

# Identification and distribution of endosymbiotic diatoms in larger Foraminifera

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**ABSTRACT:** The diatom endosymbionts have been isolated in culture from over 2,000 specimens of larger foraminifera (*Amphistegina lessonii*, *A. lobifera*, *Heterostegina depressa*, *Borelis schlumbergi*, *Operculina ammonoides*, *Calcarina calcar*) harvested from Red Sea, Indian Ocean, Hawaii, Palau, and the Great Barrier Reef stations. Twenty different species or varieties of diatoms were collectively isolated from the 6 host species in the total samples. *Nitzschia frustulum* var. *symbiotica* was the most commonly (33.7% of total hosts) isolated endosymbiont. Next most common were *Nitzschia panduriformis* var. *continua*, *Fragilaria shiloi*, *Nitzschia laevis*, and *Amphora roettgerii*, respectively, representing 14.5, 9.9, 8.7, and 6% of the isolations. More than half of the isolations were made in the vicinity of the H. Steinitz Marine Biology Laboratory on the Gulf of Elat, Red Sea so that the results have to be interpreted with that bias in mind. *N. frustulum* var. *symbiotica* was the most frequently isolated endosymbiont at all sites where more than 75 organisms were studied.

Significant numbers of *N. frustulum* var. *symbiotica*, *N. laevis* and *N. panduriformis* var. *continua* were isolated from hosts collected at every depth. *Fragilaria shiloi*, in contrast, was rarely isolated from hosts at depths greater than 25m. *Achnanthes maceneryae*, *Amphora* sp.(J) and *Protokeelia hottingeri* were only collected in hosts harvested at depths greater than 25m.

Although host species harbored more than one endosymbiont species some bias was observed. *Nitzschia laevis* and *F. shiloi* were the most common symbionts of *B. schlumbergi*. *A. maceneryae* and *N. laevis* were isolated in over 55% of all the *O. ammonoides* studied. Most larger foraminifera were the hosts for only a single species of symbiont (71%) at a time; a second symbiont species was found in the remainder.

Studies of the microflora on substrates on which larger foraminifera were feeding showed no correlation between potential diet and the endosymbiotic diatoms present in foraminifera harvested from the same substrates. Endosymbiotic diatoms were extremely rare (<0.5%) in the habitat where the foraminifera were feeding.

## INTRODUCTION

The diversity of algal types in larger foraminifera is now a very well-documented phenomenon (Reviews by Lee 1980, 1983; Leutenegger 1983, 1984). Since many diagnostic morphological features are reduced or absent in symbionts when they are in their hosts (e.g. Berthold 1978; Leutenegger 1977, 1983, 1984; Muller-Merz and Lee 1976; Schmaljohann and Röttger 1978) specific taxonomic identification can only be deduced or inferred from light or fine structural observations. Fortunately, many of the foraminiferal endosymbionts can be isolated from their hosts in monoalgal or axenic culture and there they do develop characteristic envelopes and other structures which permit specific identification (e.g. Lee et al. 1974, 1979a,b; 1980 a,b, and c; Reimer and Lee 1988).

Several of us (John J. Lee and Marie E. McEnery) were involved in the first isolation, cultivation, and identification of four endosymbiotic diatoms in larger foraminiferal hosts, *Heterostegina depressa*, D'Orbigny and *Amphistegina* spp. in the Gulf of Elat (Red Sea) (Lee et al. 1979b). Two of the diatoms were later identified and described as

new species (*Fragilaria shiloi* and *Navicula reissi*). Having made the identifications of "The" diatom endosymbionts we might not have attempted to repeat isolations and identifications had it not been for the TEM observations of Hansen and Buchardt (1977), who suggested that the algal symbionts they observed in *A. lessonii* collected in Elat, June 1971 appeared different from those collected in September 1976 at the same place. They also noted differences observed in *A. lobifera* collected in Elat and from Rhodes, Greece. Similarities and differences among algal symbionts were also noted in TEM preparations from *A. papillosa*, *A. bicirculata*, and *Heterostegina depressa*.

Considering the limited number of collections they made it is quite remarkable that they recognized four distinctive alga types in their TEM sections. Their observations are more remarkable when one considers that in all the other known cases of algal invertebrate animal symbioses there is a specific symbiont associated with each host species. When one of us (R. Röttger) was able to collect in Hawaii specimens of several of the same host species, we isolated

TABLE 1

Percentage of occurrence of the diatom species isolated as endosymbionts from collections at different depth ranges. The total number and total percent columns are for all isolations, whereas isolations from collections without a depth measurement were not included in the depth range percentages. The depth range isolations are combined for all locations, host species, and seasons of the collections. Each value is a percentage of the number of isolates for each column.

	Total No.	Total %	Depth (m)					
			<10	11-15	16-20	21-25	26-30	>30
<i>Fragilaria shiloi</i>	200	9.9	9.1	17.4	14.4	4.9	1.2	1.7
<i>Fragilaria</i> sp. (K)	15	0.7	0	0	1.0	0.4	0	0
<i>Achnanthes maceneryae</i>	79	3.9	0	0	1.0	0	11.6	10.3
<i>Achnanthes</i> sp. (L)	24	1.2	0.6	0.7	0.5	1.8	4.7	0.6
<i>Cocconeis andersonii</i>	28	1.4	0.3	2.1	2.1	1.8	0	3.0
<i>Amphora roettgerii</i>	121	6.0	3.6	0	11.3	5.8	5.2	8.2
<i>Amphora tenerrima</i>	29	1.4	0.3	0	0	0.4	0.6	0.2
<i>Amphora erezii</i>	56	2.8	1.1	7.6	7.2	0.9	0.6	4.0
<i>Amphora</i> sp. (J)	31	1.5	0	0.7	0	0	0	6.3
<i>Entomoneis paludosa</i> v. <i>densistriata</i>	9	0.4	0.6	1.4	2.6	0	0	0
<i>Navicula hanseniana</i>	71	3.5	4.7	10.4	1.5	0.4	12.2	1.7
<i>Navicula reissi</i> & <i>N. muscatinei</i>	40	2.0	0.6	0.7	10.8	0.4	0	2.3
<i>Navicula</i> sp. (W)	45	2.2	3.0	6.3	2.1	2.2	7.0	0.2
<i>Protokeelia hottingeri</i>	26	1.3	0	0	0	0	2.3	1.5
<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	679	33.7	47.7	27.1	16.5	50.2	30.8	32.7
<i>Nitzschia frustulum</i> variety	44	2.2	0.8	9.0	4.6	0.4	0	1.3
<i>Nitzschia laevis</i>	175	8.7	17.1	5.6	7.7	3.6	15.1	7.4
<i>Nitzschia panduriformis</i> v. <i>continua</i>	293	14.5	8.0	11.1	16.0	20.9	8.7	15.8
<i>Nitzschia valdestriata</i>	49	2.4	2.8	0	0.5	5.8	0	2.7
Number of Isolates	2014		363	144	194	225	172	474
Percentage of Total			18.0	7.1	9.6	11.2	8.5	23.5

their diatom endosymbionts. The new isolates were indeed different species of diatoms than those we had isolated from the same hosts in the Red Sea (Lee et al. 1980 b). This finding supported Hansen and Buchardt's (1977) observations and piqued our intellectual curiosity about symbiont specificity or selectivity in diatom-bearing larger foraminifera. Additional isolations of endosymbiotic diatoms from various hosts as they became available from Elat, Hawaii, and the Great Barrier Reef (e.g. Lee and Reimer 1983) led us to conclude that populations of larger foraminifera from a single collection at one station tend to harbor the same endosymbiotic diatom species but that specimens of the same host species collected at other stations, or even the same station, months or years later, may or may not harbor the same endosymbiotic diatom species (Lee and McEnery 1983). In general we found that individual animals are usually the host for one diatom species at a time (Lee and McEnery 1983). As additional collections and isolates were made some diatom species were repeatedly reisolated but additional species and genera were added to the small but growing list of endosymbionts (e.g. Lee and Reimer 1983).

New information raised more questions about the nature of the host and endosymbiotic diatom relationship. One was the relationship of symbiont distribution in hosts as a

function of depth or season. It wasn't even known whether populations at the same depth and season which were only moderately separated (0.01-1 km), would harbor the same, or different, symbionts. If populations of larger foraminifera at the same station change endosymbiotic species, how do they do it? Do abiotic physical conditions (e.g. temperature, light) favor the retention of some potential endosymbiotic diatom species over others? Are diatom endosymbionts recruited from the natural epibenthic or epiphytic populations upon which larger foraminifera feed? If so, what are their abundance relations among the natural population? These questions and many related ones prompted us to begin a more extensive program of collection and isolation.

## MATERIAL AND METHODS

### Collections

With one exception (Mombasa, Kenya), all specimens were harvested from the sea by SCUBA divers. The results of collections from Hawaii in April 1982 and earlier were published previously (Lee et al. 1979, 1981 a and b, 1983) but are included here for the sake of completeness. Collection sites were as follows:

TABLE 2

Percentage of occurrence of the diatom species isolated as endosymbionts from collections at different locations, regardless of the depth, host species, or season of the collection. Each value is a percentage of the number of isolates for each column.

	Location								
	WTB	ELT	RBK	EHB	GZFU	HAW	GBR	PAL	MBS
<i>Fragilaria shiloi</i>	8.5	3.6	0	0	55.6	9.9	28.6	0	24.5
<i>Fragilaria</i> sp. (K)	0.3	0.2	0	0	0	0	0	42.3	0
<i>Achananthes maceneryae</i>	1.3	13.0	0	0	0	0	0	0	
<i>Achnanthes</i> sp. (L)	1.0	1.6	0	0	0	2.7	0	0	0
<i>Cocconeis andersonii</i>	1.6	1.6	0	0	0	1.1	0	0	0
<i>Amphora roettgerii</i>	4.8	8.0	33.3	0	0	6.6	20.6	0	0
<i>Amphora tenerrima</i>	0.5	0.4	0	0	6.9	0	9.5	42.3	0
<i>Amphora erezii</i>	2.9	2.8	0	0	0	1.1	12.7	0	0
<i>Amphora</i> sp. (J)	2.8	0	0	0	0	0	0	0	0
<i>Entomoneis paludosa</i> v. <i>densistriata</i>	0.8	0	0	0	0	0	0	0	0
<i>Navicula hanseniana</i>	5.1	2.2	0	0	0	2.2	0	0	0
<i>Navicula reissi</i> & <i>N. muscatinei</i>	1.6	2.2	0	0	0	0	17.5	0	0
<i>Navicula</i> sp. (W)	3.6	1.0	0	0	0	0	0	0	1.9
<i>Protokeelia hottingeri</i>	1.4	2.2	0	100.0	0	0	0	0	0
<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	32.1	40.9	0	0	0	42.9	0	0	58.5
<i>Nitzschia frustulum</i> variety	2.9	1.2	0	0	6.9	0.5	0	0	0
<i>Nitzschia laevis</i>	12.0	3.8	33.3	0	0	9.3	6.3	0	1.9
<i>Nitzschia panduriformis</i> v. <i>continua</i>	15.5	15.2	0	0	30.6	8.2	4.8	0	13.2
<i>Nitzschia valdestriata</i>	1.3	0	33.3	0	0	15.4	0	15.4	0
Number of Isolates	1095	499	9	15	72	182	63	26	53
Percentage of Total	54.4	24	8	0.4	0.7	3.6	9.0	3.1	1.3

## LEGEND:

ELT = H. Steinitz Marine Biological Laboratory, Elat, Israel.  
 EHB = El Habik, Gulf of Elat, Egypt.  
 HAW = Makapuu Tide Pool, Oahu, Hawaii.  
 GBR = Heron-Wistori Channel, Great Barrier Reef, Australia.

WBT = Wadi Taba, Gulf of Elat, Israel.  
 RBK = Ras Burka, Gulf of Elat, Egypt.  
 GZFU = Coral Island, Gulf of Elat, Egypt.  
 MBS = Mambassa Harbour, Kenya  
 PAL = Palua.

## 1) Red Sea/ Gulf Of Elat:

- H. Steinetz Marine Biological Laboratory, Elat, Israel
- Wadi Taba, Israel
- Ras Burka, Egypt
- El Habik, Egypt
- Coral Island, Egypt

## 2) Hawaii:

- Makapuu Tide Pool
- Oahu, off Kaanapali, Maui

## 3) Heron-Wistori Channel, Great Barrier Reef, Australia

## 4) Indian Ocean, Kenya:

- Mombasa Harbor
- Seaward of the Silversands Hotel, in the backreef habitat

## 5) Palau

Samples were transported from the field to the laboratories in Elat or New York in large volumes of sea water, placed in insulated container (e.g. Aladdin insulated thermo jar cat. #7000).

Are symbionts recruited from natural diatom flora on the substrates upon which the foraminifera feed? To investigate this question we collected some very small samples, consisting of small pieces of coral rubble, 1x1 pieces of canvas or 5-10 *Halophila* leaflets, from field stations with abundant foraminifera (Lee et al. 1988b). Scissors and forceps were used to carefully harvest the samples and transfer them with minimal turbulence into sterile whorl pack bags. They were sealed in situ and carefully returned to the lab.

## Isolation procedures

The methods we used for the isolation, culture and subsequent identification of diatom endosymbionts were given in Lee et al. (1980 a,b, and c) with modifications in Lee and Reimer (1983). Very briefly: Individual animals are very carefully washed, brushed and scraped to remove all adhering diatoms on their surfaces. The washed foraminifera were aseptically placed in the well of 9-hole spot plates with 1 ml of sterile local sea water (35‰). The spot plates with foraminifera were incubated overnight at 25°C in sterile petri dishes. Protists with extended pseu-

TABLE 3

Percentage of occurrence of the diatom species isolated as endosymbionts from different host species, regardless of the location, depth, or season of the collection. Each value is a percentage of the number of isolates for each column.

	Host Species					
	<i>Amphis- tegina lessonii</i>	<i>Amphis- tegina lobifera</i>	<i>Heteros- tegina depressa</i>	<i>Borelis schlumbergi</i>	<i>Operculina ammonoides</i>	<i>Calcarina calcar</i>
<i>Fragilaria shiloi</i>	10.2	12.6	0	23.1	5.2	0
<i>Fragilaria</i> sp. (K)	0	1.6	0	0	0	0
<i>Achnanthes maceneryae</i>	2.0	0	17.8	3.1	18.2	0
<i>Achnanthes</i> sp. (L)	0	2.6	0	0	0	0
<i>Cocconeis andersonii</i>	2.0	1.3	0	0	3.9	0
<i>Amphora roettgerii</i>	5.4	5.0	12.1	1.5	6.5	0
<i>Amphora tenerrima</i>	0.8	2.0	2.1	0	0	0
<i>Amphora erezii</i>	1.4	2.6	6.4	7.7	0	0
<i>Amphora</i> sp. (J)	0	0	0	1.5	39.0	0
<i>Entomoneis paldosa</i> v. <i>densistriata</i>	0	1.0	0	0	0	0
<i>Navicula hanseniana</i>	3.7	4.8	0.7	1.5	0	0
<i>Navicula reissi</i> & <i>N. muscatinei</i>	4.8	0.4	0	7.7	0	0
<i>Navicula</i> sp. (W)	2.3	2.6	0	7.7	0	3.6
<i>Protokeelia hottingeri</i>	0.8	1.5	2.5	0	0	0
<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	34.9	39.0	20.3	10.8	7.8	92.9
<i>Nitzschia frustulum</i> variety	2.3	3.0	0.7	0	0	0
<i>Nitzschia laevis</i>	10.0	7.8	5.0	20.0	15.6	0
<i>Nitzschia panduriformis</i> v. <i>continua</i>	18.2	10.3	25.3	9.2	3.9	3.6
<i>Nitzschia valdestriata</i>	1.4	1.8	7.1	6.2	0	0
Number of Isolates	650	913	281	65	77	28
Percentage of Total	32.3	45.3	14.0	3.2	2.8	1.4

dopods were examined carefully in a dissection microscope and under a water immersion lens to make sure that they were free of external adhering algae. A few specimens in every batch were picked at random for process control and fixed in 4% glutaraldehyde or formalin in sea water. Later the surfaces of these organisms were examined in the SEM. These controls checked that external adhering algae were removed completely to prevent contamination of the isolations by nonsymbiont algae. The rest of the animals were individually transferred to clean sterile wells of 9-hole spot plates. Under a dissection microscope each animal was carefully cut and crushed with the aid of two pairs of fine pointed forceps (Dumont #5). The contents of each well was aseptically transferred by pipette to liquid isolation media (Lee et al. 1980b) and incubated at 25°C near daylight fluorescent bulbs in a temperature controlled growth room (Elat) or chamber (Sheerer Cel 4-4) (New York City) for a week to ten days.

In experiments prior to 1982 our isolates were streaked onto agar solidified media. After incubation 10 colonies were picked and their symbionts were isolated. Thus, we could discern the relative numbers of different types of colonies if there were indeed more than one algal type

present, each of which produced recognizably different colonies (see: Lee et al. 1975). The disadvantage was that if two algal species with similar colony types were present in the same host, one species might have been overlooked. The colony picking method was also more time consuming.

Therefore, for these experiments, after the isolates grew, the diatoms were gently centrifuged in their tubes, and the medium was withdrawn. A small sample was aseptically withdrawn and transferred to fresh medium to continue the line in case an interesting new symbiont might be isolated and additional material might be needed for taxonomic study. To the remainder in the tube 30% H<sub>2</sub>O<sub>2</sub> was added. The oxidation of organic matter was allowed to proceed, in each tube until the contents turned transparent. Gentle heating of the reaction mixture in a water bath was used when required. The tubes were then gently centrifuged and the H<sub>2</sub>O<sub>2</sub> was replaced by distilled water. An aliquot, of each sample was sprayed on a nucleopore filter for examination with an SEM. The remainder was dehydrated in a graded series of ethyl alcohol, transferred to toluene, and mounted in Hyrax (a high refractive index mounting



TABLE 4

Percentage of occurrence of the diatom species isolated as endosymbionts at different seasons. The first half includes all isolations regardless of the depth, location, or host species of the collection, whereas the second half combines the isolations from only Wadi Taba, and the H. Steinitz Marine Biological Laboratory, Elat, Gulf of Elat, Israel. Each value is a percentage of the number of isolates for each column.

	Season (All)				Season (WTB and ELT only)			
	Winter 12/21- 3/20	Spring 3/21- 6/20	Summer 6/21- 9/20	Autumn 9/21- 12/20	Winter 12/21- 3/20	Spring 3/21- 6/20	Summer 6/21- 9/20	Autumn 9/21- 12/20
<i>Fragilaria shiloi</i>	8.4	10.9	7.1	14.1	3.9	8.0	0	15.5
<i>Fragilaria</i> sp. (K)	0.1	0	1.1	2.4	0.1	0	1.5	0.2
<i>Achnanthes maceneryae</i>	2.7	3.5	1.1	8.0	3.2	14.0	1.5	8.8
<i>Achnanthes</i> sp. (L)	0.9	2.5	0.5	1.6	1.0	0	0.8	1.8
<i>Cocconeis andersonii</i>	1.5	2.0	2.2	0.6	1.8	4.0	3.1	0.7
<i>Amphora roettgerii</i>	3.9	8.9	8.2	9.0	2.9	11.8	11.5	9.9
<i>Amphora tenerrima</i>	1.2	0	0	3.1	0.3	0	0	0.9
<i>Amphora erezii</i>	2.3	1.0	7.1	3.1	1.9	0	9.9	3.4
<i>Amphora</i> sp. (J)	2.7	0	0	0	3.2	0	0	0
<i>Entomoneis paludosa</i> v. <i>densistriata</i>	0.8	0	0	0	0.9	0	0	0
<i>Navicula hanseniana</i>	3.9	2.0	8.2	1.6	4.5	0	11.5	1.8
<i>Navicula reissi</i> & <i>N. muscantinei</i>	1.1	0	8.2	2.4	0.2	0	11.5	2.7
<i>Navicula</i> sp. (W)	2.8	0.5	1.1	2.0	3.3	2.0	0.8	2.3
<i>Protokeelia hottingeri</i>	1.9	0	0	0.8	2.3	0	0	0.9
<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	41.4	40.6	23.4	16.9	47.1	8.0	9.2	18.7
<i>Nitzschia frustulum</i> variety	1.8	5.4	0	2.4	1.7	20.0	0	2.7
<i>Nitzschia laevis</i>	8.6	5.4	20.7	5.7	9.4	8.0	28.2	4.1
<i>Nitzschia panduriformis</i> v. <i>continua</i>	12.7	12.9	10.9	20.8	12.4	22.0	9.9	23.0
<i>Nitzschia valdestriata</i>	1.1	4.5	0.5	5.3	0	2.0	0.8	2.7
Number of Isolates	1138	202	184	490	969	50	131	444
Percentage of Total	56.5	10.0	9.1	24.3	48.1	2.5	6.5	22.0

medium from Custom Research & Development, Inc., Mt. Vernon Road, Auburn, California).

#### Diatom assemblage sample

The specimens in the small samples were treated in one of three ways when they were returned to the laboratory:

1. Some samples (e.g. a piece of coral rubble) were fixed very carefully in almost their entirety 4% glutaraldehyde (in seawater) after removal of diatom bearing foraminifera. (The symbionts were isolated from the latter). After fixation the samples were criticalpoint dried, mounted on stubs, and coated with Au or Pd in a Polaron sputter coater.

2. Some substrate samples were easily divided (e.g. *Halophila stipulacea* leaflets, canvas). In these, a small part of the sample (e.g. 2 leaflets) was carefully fixed in 4% glutaraldehyde, critical point dried, and mounted on stubs for SEM examination. The rest of the samples were vigorously brushed with clean 00 and 000 sable brushes. This manipulation was done in new disposable petri dishes. The samples were held and turned with the aid of clean forceps. The outcome thus obtained was pipetted

into new disposable, plastic centrifuge tubes and then centrifuged. The overlying seawater was withdrawn from each tube and replaced with H<sub>2</sub>O<sub>2</sub>. The samples were prepared for light or scanning electron microscopy as described above.

The light microscope objectives were Zeiss Planapochromatic (1.3 NA) mounted in a Zeiss Photomicroscope II. Phase and Nomarski interference contact were the most common modes of light microscopical observation. A Cambridge Stereoscan (Model 250) equipped with a heated LaB<sub>6</sub> emitter was used for SEM observations. It was operated at an accelerating voltage of 20 kV. A Polaron sputter coater was used to coat the specimens with Au or Pd. SEM photographs were taken on Polaroid type 55 P/N film.

#### Statistical analysis

The probability of encounter of a particular diatom species isolated as an endosymbiont is the number of isolates found of each diatom species to the total number of isolates. Hence percentage of occurrence of the diatom species were calculated to estimate this probability with respect to water depth, location and season of the collec-

TABLE 5

ANOVA F-values for the diatom species isolated as endosymbionts from collections at different depths, seasons, host species, and locations. Isolations from collections without a depth measurement were eliminated from the analyses of depth, whereas all isolations were included in the other analyses. \* = significant at  $P < 0.05$ .

	DEPTH	SEASON	HOST SPECIES	LOCATION
<i>Fragilaria shiloi</i>	3.692	1.863	1.777	7.391*
<i>Fragilaria</i> sp. (K)	0.785	3.038	0.061	7.021*
<i>Achnanthes maceneryae</i>	8.910*	2.120	5.007*	0.384
<i>Achnanthes</i> sp. (L)	0.042	0.638	0.330	0.055
<i>Cocconeis andersonii</i>	0.380	0.235	0.992	0.827
<i>Amphora roettgerii</i>	1.136	0.198	0.078	0.278
<i>Amphora tenerrima</i>	0.121	0.999	0.118	14.972*
<i>Amphora erezii</i>	0.343	1.779	0.097	0.008
<i>Amphora</i> sp. (J)	4.690*	1.683	20.384*	1.157
<i>Entomoneis paludosa</i> v. <i>densistriata</i>	1.871	1.184	0.079	0.815
<i>Navicula hanseniana</i>	0.354	0.020	1.554	1.246
<i>Navicula reissi</i> & <i>N. muscantinei</i>	0.093	3.704	2.225	0.652
<i>Navicula</i> sp. (W)	1.978	1.274	0.723	3.908
<i>Protokeelia hottingeri</i>	1.602	0.341	0.626	0.066
<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	1.293	2.866	3.749	0.128
<i>Nitzschia frustulum</i> variety	0.688	0.070	1.681	0.549
<i>Nitzschia laevis</i>	0.006	0.969	0.605	1.083
<i>Nitzschia panduriformis</i> v. <i>continua</i>	0.024	2.005	1.766	0.147
<i>Nitzschia valdestriata</i>	0.306	2.825	0.740	4.931*
Degrees of Freedom	1,113	1,135	1,135	1,135

tion of the foraminifera, as well as within each host species.

The data were fitted by means of least squares to regression analyses to test the effect of depth, location, season and host species to the number of encounters of a particular diatom species. Analysis of variance were used to see whether the estimated regression coefficients departed from zero by more than could be expected by chance. Also, the number of encounters of a particular diatom species was treated as a  $2^4$  factorial design (SAS Institute Inc., Cary, North Carolina) to test possible interactive effects. Host species, depth, season, and location were designated as factors with 6, 6, 4 and 9 levels, respectively.

## RESULTS AND DISCUSSION

Scanning electron microscopic examination of the surfaces of washed and brushed specimens showed that the methodology was quite effective in removing flora from the external surfaces of the foraminifera examined (e.g. Lee 1983, Fig. 5 and 6). An exception was *Calcarina calcar*. Since we collected very few of these on our brief stay in Mombasa we did not have "brushed controls" for this species.

During the course of our studies we isolated about 20 different species or varieties of diatoms from the 6 species of diatom-bearing larger foraminifera. Of these, *Nitzschia frustulum* var. *symbiotica* was the most commonly isolated species (Plate 1, Fig. 2, Table 1). It was isolated in 33.7% of the total 2014 isolations. Next most common were

*Nitzschia panduriformis* var. *continua*, *Fragilaria shiloi*, *Nitzschia laevis*, and *Amphora roettgerii* respectively representing 14.5, 9.9, 8.7, and 6% of the isolations (Plate 1, Fig. 3, 16 Plate 2, Fig. 10, Table 1). Generally these same species were also the most commonly isolated at all the stations (Table 2). Most of the factorial analyses performed on the distributions of the endosymbiont diatoms were not significant ( $P \leq 0.05$ ). However, there were third order interactions between *Fragilaria shiloi*, *Amphora tenerrima*, *A. erezii*, *Amphora* sp. (J), *Entomoneis paludosa* var. *densistriata*, *Nitzschia laevis*, *N. panduriformis* var. *continua* and *N. valdestriata*, and host species, depth, season and location ( $F = 21.42, 39.65, 1.62, 2.37, 8.82, 1.80, 1.98$  and  $2.34$ , respectively, with  $df = 76, 60, P \leq 0.05$ ). All other significant interactions are discussed below.

Since more than half (1095) of the isolations were made from collections at Wadi Taba, total numbers are biased toward the results of that station. The results from the next most frequently sampled site (499 isolations), seaward of the H. Steinitz Marine Biology Laboratory in Elat, were quite similar to those obtained at Taba, 6 km further south on the Gulf of Elat. The major differences were that *Fragilaria shiloi* was less frequently isolated (3.6%) and *Achnanthes maceneryae* (Plate 2, Fig. 11) was quite abundant (13%) as an endosymbiont. This in turn, can be explained by the distribution of foraminifera at the two sites. At the H. Steinitz Marine Biology Laboratory we tended to collect *Heterostegina depressa* and *A. lessonii* at depths greater than 30m. The distributions of *A. maceneryae* and *Amphora* sp. (J) were significantly corre-

TABLE 6

The effect of starvation on the isolability of symbionts from host populations. The values represent the number of isolates of each symbiont. When two values are present, the first value denotes a single diatom species isolated, whereas the + indicates symbionts isolated with another species (i.e., two endosymbionts within one individual host).

Host	Symbionts Isolated	Directly After Capture	Starved 2 Days	Starved 12 Days
<i>Amphistegina lobifera</i> Population A	<i>Fragilaria shiloi</i>	0	1,0	0
	<i>Cocconeis andersonii</i>	0,3+	0,1+	0
	<i>Amphora erezii</i>	0,3+	0	0
	<i>Navicula</i> sp. (W)	8	2,2+	4
	<i>Nitzschia panduriformis</i> v. <i>continua</i>	0	0	0,2+
<i>A. lobifera</i> Population B	<i>Fragilaria shiloi</i>	1	4	0
	<i>Amphora erezii</i>	0,1+	0	0
	<i>Navicula</i> sp. (W)	0	1	0
	<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	5,2+	4	4,4+
	<i>Nitzschia laevis</i>	1	6	2
	<i>Nitzschia panduriformis</i> v. <i>continua</i>	0	0	1,1+
	<i>Rhopaloid</i>	1	0	0,2+
<i>A. lessonii</i> Population B	<i>Navicula</i> sp. (W)	0	1	2
	<i>Nitzschia frustulum</i> v. <i>symbiotica</i>	5,3+	6,3+	8,4+
	<i>Nitzschia laevis</i>	0,3+	1,3+	0,4+

lated with two deeper dwelling hosts, *H. depressa* and *Operculina ammonoides* (Table 3, and 5). The factorial analysis on *Amphora* sp.(J.) yielded two significant first order interactions between host species and depth ( $df = 12$ ,  $F = 2.37$ ,  $P \leq 0.05$ ) and between depth and location ( $df = 3$ ,  $F = 4.74$ ,  $P \leq 0.01$ ).

With the exception of the collection made at Coral Island, *N. frustulum* var. *symbiotica* was the most frequently isolated endosymbiont at all sites where more than 75 organisms were studied (Table 2). An almost spherical variety of *N. frustulum* was recovered from the Coral Island Collection. Some of the species less frequently isolated at Taba and the H. Steinitz Marine Biology Laboratory Stations were quite abundant at other stations (e.g.: *Amphora roettgerii* at Ras Burka, and Heron-Wistari Channel, G.B.R. and Palau; *Amphora erezii* in hosts from Coral Island, Heron-Wistari Channel, G.B.R. and Palau; *Navicula muscatinei* (Plate 2, Fig 8) in hosts from the Heron-Wistari Channel, G.B.R.; *Protokeelia hottingeri* (Plate 2, Fig. 12) in hosts from El Habik; and *Nitzschia valdestriata* (Plate 1, Fig. 4) in hosts from Ras Burka, Hawaii, and Palau (Table 2)). Some of these distributions with respect to location were statistically significant (Table 5).

In particular, the distributions of *Fragilaria shiloi*, *Nitzschia panduriformis* *N. valdestriata* and *Amphora*

*tenerrima* were correlated with location and host species ( $F = 16.56$ ,  $3.72$ ,  $2.56$ , and  $98.69$ , respectively with  $df = 5$ ,  $P \leq 0.05$ ). Also, the distribution of *F. shiloi* was correlated to location and depth ( $F = 13.61$ ,  $df = 3$ ,  $P \leq 0.001$ ), and the distribution of *N. panduriformis* correlated to location and season ( $F = 8.33$ ,  $df = 1$ ,  $P \leq 0.001$ ).

Significant numbers of *Nitzschia frustulum* var. *symbiotica*, *N. laevis* and *N. panduriformis* var. *continua* were isolated from hosts collected at every depth (Table 2) whereas *Fragilaria shiloi* was only rarely isolated from hosts harvested at depths greater than 25m (Table 1). As mentioned above, *Achnanthes maceneryae* and *Amphora* sp.(J) were only collected in hosts harvested at depths greater than 25m. The same was true for *P. hottingeri* (Table 1).

Without regard to the location from which the hosts were harvested, *Nitzschia frustulum* var. *symbiotica* was the most frequently isolated symbiont in half the hosts studied, *A. lessonii*, *A. lobifera*, and *C. calcar*, and the second or third most frequently isolated symbiont in two other hosts, *H. depressa* and *B. schlumbergeri* (Table 3). *Nitzschia panduriformis* var. *continua* was the most frequent endosymbiont isolated from *H. depressa* and second most frequent species isolated from *A. lessonii* and *Calcarina calcar*. It was also frequently isolated from *A. lobifera* (Table 3). *Nitzschia laevis* and *F. shiloi* were the

most common symbionts of *B. schlumbergeri*. *Achnanthes maceneryae* was the most common species isolated from *O. ammonoides* (Table 3). *Amphora* sp. (J), *A. maceneryae* and *N. laevis* were isolated in over 72% of all the *O. ammonoides* studied.

Although we usually isolated a single species of symbionts from each host (71% of the specimens), we were able to isolate a second species from approximately a quarter (27%) of the hosts. The second method of whole organism liquid culture we used for most of the isolations emphasized this aspect of the symbiosis but did not give us the quantitative results that we were able to get from our first methods of cloning the isolated symbionts on agar. The frequency of individual species in isolations in which there were two, or rarely, three endosymbiont species was not perceptively different from the frequency of the same species in single symbiont isolations. There was no statistically meaningful difference between the frequency of isolation of each of the species in the entire sample.

Since more than half (57%) of the samples were collected in the winter, our isolations are quite biased with respect to season. Taking the data as a whole, no seasonal trends in the distribution of particular symbionts were found (Table 4 and 5). This is more evident when we compare only the samples from the Red Sea (Gulf of Elat) the most frequent sampled sites. Those species most frequently isolated, were recovered in roughly the same order of abundance through out the year (Table 4). Nevertheless, there were significant second interactions between *Fragilaria shiloi*, *Fragilaria* sp.(K) *Amphora roettgerii*, and *A. erezii*, and host species, depth and season ( $F = 8.59, 12.13, 3.47$  and  $3.66$ , respectively with  $df = 3, P \leq 0.05$ ). The distribution of both *Amphora roettgerii* and *A. erezii* was also significantly correlated with populations of *Amphistegina lobifera* at particular depths (seasons ( $F = 24.24$  and  $2.98$ , respectively, with  $df = 17, P \leq 0.01$ ). We did not find similar correlations with either *Fragilaria* species ( $F = 1.29$  and  $1.01$ ). The distribution of *F. shiloi* and *A. erezii*, however, were significantly correlated with hosts (*Amphistegina* spp.) at relatively shallow depths in the autumn ( $F = 3.51$  and  $14.77$ , respectively, with  $df = 12, P \leq 0.01$ ). Looking at

the same data as a correlation between *Amphistegina* and season the distribution of *F. shiloi* with *Amphistegina* spp. at a depth of 11-15 meters was highly significant ( $F = 242.93, df = 5, P \leq 0.001$ ). At this depth, 92% of the *F. shiloi* were isolated from *Amphistegina* spp. in the autumn. Two thirds of *A. erezii* isolated in the autumn came from the same host species and depth.

There was a first order interaction between the distribution of the rarely isolated symbiont *Entomoneis paludosa* v. *densistriata*, and host species and season ( $F = 33.81, df = 7, P \leq 0.001$ ), as well as with depth and season ( $F = 40.31, df = 9, P \leq 0.01$ ). The distribution of *N. panduriformis* was similarly correlated ( $F = 2.13, df = 9, P \leq 0.05$ ). *Entomoneis paludosa* v. *densistriata* was isolated only from *Amphistegina lobifera* at depths less than 20 meters in the winter whereas *N. panduriformis* was isolated from all host species, at all depths and seasons (Tables 1,3,4). However, of all the isolates of *N. panduriformis* 36% were from shallow depths (<20 meters) and 75% were collected in the autumn and winter.

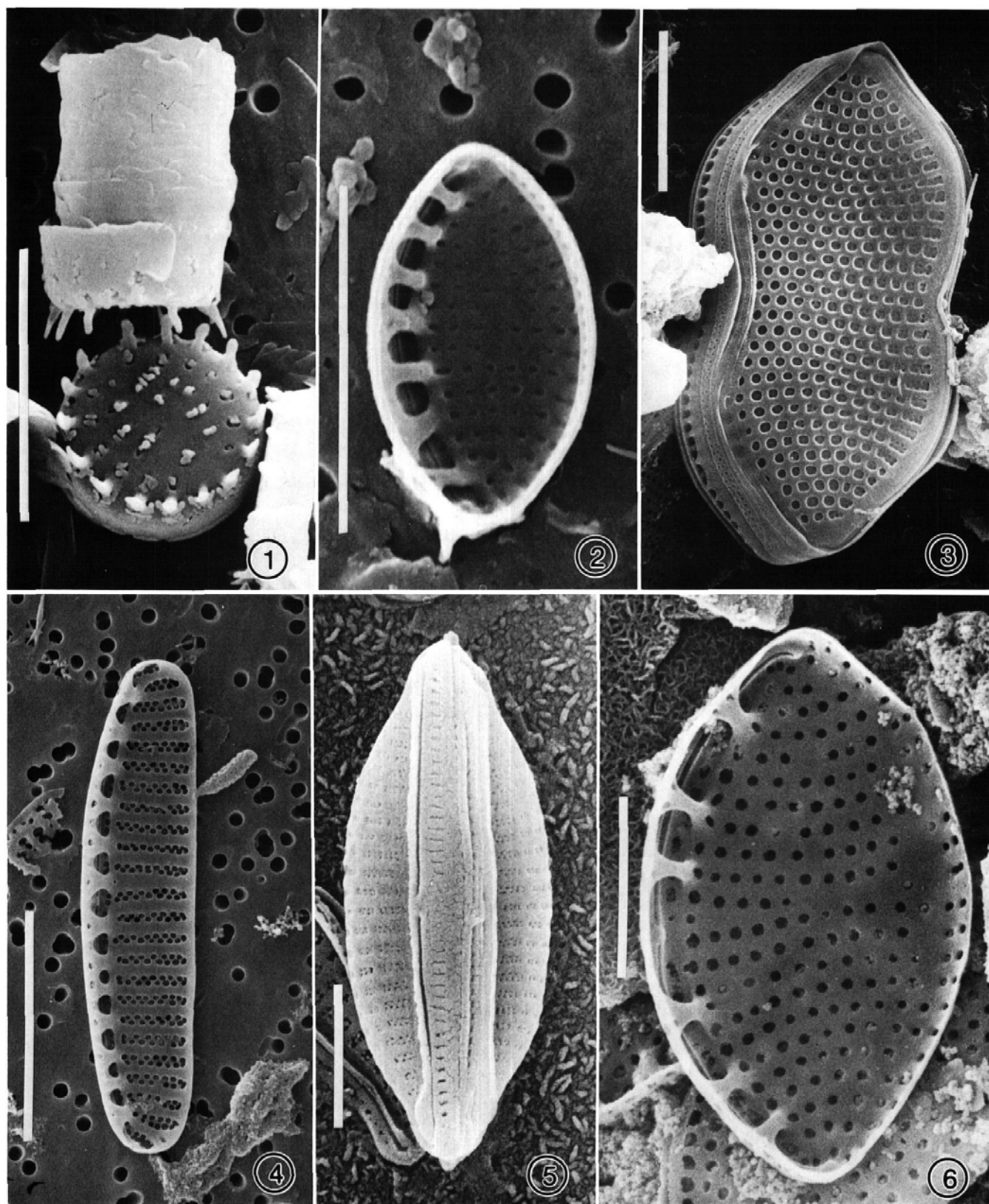
The correlation of these distributions concur with the results of Lee et al. (1982) who studied the photoadaptation of four isolated endosymbiotic diatoms. They found *F. shiloi* and *N. laevis* to be adapted to high light intensity levels whereas *N. panduriformis* and *N. valdestriata* were photoinhibited at high levels. We note, however, that the isolate of *N. panduriformis* was from a shaded tide pool and not from the habitat where most of our samples were collected. All four diatoms were found as endosymbionts in hosts collected over the entire depth range studied, with one third of all isolates of *N. panduriformis* and *N. valdestriata*, and over 80% and 55% of the isolate of *F. shiloi* and *N. laevis*, respectively, collected at depths of less than 20 meters (Table 1). This anomaly may suggest a high degree of light attenuation in the foraminiferal test of those hosts found at shallower depths (Lee, et al. 1982). The Lee et al. (1982) study involved only one clone of each isolated endosymbiotic diatom and the life history of the foraminiferal host as well as the endosymbiont may affect these results, in that clones isolated from hosts at different depths may exhibit varying photophysologies.

## PLATE 1

The most common endosymbiont species. All figures are scanning electron micrographs. Scale bar for figures 1-4 equals 4 $\mu$ , scale bar for figures 5 and 6 equals 2 $\mu$ .

- 1 *Fragilaria shiloi*
- 2 *Nitzschia frustulum* var. *symbiotica*
- 3 *Nitzschia panduriformis*
- 4 *Nitzschia valdestriata*
- 5 *Amphora tenerrima*
- 6 *Nitzschia laevis*







At the onset of the study we recognized the possibility that some of the diversity found in our isolations from specimens of larger foraminifera might be due to the isolation of living diatoms which might be surviving in food vacuoles. We designed a small experiment to test this hypothesis. We care fully collected two small, possibly clonal populations of *Amphistegina* living on clusters of 15 *Halophila* leaflets, one at 15m and the other at 25m. The populations of foraminifera were divided into three sets. One third of each population was brushed and washed for isolation of their symbionts directly after harvest from the sea. The symbionts from another third of population were isolated two days after capture. The remaining foraminifera were starved twelve days before their symbionts were isolated. We found no meaningful differences between isolations made immediately after capture or after starvation for twelve days (Table 6).

With the aid of a SEM we completed the study of 46 separate *Halophila stipulacea* leaflets, six small pieces of granite rubble, three 1 x 1 cm pieces of canvas and 55 H<sub>2</sub>O<sub>2</sub> cleaned diatom preparations from Wadi Taba or seaward of H. Steinitz Marine Biology Laboratory. We also mounted the cleaned diatom preparations in Hyrax and studied them by phase microscopy. Several typical foraminifera habitats are illustrated (e.g. Lee, 1983, Fig. 18 and 19). All of the samples were associated with collections of large numbers of diatom bearing larger foraminifera. At least 2,000-5,000 diatoms were examined in each sample; approximately  $3.25 \times 10^5$  diatoms in all. Species of diatoms morphologically identical with symbiont species were extremely rare in natural communities (Lee et al. 1988b). The most abundant of the free species was *Nitzschia panduriformis* var. *communis*; 46 specimens were found in the search. *Nitzschia frustulum* var. *symbiotica* was the next most common (33 specimens). Seventeen *Fragilaria shiloi*, 15 *Navicula reissi*, 15 *Amphora roettgerii*, 11 *Amphora erezii*, and 10 *Nitzschia laevis* were also found. None of the other species of symbionts isolated were found as free-living in the sample we observed. We can only conclude from our survey that species of diatoms we isolated as endosymbionts are extremely rare in the natural communities we studied. There is no lingering doubt that the diversity we find in endosymbiotic diatoms

is a reflection of species abundance in the natural community.

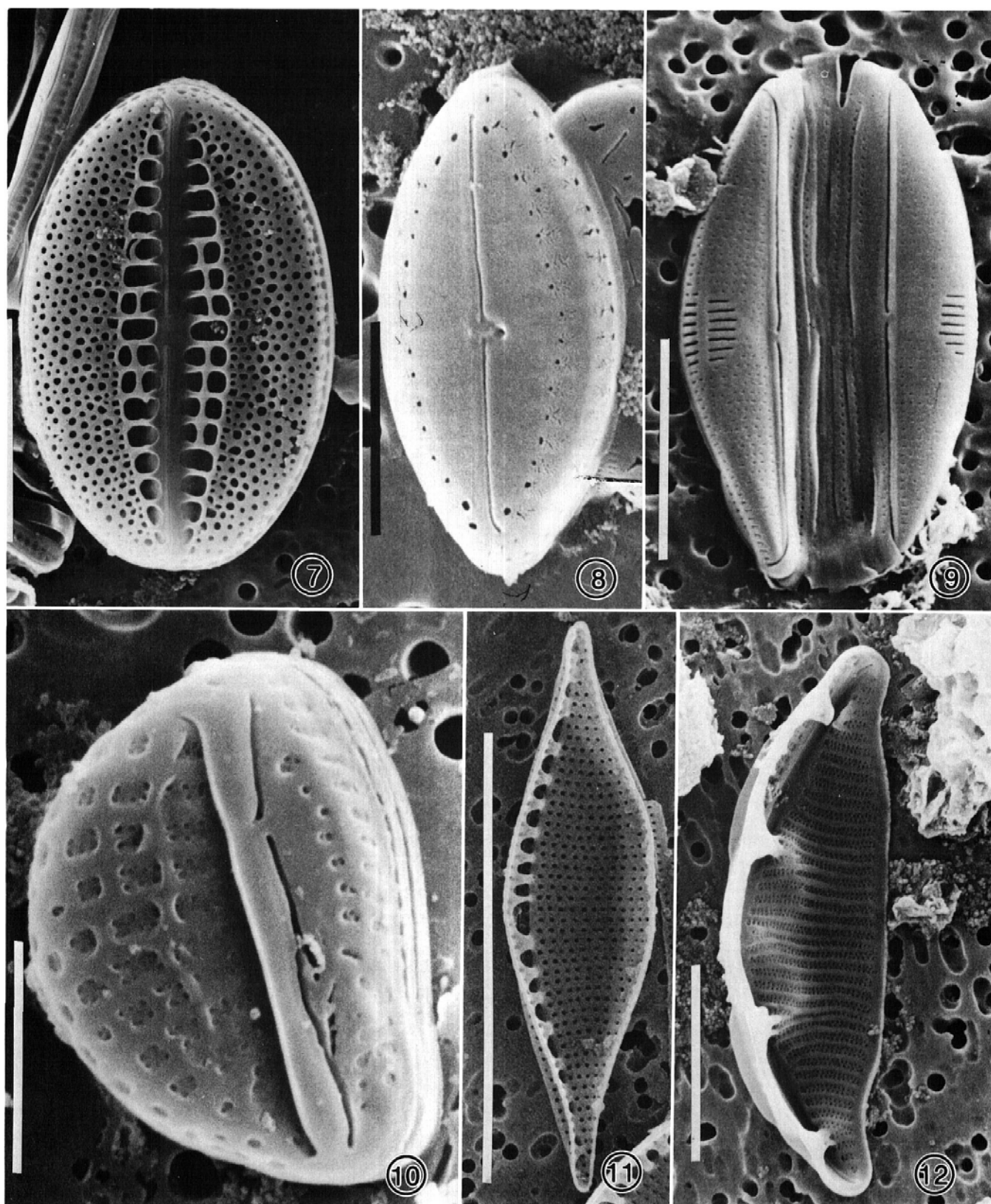
The result of this study seems consonant with the earlier studies of Hansen and Buchardt (1977), Lee et al., (1988a), and Leutenegger (1983, 1984). Even though the latter concluded that particular symbiont species were found in the various hosts she examined, she noted pyrenoid diversity among the symbionts in different hosts. She (Leutenegger, 1984) recognized three different pyrenoid types among the symbionts in situ (simple, compound, and invaginated). Two different pyrenoid types were represented in the three cultures of both *Nitzschia panduriformis* and *Fragilaria shiloi*. We must recognize that the techniques we used, and she used, have their limitations. Leutenegger based her conclusions on fine structural differences of the pyrenoids of naked endosymbiotic diatoms within their host. As noted and illustrated by Leutenegger (1983), there are no cellular characteristics of diatoms (e.g.: chloroplast number, chloroplast ultrastructure, pyrenoid ultrastructure) which are usefully applied at lower taxonomic levels. Their taxonomy is based on frustule structure, a feature not formed when the diatoms are found within their hosts. Fortunately the diatoms do form frustules when they are released from their hosts and inoculated into suitable culture media (Lee et al. 1979, 1980a, b, and c, 1983). TEM has the advantage that the algal cells being examined were living within the host species at the locations where the symbionts are found (Leutenegger, 1984). It has the disadvantage of long preparation and examination time. We note by comparison with the present paper, Leutenegger (1984) was able to study in the TEM only 11, 5, 3, 1, 9, and 6, specimens respectively of *Amphistegina lobifera*, *A. lessonii*, *Borelis schlumbergeri*, *Calcarina calcar*, *Heterostegina depressa*, and *Operculina ammonoides*.

The advantage of our isolation approach is that the cultured diatoms form frustules for definitive identification. We recognize, however, that there will always be some questions as to whether all the organisms isolated from within a diatom-bearing larger foraminifera are symbionts from characteristic locations or surviving ingested food. We can point to our own work (Koestler et al. 1985) to show that some diatoms (only endosymbiotic diatom

## PLATE 2

Rarer species of endosymbiotic diatoms. All figures are scanning electron micrographs. Scale bar for figures 7, 9, 12 equals 4µ. Scale bar for figures 8 and 10 equals 2µ. Scale bar for figure 11 equals 10µ.

- 7 *Navicula reissi*
- 8 *Navicula muscatinei*
- 9 *Amphora* sp. (J)
- 10 *Amphora roettgerii*
- 11 *Nitzschia* sp.
- 12 *Protokeelia hottingeri*



species were tested) do escape host digestive activity. This fact and the evidence obtained in this study on the frequency of species isolated as endosymbionts compared to their abundance in natural freeliving diatom assemblages from the same habitat, leave us to conclude that the probability that we are isolating the actual symbionts is much greater (by several orders of magnitude) than that we are isolating retained living food or surface contaminants.

Diversity of symbiont types in the same host species was also found by an immunochemical approach (Lee et al., in press). Since the multivalent antisera first had to be absorbed by reaction with a different symbiont species before they were specific, it is possible that symbiont species share common surface antigens. Certainly further work along these lines should be very fruitful.

We note in conclusion that although we have resolved questions about symbiont diversity: 1) in the same host species; 2) as a consequence of the communities in which the foraminifera are feeding; 3) as a function of depth, season and location, we actually know very little about the specificities of the host diatoms endosymbiont relationship.

The diversity of endosymbiont species in the same host is not endless. When we compare the score of symbiont species isolated against more than 400 diatom species found in the natural community in which foraminiferal hosts feed we see quite clearly that the hosts/symbiont relationship in strongly restrictive, even if not specific, in the hosts we examined in this study. The fact that various species of larger foraminifera form symbiotic relationship with so many different algal types (diatoms, chlorophytes, dinoflagellates rhodophytes,) and that the diatom-bearing species do not form uniquely specific relationships with their endosymbionts, points out the need to study digestive processes and foreign antigen recognition at the molecular level in this group of protists.

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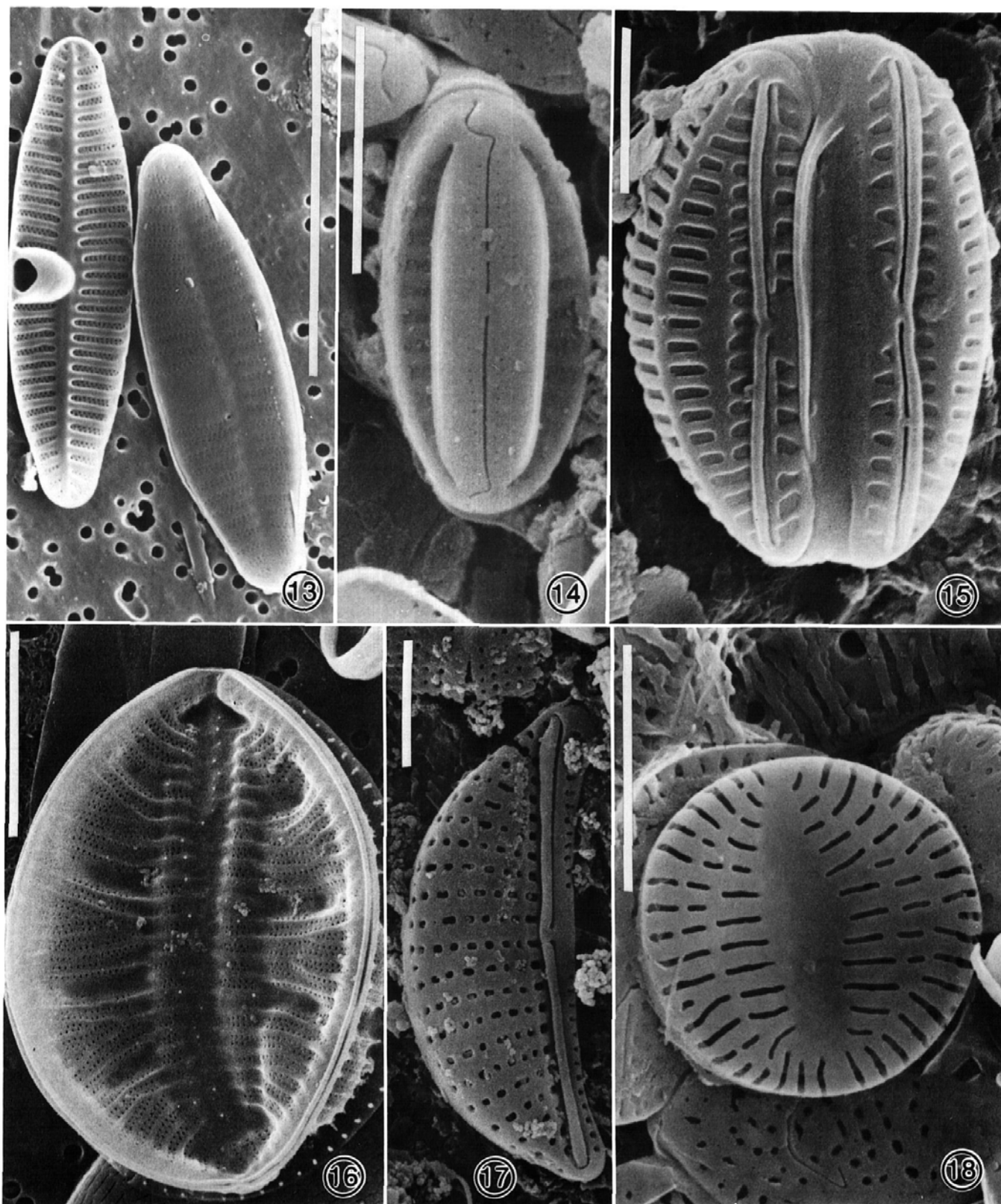
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#### PLATE 3

Rarer species of endosymbiotic diatoms. All figures are scanning electron micrographs. Scale bar for figure 13 equals 10µ, scale bar for figure 16 equals 4µ, scale bars for figures 14, 15, 17, 18 equal 2µ.

- 13 *Achnanthes maceneryae*
- 14 *Navicula* sp. (W)
- 15 *Amphora erezii*
- 16 Unidentified Rhopaloid
- 17 *Amphora* sp.
- 18 *Cocconeis andersonii*





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