevis* and Ci= *Cylindrotheca closterium.*

Diet	**Diatoms**	**Mixture**	**Ah**	**Na**	**Ni**	**Ci**
Chl/ind	3.7×10^4	2.9×10^4	3.9×10^4	3.6×10^4	3.8×10^4	3.6×10^4

When the values obtained from the counts are converted to absolute numbers of chloroplasts/individual foram (Table II.1), the are larger than the numbers obtained previously by other authors who studied this phenomenon. When compared to Lee and Lee (1989), the results obtained in this study differ by two orders of magnitude when *Amphora* and *Nitzchia* are the diets and by four orders of magnitude when *Navicula* is considered. In the studies published by Lopez (1979), only values for field samples are provided. 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 directly through microscopy the number of chloroplasts per individual. In this study and in Lopez (1979) estimations of the absolute numbers of chloroplasts per individual were made. Lopez (1979) crushed 100 forams to determine an average number of chloroplasts per ml converting it to chloroplasts per individual. Estimates are always subject to error and if these errors are in two different directions (under and over estimations), differences between the values are magnified.

The studies done by Lopez (1979) and Lee and Lee (1989) also used regular epifluorescence microscopy in their counts. This approach presents two major problems. First, the thickness of the specimens, in concert with the characteristically bright background fluorescence observed, make accurate counts of the individual chloroplasts very difficult and probably lead to underestimations of the chloroplasts. Lopez (1979) solved this problem by making "extracts" of 100 forams. This, however, increases the chances of loosing chloroplasts by lysis and bleaching of the fluorescence. THe bleaching of autoflurescence is a second source of underestimation. When regular epifluorescence is used, the cytoplasm is flooded with light from a mercury lamp, which causes the fluorescence to fade extremely fast.

These two reasons prompted the use of the Confocal Scanning Laser Microscope (CSLM) which uses laser as the light source and collects images using a pinhole method. This decreases the bleaching because the UV light from the laser is less aggressive and can be focused only on the point being scanned. The pinhole apertures limits to great extent the amount of light which floods the preparation. Furthermore, it is possible to do optical sectioning of the specimens. In this way, the thickness problem is solved without crushing the individuals. Together with the digitalization of the images, CSLM makes the counts more accurate. All of these reasons may explain why algal counts in this study are greater than those obtained by Lopez (1979) and by Lee and Lee (1989).

Effect of light on chloroplast husbandry

The results of the present experiments give a mixed message. There were no statistical differences between the number of chloroplasts retained by foraminifera incubated in a light/dark cycle or in the dark when the foraminifera were fed diatoms (with the exception of *Amphora*), the mixture (Figure II.3) or starved controls (Figure II.7). This suggests that energy captured by photosynthesis, or photosynthetates, did not affect the retention process. However, on diets of *Amphora coffaeformis*, all the green algae and the *Amphidinium* (Figure II.3) chloroplast retention values obtained from 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 degradation of the retained chloroplasts in the light. Lopez (1979), who 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, suggested that 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 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?

In plastid retention, it is known that the plastids eventually get digested or undergo autolysis (Lee *et al*., 1988) and therefore need to be replaced. Therefore, an abundance of donor species may be important in permitting the foraminifera to find the suitable algae. Diatoms are the most abundant microalgal group (diatoms) in the salt marshes where many species of *Elphidium* abound. It comes as no surprise that they would be an important component of the diet of *Elphidium excavatum* (Terquem), 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*., 1995). This rarity 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 (1999, 2000) show that diatoms that are endosymbiotic have a surface antigen not found on the surfaces of diatoms that 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. That plastid retention in *Elphidium excavatum* seems to be a selective process suggests there may be characteristics of either the host, the chloroplasts, or both in combination, that make diatom plastids more suited for retention. We could imagine transfer to the host genome of genes need for diatom chloroplast maintenance. 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 cell is degraded? Does the canal system of *Elphidium* play any role in the chloroplast husbandry? These questions, and many more, pique curiosity and challenge us to design experiments aimed at better understanding of the factors which underlie the chloroplast retention phenomenon.

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Chapter III

Chloroplast Retention By *Elphidium excavatum* (Terquem). How long do the kleptochloroplasts last?

Co-author:
John J. Lee

Symbiosis (submitted)
2000

Abstract

Intact chloroplasts are retained by *Elphidium excavatum* (Foraminifera). There is preferential retention of diatom plastids. Samples of *Elphidium excavatum* were fed diets of particular algae for a week, then starved for 8 weeks to assess the half lives of chloroplasts originating from different types of algae. The number of chloroplasts retained per individual was measured weekly using a Confocal Laser Scanning Microscope. Results indicate that chloroplasts remain in the cytoplasm of *Elphidium* for at least 8 and possibly up to 20 weeks. In a light:dark cycle, it takes 9.5 weeks the diatoms chloroplasts to be reduced to one half. Although we observed chloroplasts in the cells of Foraminifera fed chloroplytes or dinoflagellates, the numbers of chloroplasts retained were the same or less than starved controls, suggesting that no new chloroplasts had been added to the symbiont population during the week of feeding. The results are in consonance with what is known about a kleptochloroplasty occurring in the Ascoglossan (=sacoglossan) molluscs.

Keywords: Foraminifera, Elphidium, chloroplast retention, half- life, kleptochloroplasts

Introduction:

Symbioses between larger foraminifera and a diversity of microalgae (diatoms, dinoflagellates, chlorophytes and rhodophytes) are well known (reviewed by Lee, 1998). Less well researched is the phenomena of chloroplast husbandry (retention of chloroplasts from partially digested algae) by five families of foramminifera (reviewed by Berhard and Bowser, 1999). *Elphidium* is one of the genera in which isolated chloroplasts have been described in the cytoplasm of the foraminifera (Lopez, 1979; Leutenegger, 1984; Lee and Lee, 1989; Cedhagen, 1991; Bernhard and Bowser, 1999). Previous studies (Lee and Lee 1989; Correia and Lee, submitted) suggest that the retention of plastids by *Elphidium* is a selective process and that diatoms possess the best suited chloroplasts for this relationship. Isolated chloroplasts inside foraminifera seem to undergo digestion or autolysis, even if at a slow rate. Evidence for the gradual digestion or autolysis of chloroplasts was found at the ultrastructural level (Lee *et al.*, 1988). In consonance with this, Lee and Lee (1989) observed a general decrease in the number of chloroplasts with time further indicating that they have a finite viability in their host foraminifera. In this study, we try to assess two aspects of the phenomenon: 1) How long do sequestered chloroplasts last if the host is starved? 2) Are there qualitative aspects for retention longevity (does it make a difference which type of alga was the chloroplast donor)?

Materials and Methods:

Sample collection

Samples were collected at Lake Tashmoo (Martha's Vineyard N41°32' W 70°40') in the first week of August 2000. The sampling protocol has been described by Correia and Lee (submitted). Individual specimens of *Elphidium excavatum* were picked from the sediment with sable artist brushes and divided into 8 aliquots of 50 foraminifera each. Each aliquot contained individuals of approximately the same size selected at random.

Algae

The three algae used as food in the experiments were isolated from littoral benthic communities and cloned using the methods described in (Lee *et al.*, 1975). *Amphora coffaeformis* and *Dunaliella salina* were isolated from the sub-littoral epiphytic community of Lake Tashmoo. *Amphidinium* sp. was isolated from the benthos of sedimentation ponds at the National Center for Mariculture in Eilat, Israel.

Experimental setup

Eight experimental groups were set up, two groups of foraminifera were starved in sterilized seawater after being harvested from the marsh and the other six, in paired groups, were fed monoalgal diets of either *Amphora coffaeformis*, *Dunaliella salina* or *Amphidinium sp.*. The final concentration of the algal cells in the incubation medium was $\approx 10^6$ cells /ml. The cultures fed monalgal diets and the starved controls were incubated at

25°C with a 12 hours light:12 hours/dark cycle for a week. After a week of incubation, the foraminifera in each bowl were individually brushed and transferred to sterilized seawater. All organisms were placed in algal free sterilized seawater and were starved from this point forward in the experiment. Half of the flasks were incubated in a 12 hours light/12 hours dark cycle the other half were incubated within boxes in complete darkness. Each week, five individuals were selected from each flask, placed on a slide with a drop of SlowFade® (Molecular Probes) and frozen. The number of chloroplasts per individual frozen foraminifera was estimated using a Confocal Laser Scanning Microscope (Molecular Dynamics Multiprobe 2001 with an argon/Krypton laser). Scans were done using a 590 nm λ filter. Optical sections, 1 micron thick were done at 1 micron intervals. At least three sections were chosen to represent each individual and the number of chloroplasts in each section was counted. That value was multiplied by a factor representing the number of possible sections in a whole individual to calculate the number of chloroplasts in the entire volume of the organism.

Statistical analysis

All ANOVA calculations were done using the SAS® Version 6.12 statistical package. The values obtained were compared using a sequential BonFerroni analysis in order to minimize the inflation of the error associated with multiple pairwise comparisons (Rice, 1989). The alpha value used for the comparisons was 0.05.

The half-life of the chloroplasts was calculated by doing a regression of the number of chloroplasts versus the number of weeks the foraminifera were starved. An equation was obtained which related the number of chloroplasts retained to the number of weeks of starvation. The number of weeks it took for the number of chloroplasts to be reduced to one half was estimated using that equation. Confidence limits (at the 95% level) were calculated for each predicted value. All regression calculations were done using the SAS statistical package Version 8.0.

Results:

The number of chloroplasts retained by each foraminifer decreased with the length of incubation (Figure III1). The number of chloroplasts per individual was always greater in the foraminifera that had been fed *Amphora* (diatom), but the differences became statistically significant only after the fifth week in the light and sixth in the dark. When the numbers of chloroplasts retained by foraminifera incubated in a light/dark cycle were compared to those for foraminifera incubated only in the dark, two trends became evident (Figure III.3). The number of chloroplasts remaining in the cytoplasm of each individual foraminifera was significantly higher in the groups of foraminifera fed *Amphora* and the starved controls incubated in the light/dark cycle than in those incubated continuously in the dark. In contrast, those fed *Amphidinium* and *Dunaliella* hosted the same numbers of chloroplasts regardless of whether they were incubated in a light:dark cycle or in complete darkness.

The rates of the dcline in choroplast numbers and the chloroplast half-lives were calculated from regression lines fitted to the data (Table III.1). The half-life of the plastids was greater when the foraminifera were kept in the dark regardless of which type of algae they were fed. This was also true for the starved controls which retained their chloroplasts longer when they were incubated in the dark. The chloroplasts originating from diatoms had longer half-lives when they were incubated in a light/dark cycle.

TABLE III.1 – Estimate of the time in weeks it took for the chloroplasts initially retained to be reduced to one half. Values are based on observations over a period of 8 weeks.

	LIGH REGIME			
DIET	Light:Dark		Dark	
	Estimate	*95%LCL -95%HCL	Estimate	*95%LCL-95%HCL
Amphora coffaeformis	9.5	8.2-11.0	9.3	6.8-14.2
Amphidinium sp.	5.3	3.4-7.6	5.1	3.9-6.5
Dunaliella salina	7.27	5.6-9.3	8.6	7.6-9.7
Starved Control	6.8	3.6-7.9	10.9	9.2-13.0

*95%LCL=95% Lower Confidence Limit, 95%HCL=95% Higher Confidence Limit

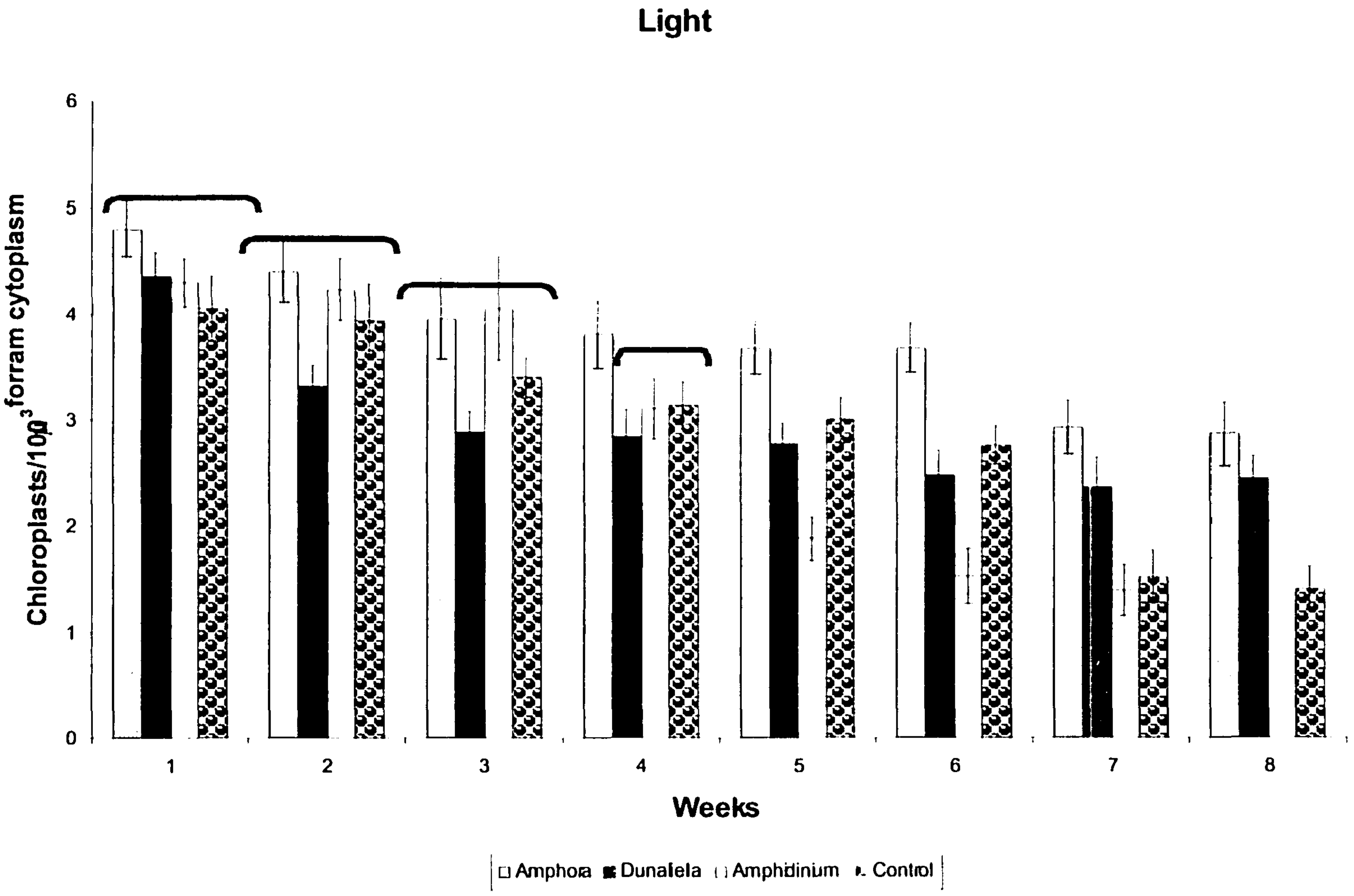

Figure III.1 – Change over time in the number of choloroplasts retained by *Elphidium excavatum* (Terquem) kept under starvation after being fed different algal diets. Light:Dark cycle incubations. Brackets indicate values not statiscally different considereing an α value of 0.05

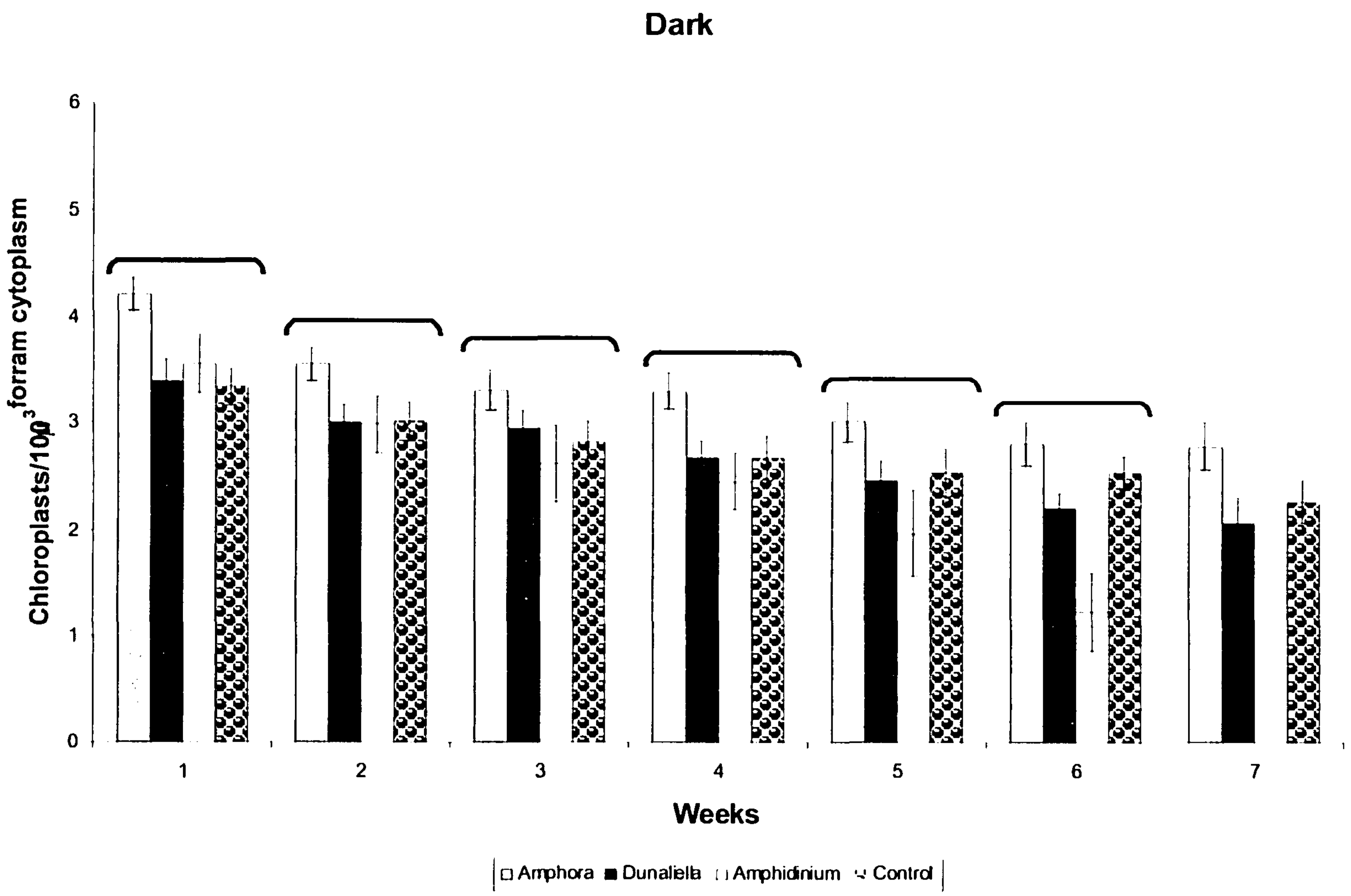

Figure III.2 – Change over time in the number of chloroplasts retained by *Elphidium excavatum* (Terquem) kept under starvation after being fed different algal diets. Dark incubations. Brackets indicate values not statistically different considering an α value of 0.05.

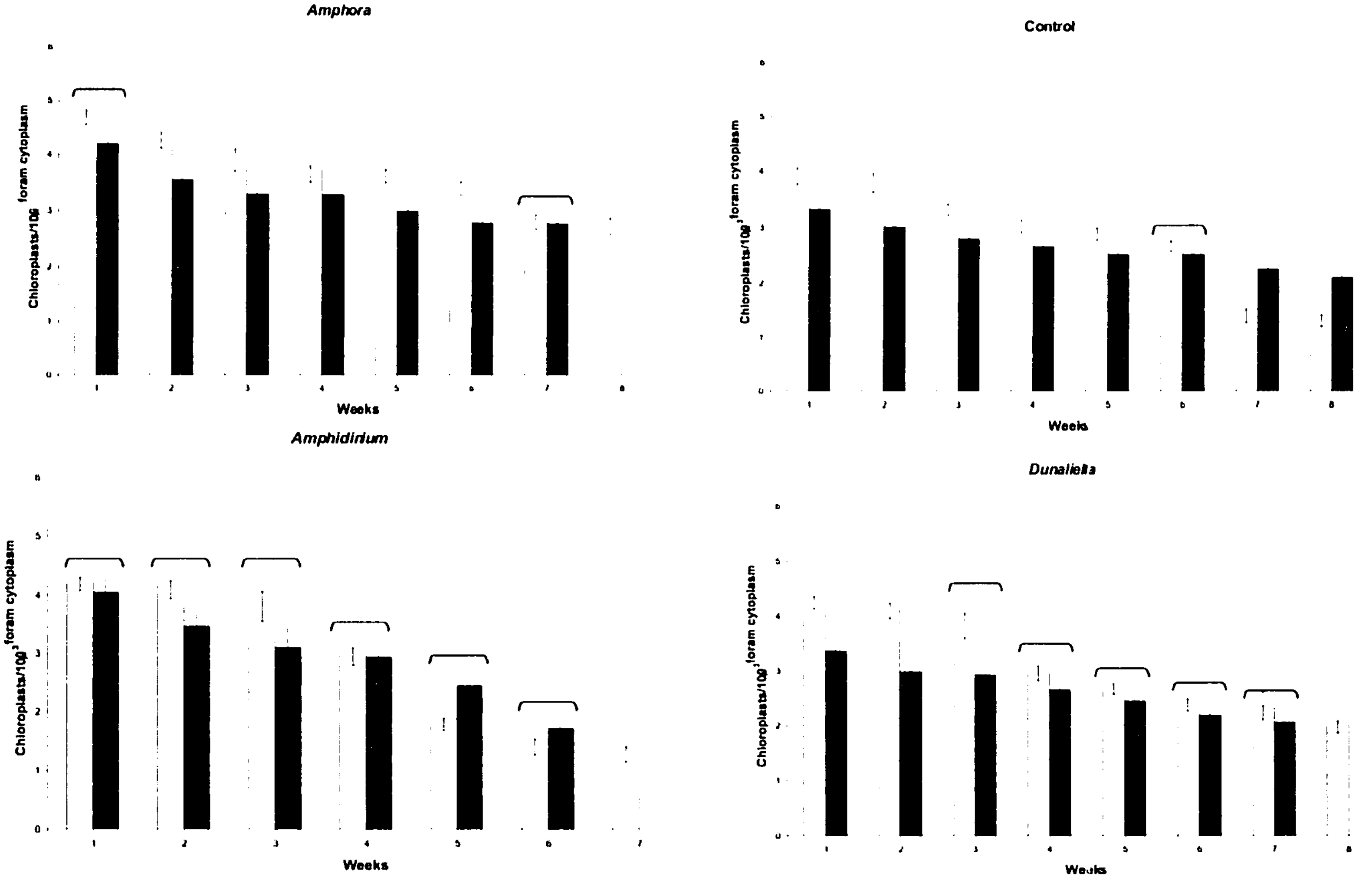

Figure III.3 – Comparison between the number of chloroplasts retained by *Elphidium excavatum* (Terquem) fed different algae and incubated in a light : dark cycle (light bars) and in complete darkness (dark bars). Square brackets indicate statistically identical values considering an α value of 0.05.

Discussion:

The results of our study on the kleptochloroplast viability within *Elphidium excavatum* are in consonance with the data emerging from the more intensively studied sea slug system (reviewed by Trench, 1975 and Rumpho *et al.*, 2000). In recent years it has been reported that photosynthetic animals (*Elysia chlorotica*) could be maintained in the laboratory for at least nine months in the absence of algal food (Rumpho *et al.*, 2000). However, Green and others (2000) note that the sea slugs lose pigmentation similar to the senescence of a leaf. These workers suggest that as the natural life cycle of the sea slug proceeds, the hosts may no longer provide the cellular environments or proteins necessary to maintain plastid photosynthetic machinery. Chloroplasts remain viable within *Elphidium excavatum* (Terquem) for at least nine weeks even when the foraminifera are starved. At this time, many foraminifera are pale in color and many have died. It would be misleading for us to draw conclusions about the viability of chloroplasts in *Elphidium excavatum* in comparison to *Elysia chlorotica* because we may not have met all the environmental requirements of the former at this time. The first chloroplast longevity experiments with *Elysia chlorotica* suggested that these animals could be maintained for only four months in the absence of algal food (*Vaucheria litorea*) (Gibson *et al.*, 1986).

The results of our present study, the one submitted (Correia and Lee), and fine structural studies in progress, suggest that diatoms are the major (and perhaps the only) contributors to the population of chloroplasts that are retained. Our experiments show that when the foraminifera were fed *Amphidinium* sp. or *Dunaliella salina* they retained no more plastids per foraminifer than starved controls. The most reasonable explanation is that they do not retain any chloroplasts of these types of algae.

Nothing is known about the factors that sustain long term chloroplast activity in the *Elphidium excavatum* system with which we have been working. Workers studying *Elysia* have not found algal nuclei or nucleomorphs in the animal's tissues. Our own fine structural studies of various species of *Elphidium* have revealed that algal organelles are only rarely retained (Lee *et al.*, 1988). In agreement with microscopic observations, Southern analysis and polymerase chain reaction did not detect an algal nuclear genome in the sea slug (Green *et al.*, 2000). The key question then becomes how do the chloroplasts remain functional for several months in the absence of any algal nucleo-cytosolic influence? Studies of the largest chloroplast genomes known suggest that they have code for only 13-25% of the 120-130 gene products necessary for plastid function (Reith and Munholland, 1995; Martin and Herrmann, 1998 cited by Rumpho *et al.*, 2000).

Knowledge gleaned from studies of sea slugs and their kleptochloroplasts suggest many potential future studies of the phenomenon in foraminifera. Using a radionuclide tracer ([^{3}H] leucine) to measure chloroamphenicol-sensitive protein synthesis Trench (1975) inferred protein synthesis in the chloroplasts. Mujer and others (1996) were able to demonstrate transcripts of two chloroplast genes, *psb* A and 16SrRNA in *E chlorotica* cultured for eight months in the absence of algae. Photosystem proteins D1, D2, Rubisco LS, and CP43, which are synthesized by plastids, were also present. Pierce and others (1996) found that [^{35}S] labeled Methionine, as part of protein synthesis in the cytosol, was translocated into the plastids. Similar studies could be attempted in the foraminifera if an efficient chloroplast extraction protocol is devised. Although chloroplasts are abundant in the foram's cytoplasm, so are contaminants on the test surface and the hypothetical genetic signals sent between the host and the plastids may be too small to be detected.

In the context of chloroplast gene autonomy, Rumpho and others (2000) suggest that while the plastid genomes of chromophytes are sometimes smaller than those of chlorophytes, they typically have an increased coding capacity due to more compact coding regions and reduced inverted repeat size. They also suggest that proteins destined for mitochondria (glucogenic and pentose pathways) could be directed to and function in the plastids. On the basis of a comparison of the stability of proteins isolated from the chloroplasts of spinach and *Codium fragile* they suggest that the latter are much more stable and raise the possibility the proteins in the chloroplasts retained by *E. chloritica* may be even more stable. This reasoning could also be applied to the *Elphidium excavatum* kleptoplastidy phenomenon because diatom plastids seem to be retained for longer and in greater numbers than green algal ones.

The chloroplasts retained by *Elphidium excavatum* seem to be retained longer when the foraminifera are kept in the dark regardless of the origin of the chloroplasts (Table I). This could be due to slower turnover of plastid proteins when the photosynthetic machinery is not active, however this remains to be demonstrated. Differences in the number of plastids retained by foraminifera kept in different light regimes are not always statistically significant (Figure 2). One possible way to better understand if light affects the kleptoplastidy phenomenon is to assure that the half-lives measured really correspond to plastids originating from the microalgae the foraminifera were fed. This can only be done by ultrastructural studies. Diatoms, Chloroplhyte and Dinoflagellate plastids can be distinguished at the fine strucural level and Transmission Electron Microscopy would enable us to confirm if the plastids of the chlorophyte and dinoflagelate are being retained as isolated organelles or if the whole algae are just being ingested and digested. The plastids observed under the confocal microscope look the same regardless of the algae they originated from. It is possible that the plastids counted in some cases are chloroplasts in the foram's cytoplasm from the start of the experiment. This could be the case because, as noted in the starved controls, the chloroplasts can remain in the cytoplasm of the foraminifera for months. Fine structural studies are proceeding in our laboratory. The dynamic aspects of plastid protein turnover in kleptochloroplast husbanding foraminifera beg future probing. Such work as with the studies of ascoglossan slughs, is hampered only by the availability of suitable techniques to isolate the retained plastids.

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Chapter IV

Chloroplast Retention By *Elphidium excavatum* (Terquem). Fine structure of the retained plastids.

Co-author:

John J. Lee

Symbiosis (in preparation)

2000

Abstract: Previous studies have demonstrated that when fed diatoms *Elphidium excavatum* (Terquem) keeps more kleptoplastids and survives longer during starvation. In order to find out which algae the kleptoplastids originated from, foraminifera were fed different diets and studied by Transmission Electron Microscopy (TEM). Controls consisted of preparations from algae provided as food to the foraminifera (*Amphora coffaeformis*, *Amphidinium* sp., and *Dunaliella salina*). Regardless of the algae used, the kleptoplastids in all sections had an ultrastructure similar to diatom plastids. The retained plastids were characterized by 2-3 thylkoid stacks, a simple internal pyrenoid with a thylakoid crossing it, and a girdle lamella surrounding the outside of the chloroplast. Some of the plastids are thought to be undergoing digestion due to a loss of organization and fusion of vesicles thought to be lysosomes. Cytoplasm within the inter chamber canals showed numerous vacuoles and vesicles. Some of the vacuoles contained remains from digestion as well as isolated plastids. The chloroplast inside the chambers showed protrusions on the edges corresponding to pores possibly leading to cannaliculli connected to the interchameral canals. These pores were plugged by an electron dense material and no cytoplasmic connection was observed between the pores in the test and the interchameral cytoplasm.

Keywords: Foraminifera, Elphidium, kleptoplastids, fine structure, algal chloroplats, TEM

Introduction

Recently we studied the phenomenon of chloroplast husbandry by the salt marsh foraminifer *Elphidium excavatum* (Terquem). These studies included experiments aimed at elucidating whether the husbadry was selective for particular types of algal chloroplasts and at finding the longevity of the sequestered chloroplasts (Correia and Lee a, b submitted). Studies in which the foraminifera were fed different algal diets suggest that the diatom chloroplasts are retained in greater numbers and for longer periods of time than chloroplasts originating from green algae or dinoflagellates (Lee and Lee, 1989; Correia and Lee submitted). However, those studies were done with the intention of counting the plastids, and epifluorescence and confocal scanning laser microscopy were used. Light microscopy, does not allow for the observation of the fine structure of the plastids and it is impossible to be sure if the plastids counted belong to a particular algal group. The results of the study of longevity study suggested that the sequestered chloroplasts remain fluorescent for at least 8-20 weeks. After that time, the starved hosts seem to loose their viability as the fluorescence of the chloroplasts fade away. It is unclear whether the retained chloroplasts run out of needed metabolites and undergo autolysis or whether they produce or withdraw molecular signals which renew host digestive activity in the symbiosome.

In studies where the fine structure of these forams was investigated, the plastids were thought to originate from partially digested diatoms or chrysophytes (Lopez, 1979; Leutenegger, 1984; Lee *et al.*, 1988 and Bernhard and Bowser, 1999). With few exceptions (Lee *et al.*, 1988) no other algal organelles were found in the symbiosomes of the foraminifer. This makes it hard to specify the particular algal group the plastids originated from. Most of the authors base their conclusions on the number and structure of the thylakoids and pyrenoids, and the number of membranes surrounding the plastid.

The aim of this study was to complement the feeding experiments done previously, by using Transmission Electron Microscopy to look at the fine structure of the chloroplasts retained by individuals of *Elphidium excavatum* (Terquem) while they were being fed different algal diets.

Material and Methods:

Sample collection

Samples were collected at Lake Tashmoo (Martha's Vineyard N 41°32' W 70°40') in the first week of August 2000. In this season it is easiest to collect large numbers of individuals of *Elphidium* spp. and *Haynesina germanica* in this habitat. The procedures followed for the collection of specimens were described in Correia and Lee (submitted a). Individual specimens of *Elphidium excavatum* were picked from the sediment with sable paintbrushes and divided into groups of 20-25 foraminifera. Each aliquot contained individuals of approximately the same size selected at random.

Experimental setup

Each group of foraminifera was fed one of three monoclonal algal cultures and incubated at 25°C with a 12 hours light : 12 hours dark cycle, or in complete darkness. The algae used were a diatom (*Amphora coffaeoformis*), a chlorophyte (*Dunaliella salina*) and a dinoflagellate (*Amphidinium sp.*). The amount of algae added to each culture in a 250 ml tissue culture flask brought the final concentration of algae to $\approx 10^6$ cells/ml. Starved controls were maintained under the same conditions as experimental cultures. Half of the controls were incubated in the dark, the other half were kept in a 12 hours light /12 hours dark cycle. Every week, ten individuals were selected from each flask and prepared for Transmission Electron Microscopy (TEM).

Preparation for TEM

Algae

Specimens were fixed in 4% glutharaldehyde in seawater for 1 hour at 4°C. After several washes in sterile filtered seawater, the cells were post fixed with 2% OsO_4 in sterile filtered seawater for 5 hours at 4°C. The specimens were then gently washed with distilled water and stained with a 2% solution of uranyl acetate in ethanol for 15 minutes. A progressive series of ethanol was used for dehydration, followed by two washes with propylene oxide, and embedding in Embed 812® (Electron Microscopy Sciences). For embedding, the samples were infiltrated with 1:2, 1:1 and 2:1 mixtures of resin and propylene oxide for at least 1 hour each. Before curing, the samples were further infiltrated with pure resin for 2.5 hours and overnight with the resin plus the polymerizer. The embedded specimens were then cured for 24-48h until the resin achieved the desired hardness.

Forams

Specimens were fixed in 4% glutharaldehyde in seawater for 1 hour at 4°C. After several washes in sterile filtered seawater, the cells were post fixed with 2% OsO_4 sterile filtered seawater for 30 minutes at 4°C. The specimens were then gently washed with distilled water and decalcified in a 1% solution of EDTA until the test appeared transparent. A progressive series of ethanol was used for dehydration, followed by two washes with propylene oxide and embedded in Embed 812® (Electron Microscopy Sciences). For embedding, the samples were infiltrated with a 1:2, 1:1 and 2:1 mixtures of resin and propylene oxide for at least 1 hour each. Before curing, the samples were further infiltrated with pure resin for 15 hours with the resin plus the polymerizer. The embedded specimens were then cured for 24-48h until the resin achieved the desired hardness.

The blocks obtained in the procedures described above were trimmed and sectioned using a Reichter-Jung Ultracut E microtome and a diamond knife (Dupont). The sections were collected in copper grids and some were stained with a 1% solution of uranyl acetate and 5% lead citrate. Observations of the sections were made using a Zeiss 902A TEM. Pictures were taken on Kodak ISO163 film and printed on Illford multigrade paper.

Results:

Micrographs of the chloroplasts from the algae fed to *Elphidium* were studied as controls, so that their fine structure could be compared to that of the plastids found in vacuoles within the host. *Amphora coffaeformis* was typical for a diatom (Round *et al.*, 1990). It had two plastids, surrounded by four membranes. Inside the plastid were a series of more or less parallel lamellae, each composed of two and sometimes three stacked thylakoids which ran the length of the plastid (Figs. IV.1 A-C). Surrounding these was a girdle lamella, that was continuous around the ends of the plastid (Fig. IV.1A). In good sections we could recognize a membrane enclosing the chloroplasts and

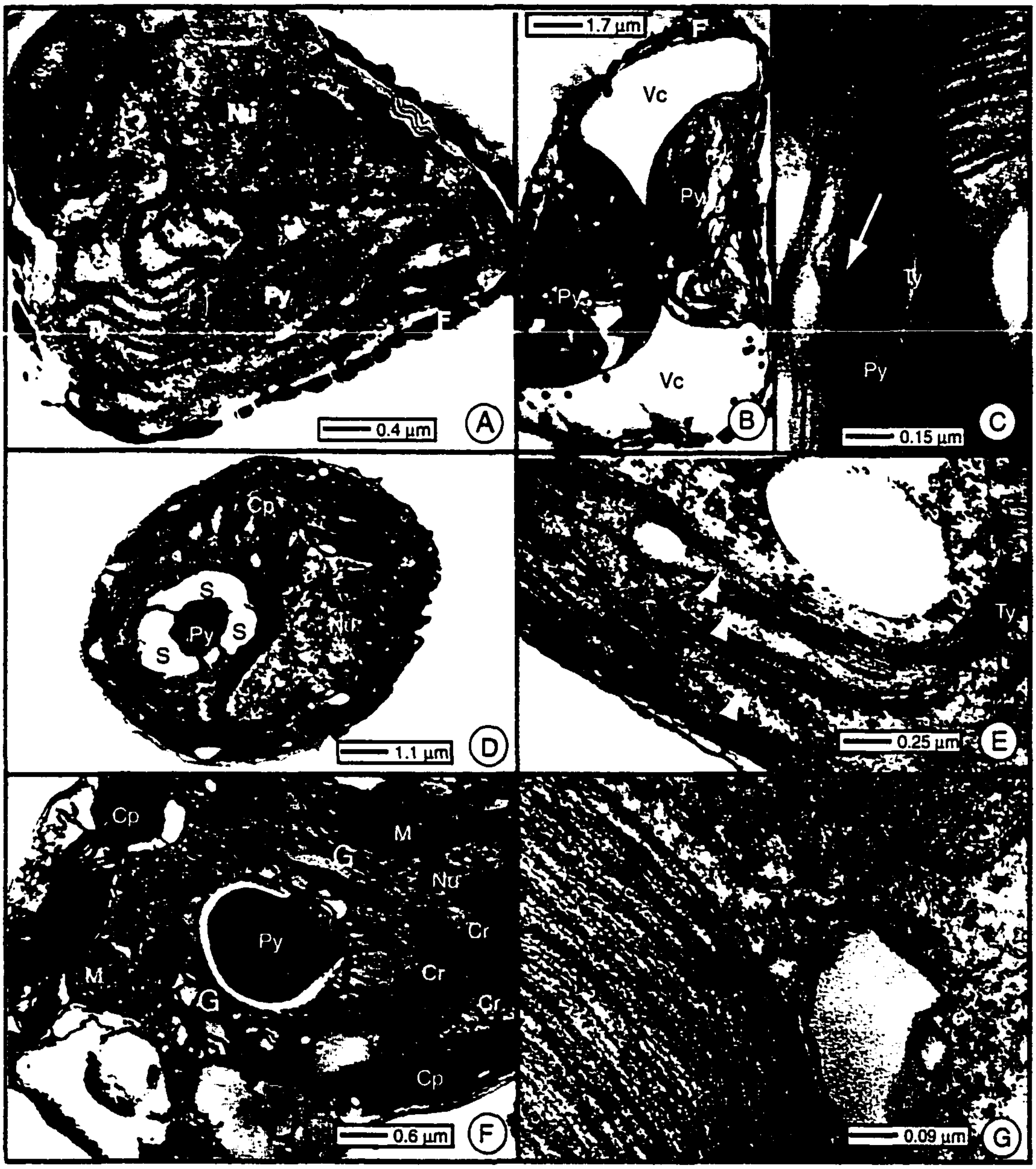

Figure IV.1 - Algae used to feed *Elphidium excavatum*(Terquem). A,B and C- *Amphora coffaeformis*; D and E - *Dunaliella salina*; E and F - *Amphidinium* sp.Cp-chloroplast; Cr-cromossome; F-frustule; G-Golgi; M-mitochondria; Nu-nucleus; Py-pyrenoid; S-starch; Ty-thylakoids; Vc-vacuole. Black arrows - membrane surrounding chloroplast; white arrows - membrane surrounding pyrenoid; black arrowhead - membrane enclosing nucleus and chloroplast; white arrowheads - stacks of different numbers of thylakoids; asteriscs - girdle lamella). Scale bars as indicated in each figure.

the nucleus (Fig. IV.1A). Each plastid contained a simple internal pyrenoid, lenticular in shape and crossed by a single thylakoid (Fig. IV.1A-C). The pyrenoids were separated from the lumen of the plastid by a membrane (Fig. IV.1C). Pyrenoids varied in length from half the length of the plastid to approximately the full length. There were no large electron transparent vacuoles in log phase cells (Fig. IV.1A), but they appeared as cells began stationary phase (Fig. IV.1B). The nucleus was central in the cell and varied from spheroidal to oval (Fig. IV.1A).

The structure of *Dunaliella salina* was typical of volvophyceans (Pickett-Heaps, 1975). In this alga the chloroplast was cup shaped and surrounded the central nucleus (Fig. IV.1D). The chloroplast had a typical invaginated pyrenoid surrounded by platelets of starch (Fig. IV.1D). Chloroplast lamellae enter the pyrenoid matrix (Fig. IV.1D). We did not see any suggestion of an eyespot (stigma) within the chloroplast membrane. Chloroplasts had thylakoids stacked in groups of two to six (Fig. IV.1E).

Amphidinium had a typical dinokaryon with condensed chromosomes, which have a fibrillar and banded appearance (Fig. IV.1F and G). The chloroplast of *Amphidinium* sp. had a stalked pyrenoid (Fig. IV.1 F). The thylakoids in this type of chloroplasts were stacked in groups of three, but in most of the sections the thylakoids was so compressed it was difficult to count them (Figure IV.1G).

Choloroplasts were abundant in the cytoplasm of *Elphidium excavatum* (Terquem) (Figure IV.2A and B). Their distribution was random within the chambers. Part of the cytoplasm of each chamber of the foraminifera appeared vacuolated, even in the sections where other parts of the cytoplasm was denser (Figure IV.2A and B). In

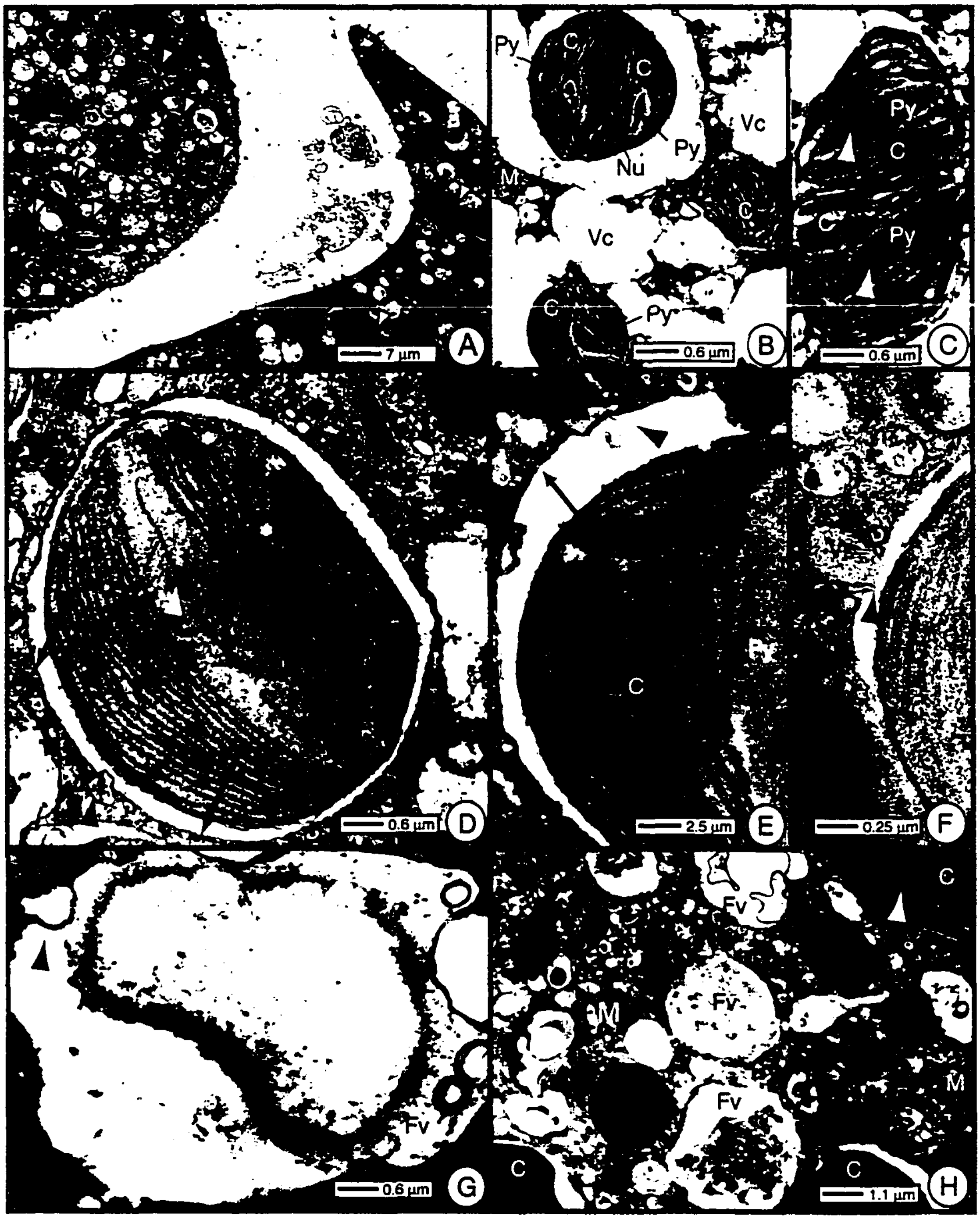

Figure IV.2 - Cytoplasm of *Elphidium excavatum* (Terquem). A-Low magnification showing numerous chloroplasts (arrowheads). B to F-Algal plastids retained in the foraminifer's cytoplasm. Note thylakoid and pyrenoid structure; abundant vesicles around the plastids; some of the vesicles are fusing with the membrane(s) encosing the plastid (black arrowheads). G and H-Food vacuoles with residues from digested material. C-chloroplast; F-food vacuoles; M-mitochondria; Nu-nucleus; Py-pyrenoid; Vc-vacuole and Vs-vesicles. White arroheads-thylakoids; large black arrows-membranes separating chloroplasts from host cytoplasm; small black arrows-pyrenoid membrane; asterisc-girdle lamella.

some cases, remnants of the algal cytoplasm were present and remained around the plastids (Figure IV.2B). These algal remnants occasionally included the algal nucleus (Figure IV.2B) and/or mitochondria, but most frequently only a dense cytoplasm was visible (Fig. IV.3D). It was significant to note that diatom frustules were never observed inside the internalized cytoplasm of the foram. The fine structure of every chloroplast retained within a vacuole in each section was examined with respect to thylakoid numbers, girdle lamellae, surrounding membranes and pyrenoid structure. The typical shape of the retained chloroplasts was oval to round and in some cases two lobes or plastids were evident (Figure IV.2 B-D). In the majority of the plastids, the pyrenoid was visible as a central structure separated from the lamellae of the chloroplasts by a membrane (Figure IV.2 C). In some sections, a single thylakoid could be seen crossing the pyrenoid longitudinally (Figure IV.2C and D). The lamellae were formed in most specimens by two or three stacked thylakoids (Figure IV.2 D). The girdle lamella, was usually thicker and could have up to four stacked thylakoids (Fig. IV.2D). The lamellae, were longitudinally oriented following the outline of the plastid, sometimes interrupting or curving to accommodate the pyrenoid (Figures IV.2 B-D). We found no fine structural evidence that the plastids in the foraminifera harvested from the field and those fed experimental diets of *Dunaliella salina* or *Amphidinium* sp. retained plastids from any group except the diatoms.

There was quite a range of materials found in vacuoles that did not contain robust plastids. Some clearly contained residua from the digestion of food (Fig. IV.2G and IV.2H). Some of them were clearly algal cells in various stages of digestion (Fig. IV.2F)

Figure IV.3 - Test and pseudpods possibly running through the canal system. A,C and E - Micrographs showing the numerous pores found on the test of *Elphidium excavatum* (Terquem). B,D and F - Micrographs of pseudopods found in between chambers and though to run through the canal system. D-Vacuole with remains of digestion and F-VAcuole with isolated plastid. Black arrowheads-pores; White arrowheads-cell membrane; Cb-chamber; Cp-chloroplast; Fv-Food vacuole; Fz-fuzzy coated vesicle; Nu-Nucleus; P-Pseudopod; Ty-thykakoids. Sclae bars indicated in each figure.

and others seemed to contain bacterial remnants (Fig. IV.2G). Sections obtained from foraminifera kept in culture for three weeks, as well as those not fed *Amphora*, showed more vacuoles (Fig. IV.2H). Some of the plastids were also less organized and their thylakoid lamellae were becoming more faint (Fig. IV.2B and F).

The plastids were separated from the foraminifer's cytoplasm by a symbiosome (vacuolar) membrane (Fig. IV.2C-E). We found several sections where the vacuoles were surrounded by vesicles that appeared morphologically to be lysosomes. In several instances we observed the "lysosomes" fusing with vacuoles containing the chloroplast, possibly converting them into phagolysosomal vacuoles (Figure IV.2D, E and F).

In sections going through the edge of the chambers, structures similar to the pores found in some larger foraminifera were observed (Figs IV.3A, C and E). A membrane separate from the cell membrane lines these apertures (Fig. IV.3D). In several sections, around the inner cytoplasm from the chambers and in between chambers, less dense cytoplasm was observed (Fig. IV.3B, D and F). This less dense cytoplasm consisted mainly of vesicles among which fuzzy coated vesicles were found (Fig. IV.3D). Vacuoles sometimes containing isolated chloroplasts (Fig. IV.3F) and other times remains of digested materials (Figure IV.3D) were also found.

Discussion

The fine structure analysis of the plastids found in the samples fixed after collection indicates that the plastids are probably all from diatoms. They had lamellae made of two or more stacked thylakoids, no starch accumulations inside the plastids, a girdle lamella and a simple internal pyrenoid bound by a membrane. These are common characteristics of diatom plastids (Dodge, 1973; Round *et al.*, 1990). Earlier reports of the fine structure of retained chloroplasts by *Elphidium* species refer to lamellae made up of three thylakoids, but only the micrographs published by Lopez (1979) and Bernhard and Bowser (1999) have the necessary magnification to count the thylakoids.

Lee and others (1988) had found algal organelles nearby the chloroplasts within symbiosomes. This was also the case in our study (Figures IV.3A). It is possible that these are intermediate stages of the digestion of the algae up to the isolation and retention of the plastid.

The fact that the fine structure of the plastids in the cytoplasm from all the foraminifera looks the same, regardless of the diet they were fed, seems to indicate that chlorophyte and dinoflagellate plastids, were not retained by the cultured foraminifera. Even in specimens fixed right after collection there seems to be little or no difference in the type of plastid retained by the foraminifera. These results are in consonance with what we had observed in the feeding and half-life experiments (Correia and Lee, submitted). We concluded then, that chloroplasts from green algae and dinoflagellates were retained in lesser numbers and had shorter half-lives than those fed diatoms or than the starved controls. Cedhagen (1991) had reported that the retained plastids in *Elphidium* could originate from dinoflagellate algae. In our opinion, and based on the micrographs published in that article, this interpretation is erroneous because the structure of the plastid as well as the pyrenoid structure seem similar to the plastids observed in this study. Lee and Lee (1989) had suggested that Elphidium retained chloroplasts from green algae even if in lesser numbers than those of diatoms. The fine structure of *Elphidium excavatum* (Terquem) fed *Dunaliella salina* does not support these observations. Probably what the authors in that study counted were chloroplasts that persisted inside the foram from the time it was collected.

The results of the fine structure analysis are in agreement with those obtained previously in establishing that the most likely origin of the plastids are diatoms. This had been observed in other fine structural (Lopez, 1979; Leutenegger, 1984; Lee *et al.*, 1988; Bernhard and Bowser, 1999) and pigment analysis (Knight and Mantoura, 1985). Furthermore, from the fine structure analysis of these foraminifera fed in the laboratory we can conclude that chloroplasts originating from the dinoflagellates and green algae are not suitable for retention by *Elphidium excavatum* (Terquem). Even after three weeks of feeding, these plastids never appeared in the cytoplasm of *E. excavatum* (Terquem).

The cytoplasm found in between the chambers of *Elphidium excavatum* (Terquem) is probably filling the septal canals characteristic of the rotalid foraminifera. It most likely corresponds to the septal canal cytoplasm described by Alexander and Banner (1984) for *Elphidium williamsoni*. This cytoplasm is similar to the pseudopods in ultrastructure. In fact, the fuzzy coated vesicles observed (Fig. IV.3D) are usually found in pseudopods and not in the internal cytoplasm (Travis and Bowser, 1991). Hottinger (1978) had also described "the living cytoplasm filling the interlocular spaces of nummulitids" as having a "pseudopodial ultrastructure". It is interesting to note that in the "pseudopodial cytoplasm" found in the inter-chamber canals isolated plastids, as well as remains from digestion, were sometimes found (Figs. IV.3B, D and F), indicating that digestion and eggestion take place.

The pores in the test walls of *Elphidium excavatum* (Terquem) seem to be sealed by an organic layer (Figs. IV.3C and E). Although in some sections these pores were in the vicinity of the interchameral pseudopodial extensions (Fig. 3C), we found no evidence that there is a connection between the two structures. Alexander and Banner (1984) have suggested that the organic "plug" covering the pores allows only the exchange of dissolved organic material, inorganic cations and dissolved gases. These pores probably connect to the intercannaliculi leading to the canals in-between the chambers (Banner, *et al.*, 1973).

Although we did not do the appropriate cytochemistry to obtain definitive evidence, the micrographs we obtained strongly suggest that the symbiosomes containing retained chloroplasts eventually are converted to phagosomes, which allow lysosomal fusion. The events leading to the digestion of the retained plastids are an enticing prospect for the next stage in this research.

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Chapter V

Summarizing Discussion

SUMMARIZING DISCUSSION

Elphidium excavatum (=*clavatum*) (Terquem) seems to prefer diatoms and mainly *Amphora coffaeformis* as its food source and, most likely, chloroplast donor. This conclusion is supported by the results from the feeding experiments performed. When the diet provided to the foraminifera consisted of green algae or a dinoflagellate (*Amphidinium* sp) the number of plastids retained by the foraminifera was less than if *E. excavatum* was not fed at all. It should be noted that the method used to count the number of plastids in each individual (Confocal Scanning Laser Microscope, CSLM) only allows the identification of what is a plastid, but not the algae it originated from. Furthermore, it is impossible to be certain whether the plastids counted were already present in the foraminifer's cytoplasm at collection time. For this reason, the average numbers of chloroplasts per foraminifer in the starved controls was subtracted from the numbers obtained for the foraminifera fed the different diets. With this measure, it is expected that the final values obtained represent a change in the number of chloroplasts retained by the foraminifera kept in culture.

The results obtained for this experiment are in agreement with the literature in establishing the diatoms as a preferred food organism. Nevertheless, when the absolute numbers of plastids per individual are considered, the results obtained in this thesis are greater than what had been previously reported (Lopez, 1979; Lee and Lee, 1989). This finding result can be attributed to the different methods used in quantifying the chloroplasts and the extrapolation methods used to transform chloroplasts per volume or unit area into chloroplasts per individual. With the advent of CSLM, problems such as the thickness of the specimen as well as the bleaching of the fluorescence are reduced or eliminated. Observing the whole individual foraminifera with conventionl epifluorescence creates superimposition problems caused by the background fluorescence produced by several layers of highly fluorescent cytoplasm. Furthermore, the focusing through the test also decreases the resolution. As an alternative, Lopez (1979) crushed several foraminifera and observed cytoplasmic extract under an epifluorescence microscope. This technique has the problem of possibly losing the chloroplasts by lysis. Using a CSLM allows optical sectioning that solves these practical problems. The CSLM also decreases the bleaching of the plastid fluorescence by focusing the laser beam on a very narrow part of the sample instead of flooding the whole individual with ultraviolet light. Both of these technical solutions allow more accurate counts of the numbers of plastids, which probably explains the greater numbers obtained in this study.

Chloroplasts retained by *E. excavatum* (Terquem) retain their fluorescence for at least eight weeks and possibly up to twenty even when the foraminifera are starved. The actual maximum survival time of the foraminifera with the retained plastids as their only carbon source is not known because the ideal culturing conditions have not yet been established. After the eighth week of incubation, some foraminifera are still alive but others start to look bleached even if they are fed. At this point it is premature to say if this bleaching is due to autolysis/digestion of the plastids or to the lack of specific requirements the foraminifera may have for surviving in culture. The type of alga fed to the foraminifera before they were starved seems to make a difference in how long the plastids are maintained and whether the hosts survive. This difference, however, is only statistically significant after a couple of weeks of incubation. Once again, when the foramifera were previously fed *Amphora coffaeformis*, a slower decrease of the number of plastids retained was observed, as well as slightly higher number of plastids retained than when the foraminifera had been fed *Dunaliella salina* and *Amphidinium* sp. This fact again, suggests that diatoms are better plastid donors than green algae or dinoflagellates for *E. excavatum*.

Incubation in complete darkness instead of a light:dark cycle does not change the results. Foraminifera with isolated plastids in their cytoplasm have been collected from regions below the photic zone (reviewed by Bernhard and Bowser, 1999). This implies that the retention of the plastids occurs regardless of their photosynthetic activity. In spite of the fact that the retained chloroplasts continue fixing carbon, and therefore are photosynthesizing (Lopez, 1979; Lee *et al.*, 1988; Correia, unpublished), it seems that the photosynthetic ability of the plastids may not be required for their retention.

As mentioned above, the count of chloroplasts using the CSLM does not allow the observation of the fine structure of the retained plastids. In order to supplement the data obtained by CSLM regarding the selectivity of the plastid selection in *Elphidium excavatum* (Terquem), an analysis of the fine structure of the plastids retained by the foraminifera, as well as the algae used as food, was completed. The fine structure of the plastids becomes important in establishing from which alga these plastids originated. Certain characteristics, such as structure and arrangement of the thylakoids, presence or absence of a girdle lamella, presence and structure of the pyrenoid, and the number of membranes separating the chloroplast from the surrounding environment, are different in the major algal groups. Studying the fine structure of the retained plastids provides information on which algal group the plastids belong. It should be noted, however that the number of membranes surrounding the retained plastids may be misleading and care should be taken in interpreting those results. It is likely that membranes of host origin, as well as membranes remaining from the algal cell, surround the retained plastids. The observations of the fine structure of the retained plastids in individuals collected from the field, as well as individuals maintained in culture and fed different algal diets, did not vary. In all cases, the chloroplasts found seem to be of diatom origin. The other authors who studied the fine structure of the phenomenon also found chloroplasts that were interpreted as originating from diatoms or crysophytes (Lopez, 1979; Leuteneger, 1984; Bernhard and Bowser, 1999). When the structure of the retained plastids is compared with that of *Amphora coffaeformis* (the diatom used as one of the diets), it seems likely that the plastids from this alga are being retained. This conclusion is based on the structure of the pyrenoid and the thylakoids of the retained plastids. On the contrary, plastids with the characteristic pyrenoids found in *Dunaliella salina* and *Amphidinium* sp. were not observed in the foraminifera cytoplasm, supporting the data from the CSLM observations on plastid retention numbers.

The findings discussed above demonstrate that diatoms are the best and possibly only chloroplast donor to these foraminifera. It would seem that these chloroplasts have some characteristics that make them more suited for kleptoplastidy and that somehow the foraminifer can distinguish them from chloroplasts of other algae present in the environment. Why are chloroplasts from chromophytes found recurrently in the chloroplast-retaining phenomena? Recently, some hypotheses have been posed in order to explain why chromophytic chloroplasts seem to be preferred by species that husband chloroplasts. Most of the hypotheses have to do with relatively recent advances made in our understanding of the genetics and biochemistry of chromophytic algae, which are very different from those of green algae and higher plants which have been the best studied groups. Possible explanations as to why chromophytic algae are best suited for kleptoplastidy include: unusual chloroplast gene autonomy of these plastids; the possible extreme stability of the plastids and the essential proteins intervening in photosynthesis; a low minimum protein composition necessary to maintain chloroplast activity; the ability of the plastids to use related animal encoded proteins (such as mitochondrial ones); and finally, a possible horizontal gene transfer from the algae to the foraminifera (Rumpho *et al.*, 2000). Larger foraminifera have been shown to select between diatom species for their endosymbiotic partners. Chai and Lee (2000) found a Common Symbiotic Surface Antigen (CSSA), which was characterized as a 104kDa glycoprotein found only on the surfaces of symbiotic diatoms and absent from the non-symbiotic species. Furthermore, blocking this CSSA prevented the foraminifera from ingesting the diatoms and blocking symbiosis (Chai and Lee, 2000). We used an antibody against the CSSA and incubated it with isolated plastids of several diatoms. No binding occurred, leading us to conclude that this specific molecule is not involved in the "communication" between the foraminifer and the food/symbiont in this case.

The retained plastids may be actively digested even if at a slow rate. Evidence for the digestion of these plastids is incomplete because cytochemistry tests such as the Gomorri stain were not performed. However, there are indications that digestion is taking place because vesicles, which structurally look like lysosomes, are found surrounding and sometimes fusing with the vacuoles containing the chloroplasts. Furthermore, it was observed in some of the sections that plastids appear disorganized and with thick thylakoids. It is also interesting to notice that the chloroplasts seem to go through a sorting process by which the algae are incorporated and the individual chloroplasts are gradually separated and kept isolated. Evidence for this comes from the several intermediate stages we photographed between an almost intact alga to isolated plastids found in several sections.

From the analyses of pseudopods found in some of the sections, it becomes evident that these structures play a role in the sorting of the plastids. In fact, sections through pseudopods show that the plastids can be separated from the rest of the alga before they reach the internal cytoplasm. Due to the position of the pseudopods, it is possible that these are circulating through the canal system of the foraminifera. This could be verified by serial thick sectioning of the specimens and observation using a High Voltage Elevtron Microscope to determine the three dimensional position of the pseudopods. If these are in fact pseudopods circulating through the canal system, we provide evidence for the speculated importance of the canal system in the exchange of material between the foraminifer and the external environment.

The results of this thesis, as well as other work, prompt several questions. The study of plastid retention is important and can shed some light in mainly three areas. One of those areas is the evolution of the chloroplasts. It is generally agreed that chloroplasts originated from symbionts which were gradually reduced to plastids. Could it be that the kleptoplastidy phenomenon we are observing now is a snapshot of what happened previously when the first eukaryotic cells acquired chloroplasts? If so, under the "right" selective pressures the organisms which now have kleptoplastids could, in the future, have plastids which are not derived from food but inherited. Another interesting aspect to consider is the ecological implication of being a mixotrophic organism. Is the carbon fixed by the retained chloroplasts sufficient for the host's energetic needs? Are these photosyntetic products transferred and incorporated by the host? Finally the "mechanics" of the selective process that permit cells like *Elphidium* to retain chloroplasts as "symbionts" while digesting the rest of the alga beg further investigation.

In the ecological front, it would be interesting to sample a population of chloroplast-retaining species over a long period of time and observe if the kleptoplastidy changes seasonally. This requires an environment where individuals are easily sampled throughout the year is available. If the culture of these organisms can be done successfully, physiological approaches could be pursued in order to test what factors besides light and diet (as tested in this thesis) influence the relationship. One of the additional factors tested could be oxygen levels, which would test the hypothesis proposed by Bernhard and Bowser (1999) that kleptoplastidy might be an adaptation to low oxygen environments.

Another set of questions require the use of molecular techniques to understand the genetics of the phenomenon. One of the questions would be to determine genetically which algae are kept by *Elphidium*. Since there is good evidence that the algae retained are diatoms, probes to specific diatoms chloroplast encoded genes could be used to detect to which species the algal chloroplasts belong. This may be the only approach that allows identifying the chloroplast donors at the species level. On the other hand, tests similar to those done in *Elysia viridis* could be done to search for mRNA coding for chloroplast and algal nuclear coded protein expression in the retained plastids. A further step could be taken to test whether horizontal transfer has occurred between the algal and the foraminifera. All of these molecular approaches depend, however, on a successful extraction procedure of the retained plastid genetic material. Although the chloroplasts in the foram's cytoplasm are abundant, so are contaminants. Extracting enough suitable DNA or RNA from the foraminifera will probably not be as easy as with slugs.

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