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Experiments on Persistence of Endosymbiotic Diatoms in the Larger Foraminifer: *Amphistegina lessonii*

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Abstract

A previous experiment with *Amphistegina lessonii*, a diatom-bearing larger foraminifer, showed that the host-symbiont system was experimentally tractable and that the symbionts were fungible. New experiments were aimed at clarifying questions raised about differential persistance of endosymbiontic diatom species and the mechanisms of recolonization of hosts rendered nearly aposymbiotic. Individuals of *Amphistegina lessonii* were rendered nearly aposymbiotic by incubation with DCMU in bright light. Randomized mixtures of endosymbiotic diatoms and the bleached animals were inoculated into flasks with filter-membrane windows and incubated *in situ* at 20 meters in the Gulf of Eilat. After a week the algae from a representative sample were isolated, cultured, and identified. The remaining animals were incubated 3 weeks more with a mixture of free-living algae; again their internal algae were cultured and identified. During the experiment, two of the symbiont species present in the original experimental population, *Nitzschia panduriformis* and *N. frustulum*, were almost completely eliminated from the hosts. Many introduced symbiont algal species persisted for weeks inside the hosts and potentially could have or did indeed replace established species. With the possible exception of *Entomoneis* sp., none of the free-living algal species incubated with the foraminifera as potential food during the 3-week second incubation period were isolated in culture. This increases our confidence that the isolation methodology we have been using is a valid way to extract and identify symbionts. "Rebrowning" of nearly aposymbiotic animals involved division of cortical algae. Cell division was mostly seen in animals fixed just before dawn when approximately 15% of the surviving algae were in division. Undigested diatoms with frustules within food vacuoles were observed in some preparations.

Keywords: Endosymbiotic diatoms, *Amphistegina lessonii*, symbiont diversity.

Abbreviation: DCMU=[3(3,4-dichlorophenyl)1,1-dimethylurea]

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1. Introduction

The frustuleless endosymbiotic diatoms of larger foraminifera show little diversity as examined in the transmission electron microscope (e.g. Leutenegger 1977, 1983, 1984; Koestler, 1985), but cultivation of algae from there reveals a diversity of extremely small ($\lesssim 10\mu$m) diatoms (Lee et al., 1979, 1980a & b, Lee and Reimer, 1984, Reimer and Lee, 1984). Five species of *Nitzschia*: *N. frustulum*, *N. frustulum* var. *symbiotica*, *N. valdestriata*, *N. laevis* and *N. panduriformis*; 4 species of *Navicula*: *N. reissi*, *N. hanseniana*, *N.* sp. (strain musc.) and *N.* sp. (strain elati); 3 species of *Amphora*: *A. tenerrima*, *A. roettgeri*, and *A.* sp. (strain Er); and single species of the following genera: *Fragilaria shiloi*, *Protokeelia hottingeri*, and as yet to be named *Achnanthes* sp. (strain MEM) have been isolated from large foraminifera. Although each animal usually hosts one diatom type at a time over the years many of the diatom species have been isolated from the same host species of foraminifera (e.g. *Amphistegina* spp., *Heterostegina depressa*). This suggests that the relationship between these hosts and their symbionts, although restricted to few species, is not rigidly monospecific.

Many questions arise about symbiont diversity among the diatom-bearing larger foraminifera. The symbionts might be recruited from the natural food assemblages; some symbionts might be more adaptive for their hosts at different depths, seasons, and other environmental variables. Recently we have obtained nearly aposymbiotic hosts by bleaching them with DCMU and reestablished symbiosis by feeding the host previously isolated and cultured endo-symbiont species (Lee et al., 1983). This experiment suggested that some algal species were more eligible than others to establish themselves during "re-browning". Thus, *Nitzschia laevis* and *N. valdestriata* seemed preferred over *N. panduriformis* and *Fragilaria shiloi*. This was an unusually interesting finding since the latter two species are among the most commonly isolated species in the natural collections examined thus far. The experimental results were obtained after 1 week incubation. What if the exposure had been more prolonged, or if the hosts had initially harbored symbionts ranked higher in persistence? One could speculate that after recruitment by nearly aposymbiotic hosts the species composition of symbionts may change because different symbionts vary in their reproductive rates or physiological traits which would give some of them competitive edges in filling vacant symbiont niches.

In view of the fact that some aspects of the *Amphistegina*/diatom host/symbiont system have been shown to be experimentally tractable, we undertook new experiments with nearly aposymbiotic animals. Our aims were to see if ingested potential symbiotic algae fed to nearly aposymbi-

otic hosts would persist long after their hosts were changed to a new diet of free-living algae. We also hoped that new cytological preparations made from organisms undergoing "rebrowning" might give some indication of the replacement mechanisms occurring during the process. For example, if we found no dividing algal cells in the cortex, and many undigested algal cells throughout the animal, this might be interpreted as an indication that "rebrowning" occurs by recruitment. On the other hand, if algal cells were dividing throughout the host animal this would be good evidence for reproduction of recruited potential endosymbionts.

We also felt that the experimental design of our new experiment could give us an opportunity to check our isolation technique. Even though Koestler et al. (1985) showed that the digestion of *Chlorella* sp. (AT) and *Amphora* sp. (B1 45) began outside the animals soon after contact by the granuloreticulopods, there is always the lingering doubt that undigested food within the animals might survive and be isolated in culture.

2. Methods and Materials

Field work was at the Gulf of Eilat Heinz Steinitz Marine Biology Laboratory of the Hebrew University of Jerusalem, March and April 1984. Identification of isolated algae was continued in New York at the American Museum of Natural History. Some animals used for autoradiographic and cytological preparations were incubated and fixed in Eilat; others in New York. Embedding, sectioning, staining and radioautography were done in New York. The animals used were freshly collected at a site near Wadi Taba (20–24 m depth). As before we took a sample from each group (15 animals). The animals were washed, brushed and crushed so that their symbionts could be isolated, cultured and identified (Lee et al., 1980a & b, Lee and Reimer, 1984).

Experiments in resynthesis of symbiosis

Incubations were seaward of the laboratory at 20 m depth. A lucite rack tied to a pair of ropes anchored to the sea floor and tied to a float at the surface held the experimental vessels. These were tissue-culture flasks with windows cut in them and covered with 1μm nylon filter membranes (Coccetti and Lee, 1979). The animals were bleached, or rendered nearly aposymbiotic, by incubation for 5 days in 1×10^{-6} freshly prepared DCMU in Gulf sea water. The earlier experiment had shown that 70–90% of the symbionts were eliminated in some individuals of the experimental population (Koestler, 1985; Koestler et al., 1985). The flask wherein bleaching took place was anchored at 5 m below the surface. The flasks were retrieved each day, sea

water with DCMU decanted and replaced with fresh sterile medium. After 5-day exposure to DCMU, the animals were spread out in fingerbowls. They were observed 2 h later with the aid of a dissection microscope and those animals with extensive pseudopodial networks were picked. The animals were brushed vigorously with 0000 sable paint brushes, then inoculated in groups of 50 into flasks. Randomized mixtures of endosymbiotic algae (Table 1), (a) *Fragilaria shiloi*, (b) *Nitzschia frustulum*, (c) *Nitzschia valdestriata*, (d) *Navicula* sp. (strain elati), (e) *Amphora tenerrima*, (f) *Navicula* sp. (strain musc.) were inoculated at the same time. The concentration of the algae in the inoculum was adjusted with the aid of a haemacytometer so that the final concentration of each species in the experimental flasks was $1–3\times10^4$ algae ml^{-1}. A flask without algae (group 1) served as a "rebrown" control.

After 1 week incubation with the algal mixtures, the animals were harvested from each flask, but kept in separate groups. After 2 h ten animals with extensive pseudopodial networks were transferred to spot plates, rebrushed vigorously, washed, and then crushed so that their symbionts could be identified in culture (Lee et al., 1980a & b, Lee and Reimer, 1984). The remainder of each group was inoculated into fresh experimental flasks along with a different set of food algae: *Cocconeis placentula*, *Entomoneis* sp., *Navicula* sp. (strain E 8), *Amphora* sp. (strain E 29) and *Chlorella* sp. (strain AT) (total final concentration 1×10^6 algae ml^{-1}). These algae, very common in the natural assemblages in which the foraminifera are found, previously had been isolated by us in axenic culture from *Halophila* leaves at Wadi Taba, and are considered to be the potential major food organisms for the foraminifera. The flasks were reincubated at the same site and depth. They were retrieved each week, decanted and inoculated with fresh algal food mixtures.

After a further 3 weeks incubation in the sea (total 4 weeks) the animals were harvested from each flask, brushed, and transferred to sterile sea water in finger bowls. After 2 h 10 foraminifera with extensive pseudopodial networks were selected at random for identification of symbionts. The entire contents of a crushed animal were placed in a single tube of liquid medium. Incubations were begun in Eilat and continued in New York. Identification of isolated symbionts was done with the aid of a Cambridge scanning electron microscope (model 250).

Autoradiographic and cytological studies

The purpose of these studies was to gain more insight into the overall cellular processes which were taking place during "rebrowning". Some organisms from the same collection from Wadi Taba were placed in 9-hole spot plates

(15/spot) and divided into two groups: drug-treated and controls. Both groups were fed the following mixture of algae (previously isolated from the same collection site as the foraminifera): *Cocconeis placentula*, *Chlorella* sp. (strain AT) and *Entomoneis* sp. (possible *E. paludosa* var. *densestriata*). The experimental organisms were incubated in 10^{-5}M DCMU for five days, control group organisms were incubated in sea water (salinity–40.5 ppt). DCMU served to inhibit photosynthesis of the symbionts and lead to their digestion by their hosts (Koestler et al., 1985). The bleached animals were allowed to recover in a sea water medium for 2 days. Then half the control and experimental organisms were incubated with [^{3}H] thymidine, a tracer commonly incorporated into the DNA being synthesized by dividing cells. The other half of the organisms incubated in sea water (experimental and control) were treated with 0.25% colchimid at least 5 h before being fixed. This treatment was used to arrest cells in the process of cell division. Fixed organisms were decalcified in 15% acetic acid (1 h), dehydrated in a graded series of ethanol (50–95% 5 min; 3 changes of absolute alcohol, 15 min each), cleared in toluene (3 changes, 15 min each), embedded in Paraplast (a mixture of purified paraffin and plastic polymers; Fisher Scientific Co.) and sectioned at 7μm. Several stain techniques were applied to serial sections of the organisms that had been incubated in sea water: Himes and Moriber (1956) triple stain (8 min hydrolysis): de Tomasi modification of the Feulgen technique (Pearse, 1968); and ethanolic Fe-hematoxylin (stain 2 h in Fe-hematin; destaining with 1% HCl 10 min) (McEnery and Lee, 1981). In over-all more than 300 serially sectioned animals were examined. Observations were restricted, primarily to the study of symbiont nuclei.

Autoradiography with Kodak NTB II liquid emulsion was performed on sections incubated with [^{3}H] thymidine. The preparations were mounted on cleaned slides. Manufacturer's precleaned microscope slides were cleaned again by soaking them for 24 h in $K_2Cr_2O_7$-H_2SO_4 solution, rinsed in distilled water, then coated with gelatin/chrome alum solution (0.5% w/v), and 0.05% (w/v), respectively and stored under dust-free conditions (Rodgers, 1967). Sections were mounted and hydrated and the final bath was of distilled water at 40°C. Fresh emulsion (Eastman Kodak NTB II; Rochester, NY) was liquefied in a thermostatically controlled water bath at 40°C. Processing of preparations in the emulsion was in total darkness. The slides were slowly dipped in the emulsion and vertically withdrawn to drain off excess emulsion. Backs of the slides were wiped clean and the slides cooled to 4–10°C to permit the emulsion to gel before it dried, thereby preventing uneven distribution of silver halid crystals and reducing background. The preparations were air dried in total darkness for 45 min and transferred to trays in a

Con-Rad/Joftes light-tight box containing dessicant. They were incubated at 4°C for 40–45 days. They were then developed at 15°C in Kodak Dektol (1:1, 2 min), rinsed in distilled water (10 sec), fixed (5 min), and washed in distilled water. This procedure was also carried out in total darkness because the emulsion is more light sensitive when completely dried. Preparations were post stained with 1% toluidine blue (a post-emulsion procedure compatible with radioautography) (McEnery and Lee, 1981).

3. Results

Resynthesis experiments

The results of resynthesis with isolates from 15 individual specimens of the original collection used in the experiment are shown in Table 1. They were mixtures of symbionts: *Nitzschia laevis*, *N. panduriformis* var. *continua*, *N. frustulum* var. *symbiotica* and *Amphora roettgeri*. Only 1 of 15 forams was host for a single diatom species, *Nitzschia laevis*. With the exceptions to be noted (e.g. group nos. 2,3,4), the same species were also most often isolated at the end of week 1 of incubation. During week 1, *Nitzschia frustulum* was isolated from at least one host in each of the experimental groups (nos. 2,3,4,5 and 7) in which it was used but was rare in hosts incubated for 4 weeks. *Fragilaria shiloi* was isolated during the first week and became even more abundant in hosts from two (nos. 6 and 10) of the four groups. *Amphora tenerrima* was isolated during week 1 from 4 of 10 specimens in group no. 3 but not from the other groups (nos. 4,6,8 and 9) in which it was introduced. Neither *Nitzschia valdestriata* nor *Navicula* sp. (Eilati) were isolated from specimens incubated in mixtures in which these diatoms were present. Isolates from 4-week incubation unlike those made after 1 week showed a general reduction in symbiont diversity (Table 2). *Nitzschia panduriformis* and *Nitzschia frustulum* var. *symbiotica* were much less abundant, the latter being more abundant only in groups 6 and 7. *Amphora roettgeri* on the other hand became more abundant in many groups during 4-week incubation. A similar trend though less conspicuous, emerged in groups in which *Fragilaria shiloi* was present. *Amphora tenerrima* and *Navicula* sp. (musc.) became abundant in several groups. *Entomoneis* was isolated from specimens in a number of groups. Since it was not in the mixtures used in the experimental groups nor was it isolated in the specimens taken as representatives of the initial collection, presumably it has been present at the start of the experiment but eluded detection in our limited control sample. It was used as food for the specimens incubated for 4 weeks.

Autoradiographic and microscopic observations

Some [^{3}H] labeled thymidine was observed in food vacuoles. Some label was also scattered throughout the cytoplasm but most of the label did not enter the animal (Fig. 1). Much label was absorbed on the surface of the test (Fig. 2). That label which did enter seemed not to be concentrated in any of the hosts' organelles or in the nuclei of the endosymbiotic algae. Occasionally symbiont nuclei were clearly labeled (Fig. 3). In overall the [^{3}H] labeled thymidine technique was not useful to this animal and its symbionts. However, Feulgen-stained sections helped answer the questions raised. Cell division of the endosymbiotic algae was rare in control animals (not treated with DCMU) but observable in the cortical (peripheral) region of all the groups fixed at different times of the day. Algal division was more often observed in animals fixed just before dawn. Although it is difficult to estimate the number to algae involved in division at one time because they are at the cortex of sphaeroid animals, perhaps as many as 5% of the cortical algae were in late stages of mitosis when they were fixed. We base this estimate on the examination of ~2,000 dorsal and lateral algal cells visible in sections largely horizontally through the animal. Fig. 4 shows a tangential section through the outer cortex in which 3 near by cells were in division at the same time. Division was more frequently observed in algae of the animals recovered from DCMU treatment. Roughly 15% (3–20%) of the algae in 50 representative dorsal tangential sectioned fields were in division at the time of fixation.

Although digestion begins in the pseudopodial net in the extrathalamic cytoplasm (Koestler et al., 1985) many food vacuoles were drawn into the test. Diatoms in food vacuoles are easily distinguishable in cytological preparations from endosymbiotic diatoms: food vacuoles are larger and have a large optically less dense region surrounding a diatom undergoing digestion (e.g. McEnery and Lee, 1981). The frustule of a diatom in a food vacuole is usually easily resolved by bright field or phase contrast microscopy. Moreover, diatoms in food vacuoles are larger ($> 2\times$) than endosymbiotic diatoms. Usually there is also wide spatial separation within the animal between digestive vacuoles and symbionts (McEnery and Lee, 1981). Digestive vacuoles tend to be more abundant in the ventral medial cytoplasm while symbionts tend to be dorsal and cortical. We observed three Feulgen-stained individuals each with a food vacuole containing a diatom with a frustule and a Feulgen-positive nucleus (e.g. Fig. 5). By light microscopy these three specimens had appeared cytologically normal and were apparently not being digested at the time they were fixed.

Figure 1. Autoradiograph of a section of *Amphistegina lessonii* fixed after incubation in medium containing [^{3}H] thymidine as a label for DNA synthesis. ×40.

Figure 2. Tangential section through the periphery of *Amphistegina*. Radioautograph of a specimen incubated in [^{3}H] thymidine and stained with toluidine blue-O. Arrow points to alga with some radioactivity detected in the photographic emulsion above the nucleus. ×100.

Figure 3. Higher magnification of a cytoplasmic section (ring) shown in Fig. 1. Label appears over food vacuole. ×100.

Figure 4. Tangential section through the periphery of *Amphistegina* showing several binucleate specimens (arrows) in focus. Feulgen preparation. Taken with a green filter for contrast. ×1,000.

Figure 5. Cell of *Cocconeis placentula* in a digestive vacuole within *Amphistegina*. Taken with a green filter for contrast of pink stained nucleus. Arrow points to Feulgen-positive nucleus within cell. ×1,500.

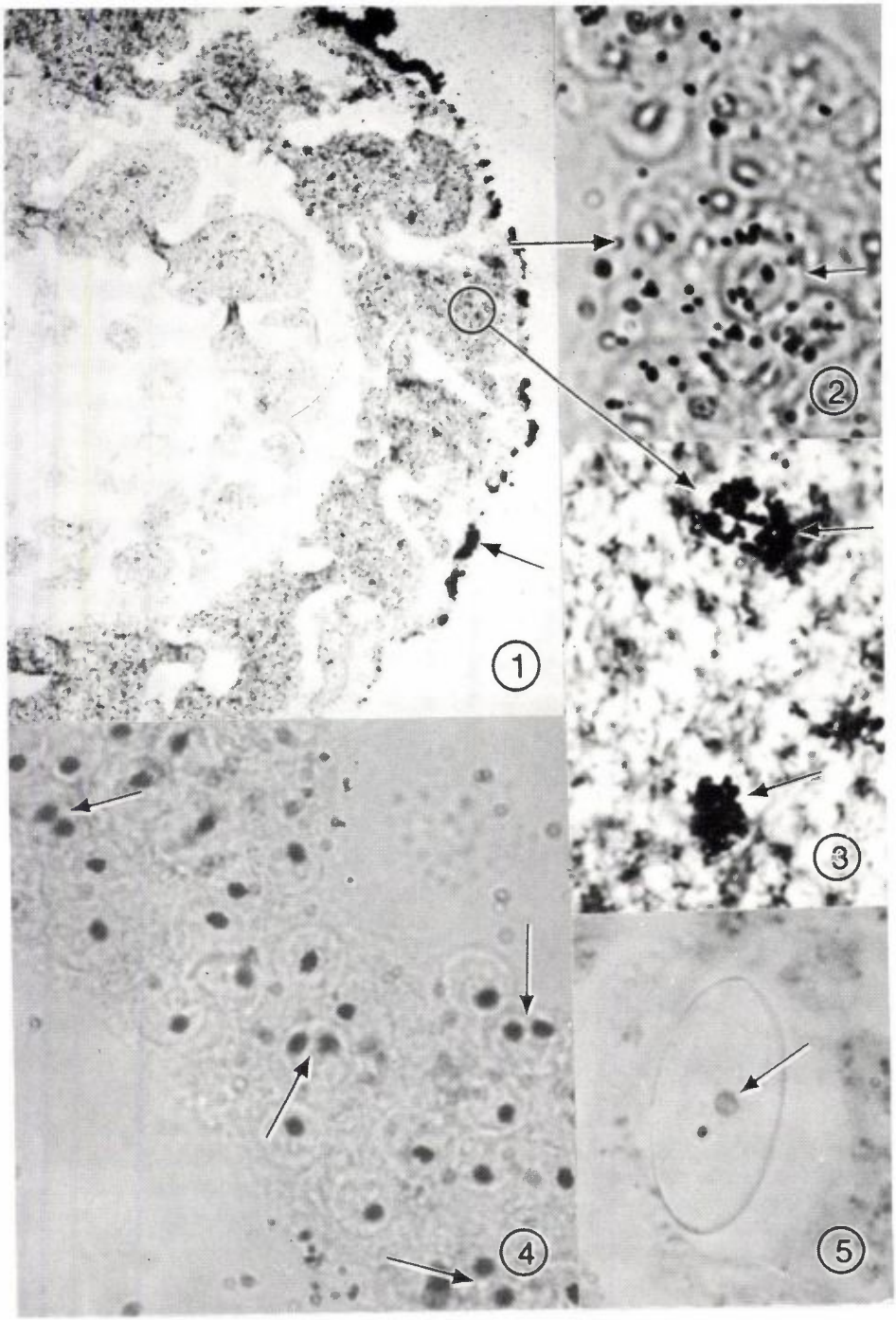
1
2
3
4
5

Table 1. Resynthesis experiment.
Algae recovered from foraminifera after 1 week incubation.

Algae introduced and recovered after incubation

a) *Fragilaria shiloi* (*Fs*)
b) *Nitzschia frustulum* (*Nf*)
c) *Nitzschia valdestriata* (*Nv*)
d) *Navicula* sp. (strain elati) (*Ne*)
e) *Amphora tenerrima* (*At*)
f) *Navicula* sp.(strain musc.) (*Nsp*)

Algae recovered but not introduced
Nitzschia laevis (*Nl*)
Nitzschia panduriformis (*Np*)
Nitzschia frustulum var. *symbiotica* (*Nfs*)
Entomoneis sp. (*E*)
Amphora roettgeri (*Ar*)

Algal mixtures for experimental group

1) No addition of "rebrown controls"
2) a,b,c
3) b,c,e
4) c,e,f
5) f,a,b
6) d,e,f
7) b,c,e
8) c,d,e
9) a,d,e
10) d,f,a

Experimental group	*Nl*	*Np*	*Nfs*	*Ar*	*Nf*	*Fs*	*E*	*At*	*Nsp*	*Nv*	*Ne*
Original	1	0	0	0							
Collection	7+	8+	2+	6+							
Mixture no.											
1	2	0	2								
	3+	3+	6+								
2	0				2						
	5+				5+						
3	0		0		1			2			
	3+		4+		4+			2+			
4		0	0	10							
		2+	2+	3+							
5	0	0			3						
	8+	9+			9+						
6	4	0	1				2	0	0		
	4+	1+	1+				3+		1+		
7	0	0	8	0	0		0		0		
	2+	3+	2	1+					1+		
8	0	0	0	2							
	4+	2+	4+	7+							
9	0	0	0	0		0	0				
	2+	1+	4+	6+		3+	8+				
10	2	0	1			0	0				
	7+	4+	2+			6+	1+				

The upper number in each pair indicates number of specimens in which only one species of alga was recovered. The lower number (+) indicates that more than one algal species was isolated from the same host specimen. The following algal species were not recovered from any host: *Cocconeis placentula*, *Navicula* sp. (E8), *Amphora* sp. (*E29*), *Chlorella* sp. (*AT*), *Nitzschia valdestriata*, *Navicula* sp. (strain elati).

Table 2. Resynthesis experiment.
Algae recovered from foraminifera after 4 weeks incubation.
(After week 1, the foraminifera were incubated for 3 additional weeks with the following mixture of free-living algae which served as food: *Cocconeis placentula*, *Navicula* sp. (*E8*), *Amphora* sp. (*E29*), *Entomoneis* sp. and *Chlorella* sp. (*AT*))

Experimental group	*Nl*	*Np*	*Nfs*	*Ar*	*Nf*	*Fs*	*E*	*At*	*Nsp*	*Nv*	*Ne*
Mixture no.											
1	8 2+		0 1+				0 1+				
2	3 5+	0 1+	0	0 6+	 1+	0					
3	0 3+			6 4+							
4	1+			9 1+							
5	0 6+			0 8+	0 1+	0 4+					
6	1 6+		2	2 2+		0 1+			6 1+		
7	3 2+		4	3 2+							
8	0 3+			8 3+							
9	0 1+					0 9+		1 9+			
10	0 1+					0 8+	0 4+	0 1+	0 9+		

Footnote as in Table 1.

Discussion

The results from the recolonization experiments here agree in the main with our earlier experiments (Lee et al., 1983), if we disregard for the moment the results we obtained when *Nitzschia valdestriata* was present in the incubation. There were methodological differences between the two sets. Isolation technique for the new experiment was somewhat different. In the first experiment the endosymbionts were streaked out on plates and then the organisms within 10–20 colonies were identified with the aid of Transmission Light Microscopy or Scanning Electron Microscopy. In the present experiment endosymbionts from a crushed animal were transferred to a liquid isolation medium. The former method has the advantage of allowing a semiquantitative evaluation of the endosymbiotic populations. Quantitative aspects are sacrificed by the latter method but it has the advantage of isolating and identifying organisms which may be present in the host at low densities, hence easily overlooked. By the latter technique we found that all foraminifera in our initial collection had mixtures of diatoms. The endosymbionts in the collection made for the present experimental application were almost exclusively mixtures of *Nitzschia laevis*, *N. panduriformis* and *N. frustulum* var. *symbiotica*. A few hosts also had *Amphora roettgeri*. Although it was not detected in sample specimens, some members in the initial collection may also have harbored *Entomoneis* sp. (possibly *E. paludosa* var. *densestriata*), because we isolated it from some specimens incubated during the experiment. *Entomoneis* sp. is abundant in the natural communities in which the animals are found and could be a potential food organism. This species is interesting because it is eaten by the tiny foraminifer, *Metarotaliella parva*, from the same habitat which digests most of the alga but retains its chloroplast (Lanners work in progress, cited and illustrated in Lee, 1983). Since we have yet to isolate *Entomoneis* sp. as the sole algal species from any diatom-bearing foraminiferal host we are inclined to regard it as a food organism which occasionally escapes digestion.

The differences in the initial populations of symbionts in the hosts used in the earlier experiment and the present one may be significant. In contrast to the present experiment when hosts initially harbored *Nitzschia laevis*, *N. panduriformis*, *N. frustulum* var. *symbiotica* and *Amphora roettgeri* almost all animals in the collection for the earlier colonization experiment initially harbored only *Nitzschia panduriformis* (Lee, et al., 1983). *Nitzschia laevis* may be far less easily replaced after aposymbiotic stress than *N. panduriformis*. We observed in both experiments that the latter species became reduced in abundance after bleaching and rebrowning.

In our earlier resynthesis experiment (Lee et al., 1983) we found that *N. laevis* and *N. valdestriata* were preferred (or preferentially persisted) over all the other species tested. *N. panduriformis* and *F. shiloi* were lowest in the order of persistence. In the present experiment *N. laevis* was already an endosymbiont in most hosts. The simplest interpretation of the data would be that little or no replacement of the original endosymbionts took place during the algal repopulation of the hosts after DCMU treatment was stopped. Changes involving losses in the diversity of endosymbionts (e.g. reduction in the number of *N. panduriformis* and *N. frustulum* var. *symbiotica* in the hosts) may have been favoured by the particular conditions of incubation.

Some introduction of recruited algae into the hosts' endosymbiotic population were observed in several groups (nos. 5,6,9, and 10) in which *F. shiloi*, *A. tenerrima* and *Navicula* sp. (musc.) were introduced. This was more evident in the hosts incubated for 4 weeks. Though lacking direct evidence that these algae replaced cortical endosymbionts we can infer that they persisted within host populations for at least the 3 weeks of further incubation despite their hosts being fed other algal species.

It should be noted that neither *N. valdestriata* nor *Navicula* sp. (strain elati) were recovered in any of the isolations. The latter species has only rarely been recovered from *Amphistegina* (work in progress) and may lack properties which favour persistence except under, as yet, unspecifiable conditions. It is more difficult to apply the same interpretation to *N. valdestriata* unless some new factor is discerned. Perhaps the strain of *N. valdestriata* in the present experiment may have lost in culture something important for persistence since being used in our experiment two years ago. In a similar experiment Colley and Trench (1983) found that only freshly isolated strains of *Symbiodinium microadriaticum* would reinfect the scyphistoma stage of the jellyfish *Cassiopeia xamanchana*. Isolates from the same host lost infectivity after prolonged culture.

Studies of endosymbiont entry, and persistence in several invertebrate systems point to prerequisites for establishment of symbiotic relationship. An obvious prerequisite is for ingested potential endosymbionts to stay alive and undigested (e.g. Karakashian and Rudzinska, 1981; Colley and Trench, 1983; Fitt and Trench, 1983). There is evidence that in organisms as diverse as *Paramecium bursaria* and *Cassiopeia xamanchana* symbiotic algae alter the membrane of the vacuole which surrounds them, thus inhibiting fusion with secondary lysosomes.

Fine-structure studies of *Amphistegina* suggest that there may be parallels in larger foraminifera (Koestler, 1985; Koestler et al., 1985). All of the

free-living diatoms gathered by the foraminifera were internalized and the resultant vacuoles quickly converted to phagolysosomes. In contrast the two species of endosymbionts studied in some detail, *F. shiloi* and *N. laevis*, were envaculated rapidly but not all vacuoles became phagolysosomes, with many more *F. shiloi* being digested than *N. laevis* (Koestler, 1985; Koestler et al., 1985). The observation in our present study that occasional *Cocconeis placentula* in food vacuoles stained Feulgen positive might be taken as evidence of shortcomings in the digestive system of *Amphistegina* permitting survival of an ingested diatom — a trait perhaps underlying the evolution of endosymbiosis in larger foraminifera. It also warns that living undigested food organisms will occasionally be isolated from within hosts in addition to symbionts. However, the fact that during 3 weeks of incubation with 5 different food organisms only one *Entomoneis* sp. was isolated from the foraminifera suggests that contaminations of symbiont isolations by food organisms is not likely when our techniques are applied.

Observations of stained histological preparations clearly indicated that many of the algae in the peripheral cytoplasm were actively dividing when fixed. It is not possible to discern by light or electron microscopy whether the dividing algae were from the resident population or recruits. It may be possible to detect if they come from newly recruited populations. A study of effect of larger foraminiferal homogenates on endosymbiont algal strains *in vitro* suggests that something in host homogenates inhibits or blocks formation of new frustules (Lee et al., 1983). If this happens *in vitro* one should be able to obtain TEM or LM sections which mirror this event. Cells with frustules would be joined to spherical frustuleless cells — a step which should precede insertion of newly recruited endosymbionts. Although we did not observe any cells of this type in the present study efforts focused on experimentally manipulated populations at this critical stage may perhaps reveal them.

REFERENCES

Coccetti, G. and Lee, J.J. 1979. Microcosms and systems for the study of the realized niches of benthic marine diatoms. *Hydrobiol.* **67:** 51–80.

Colley, N.J. and Trench, R.K. 1983. Selectivity in phagocytosis and persistence of symbiotic algae by the scyphistoma stage of the jellyfish *Cassiopeia xamanchana*. *Proc. R. Soc. Lond. B* **219:** 61–82.

Fitt, W.K. and Trench, R.K. 1983. Endocytosis of the symbiotic dinoflagellate *Symbiodinium microadriaticum* Freudenthal by endodermal cells of the scyphistomae of *Cassiopea xamanchana* and resistance of the algae to host digestion. *J. Cell Sci.* **64:** 195–212.

Himes, M. and Moriber, L. 1956. A triple stain for deoxyribonucleic acid, polysaccharides and proteins. *Stain Technol.* **31:** 61–70.

Karakashian, S.J. and Rudzinska, M.A. 1981. Inhibition of lysosomal fusion with symbiont-containing vacuoles in *Paramecium bursaria*. *Exp. Cell Res.* **131:** 387–393.

Koestler, R. 1985. Fine structural and cytological investigation of digestion and the re-establishment of symbiosis in the larger foraminifer *Amphistegina lessonii*. Dissertation. City University of New York.

Koestler, R.J., Lee, J.J., Reidy, J., Sheryll, R.P., and Xenophontos, X. 1985. Cytological investigation of digestion and re-establishment of symbiosis in the larger benthic foraminifera *Amphistegina lessonii*. *Endocyt. C. Res.* **2:** 21–54.

Lee, J.J. 1983. Perspective on algal symbionts in larger foraminifera. In: *Inter. Rev. Cytol.* Supp. 14, K.W. Jeon, ed. Academic Press, New York, pp. 49–77.

Lee, J.J., McEnery, M.E., Kahn, E.G., and Schuster, R.L. 1979. Symbiosis and the evolution of larger foraminifera. *Micropaleontology* **25:** 118–140.

Lee, J.J., McEnery, M.E., Rottger, R., and Reimer, C.W. 1980a. The isolation, culture, and identification of endosymbiotic diatoms from *Heterostegina depressa* D'orbigny and *Amphistegina lessonii* D'orbigny (larger Foraminifera) from Hawaii. *Bot. Mar.* **23:** 297–302.

Lee, J.J., McEnery, M.E., Koestler, R.J., Lee, M.J., and Shilo, M., 1983. Experimental studies of symbiont persistence in *Amphistegina lessonii*, a diatom-bearing species of larger foraminifera from the Read Sea. In: *Endocytobiology II: Intracellular Space as Oligotrophic Ecosystem.* H.E.A. Schenk and W. Schwemmler, eds. Walter de Gruyter, Berlin & New York, **2:** 487–514.

Lee, J.J., Reimer, C.W., and McEnery, M.E. 1980b. The identification of diatoms isolated as endosymbionts from larger foraminifera from the Gulf of Eilat (Red Sea) and the description of 2 new species, *Fragilaria shiloi* sp. nov. and *Navicula reissii* sp. nov. *Bot. Mar.* **23:** 41–48.

Lee, J.J. and Reimer, C.W. 1984. Isolation and identification of endosymbiotic diatoms from larger foraminifera of the Great Barrier Reef, Australia, Madapur Tide Pool, Oahu, Hawaii, and the Gulf of Eilat, Israel with the description of 3 new species: *Amphora roettgeri*, *Navicula hanseniana* and *Navicula frustulum* var. *symbiotica*. In: *Proceedings of the 7th symposium of recent and fossil diatoms. Philadelphia, 1982.* D.G. Mann, ed. West Germany, Koeltz Science Books.

Leutenegger, S. 1977. Ultrastructure de foraminiferes perfores et imperfores ainsi que de leurs symbiotes. *Cahiers de Micropaleontologie* **3:** 1–52.

Leutenegger, S. 1983. Specific host-symbiont relationship in larger foraminifera. *Micropaleontology* **29:** 111–125.

Leutenegger, S. 1984. Symbiosis in benthic foraminifera: Specificity and host adaptations. *J. Foram Res.* **14:** 16–35.

McEnery, M.E. and Lee, J.J. 1981. Cytological and fine structural studies of 3 species of symbiont-bearing larger foraminifera from the Red Sea. *Micropaleontology* **27:** 71–83.

Pearse, A.G. 1968. *Histochemistry — Theoretical and Applied.* Vols. 1 and 2. Williams and Wilkins, Baltimore.

Reimer, C.W. and Lee, J.J. 1984. *Protekeelia hottingerii*, gen. nov., sp. nov., an unusual new endosymbiotic diatom with a primitive keel. *Proc. Acad. Nat. Sci. Philadelphia.*

Rodgers, A.W. 1967. *Techniques of Autoradiography.* Elsevier, Amsterdam.