

A Laboratory-Induced Association Between the Marine Amoeba *Trichosphaerium* AM-I-7 and The Dinoflagellate *Symbiodinium* #8

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Abstract

A laboratory-induced association between the marine amoeba *Trichosphaerium* and the dinoflagellate *Symbiodinium*, originally isolated from the anemone *Aiptasia pulchella* was examined. When cultured with algae, individual amoebae incorporated approximately 260 algae within 3 weeks partitioning them intracellularly within vacuoles. Once established, the association survived for over 2 years in culture. When dinoflagellates were abundant in the medium, the apparent turnover time of the algal population was close to 30 hr, but when algae were excluded from the medium amoebae retained a stable complement of intracellular algae. Typically, about 97.8% of the dinoflagellates phagocytosed by amoebae in the light remained viable for at least one week; while in the dark only 82.2% of the algae were viable. The transfer of photosynthetic products from alga to amoeba was 9.8% of total ¹⁴C-labelled photosynthate. This may account for the slightly shorter generation times of amoebae with algae grown in the light (49.8 hr) versus those in the dark (54.6 hr). The potential for using this novel laboratory system to investigate algal/invertebrate associations is discussed.

Keywords: association, dinoflagellate, marine amoeba, *Symbiodinium*, symbiosis, *Trichosphaerium*

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1. Introduction

Trichosphaerium is a common genus of marine amoeba comprising three named species (Page, 1983). It is an unusual amoebae in that it resembles a naked form under the light microscope but in high osmoticum medium and at the EM level, a thin flexible outer test can be seen either with or without calcareous spicules. The test has many pores through which cytoplasmic protrusions, or dactylopodia, project and it is these distinctive pseudopodia which facilitate the identification of the genus *Trichosphaerium*.

Trichosphaerium has been frequently isolated by the authors and others (Angell, 1975, 1976; Page, 1983) from neritic regions throughout the world but these isolates have never been reported to harbor symbiotic algae.

Among the marine Sarcodina, those protists bearing pseudopodia of one form or another, only the foraminiferans (Lee, 1983; Lee and McEnery, 1983), radiolarians (Anderson, 1983) and acantharians (Febvre and Febvre-Chevalier, 1979) have been shown to sequester algal symbionts. In the case of foraminiferans, the retention of symbionts is a very common phenomenon and they incorporate a broad range of algal types including dinoflagellates, chlorophyceans, diatoms and a unicellular rhodophyte (reviewed in Smith and Douglas, 1988). Moreover, several marine protists, notably oligotrich ciliates and foraminiferans, have been shown to sequester the chloroplasts of the algae upon which they feed (Laval-Peuto and Febvre, 1986; Lee and Lanners, 1988).

While the aforementioned symbiotic associations are relatively common in the marine plankton, they do not lend themselves to experimentation as few of the protozoan hosts can be successfully cultivated in the laboratory. *Trichosphaerium*, on the other hand, grows with ease in the laboratory on a bacterial diet or on a variety of micro- and macro- algae (Polne-Fuller, 1987). During experiments testing for suitable food sources it was found that this amoeba readily sequestered, rather than digested cells of the symbiotic dinoflagellate *Symbiodinium* sp. #8.

It is generally accepted that there are complex recognition processes involved in the establishment of associations between microalgae and invertebrates (Trench, 1988). However, little is known about the mechanisms controlling this phenomenon. *Trichosphaerium*, in association with its dinoflagellate symbionts, may provide a unique model for future studies on protozoan/algal symbiosis and in a broader context a model for studying cell-cell recognition. Towards this end, we present a detailed description of this novel laboratory-induced association.

2. Materials and Methods

Culture

Trichosphaerium AM-I-7 was isolated from the brown alga *Sargassum muticum*, collected at Alegria Beach, Santa Barbara County, CA. Full details on culture and isolation procedures for *Trichosphaerium* are given elsewhere (Polne-Fuller, 1987). Stock cultures of axenic amoebae were maintained on ground and autoclaved algae in PES enriched seawater (Provasoli, 1968). Amoebae were also cultured on bacteria in Cerophyl/seawater (C7 medium (Page, 1983). The dinoflagellate *Symbiodinium* sp.#8 was originally isolated from the sea anemone *Aiptasia pulchella*. It was maintained in axenic culture in ASPSA (Ahles, 1967).

Establishment of association

Three methods were used to establish the amoebal/algal laboratory association. The first method mixed axenically reared *Symbiodinium* and *Trichosphaerium* in PES medium. The two other methods had bacterial prey present promoting amoebal replication. One of the bacterized procedures used organically rich C75S medium which encouraged luxuriant bacterial growth. The other method used PES artificial medium which, because of its low organic content supported only a background bacterial population. In all cases the media supported dinoflagellate growth in the light. When *Trichosphaerium* were added, they ingested, partitioned and sequestered algae. The fastest uptake rates of the algae by the amoebae occurred at the bacteria-free and low bacterial cultures. In these culture systems, and in all subsequent experiments (unless noted) the concentration of dinoflagellates free on the bottom of the culture vessel was approximately $750 \text{ cells mm}^{-2}$.

Uptake rate of dinoflagellates by amoebae

For reasons of convenience the incorporation of algae by amoebae was examined in bacterized cultures using PES and C75S media. The number of algal cells incorporated over time was determined by counting the number of algae in 25 randomly selected amoebae. In the early stages, when amoebae harbored few algae, cells were enumerated directly on an inverted microscope. Later, when amoebae contained many symbionts, cells were compressed under coverslips and algae counted at higher magnification.

Determination of growth rate

Individual amoebae were micropipetted into 0.5 ml aliquots of media in cavity slides. To prevent evaporation and contamination, a surface layer of sterile paraffin oil was added. Conditions in each experiment varied. Some amoebae were full of symbiotic algae while others were taken from cultures fed on bacteria only. Two different media were used for comparison (C75S and PES) and experiments were conducted with or without algae in the light and in the dark. For each experiment, 15 replicates were set up and the number of amoebae counted at least twice daily. Semilog regressions of cell count against time (hr) were computed for the exponential phase of growth.

Fate of ingested algae

The nature of the vacuoles with dinoflagellates, (whether they were digestive or perialgal) was examined by neutral red staining and by lectin binding. Neutral red stains food vacuoles of amoebae from red to yellow depending upon the pH of the vacuoles (Finlay et al., 1988). Free dinoflagellates in the media were examined also for stain accumulation. This gave information about the proportion of viable and non-viable algae in the media. Dead cells with completely or partially degraded cell walls stained dark red. At least 1000 algal cells, sequestered or free, were counted on each experimental occasion.

The lectins *Ricinus communis* agglutinin (RCA¹²⁰) and *Triticum vulgaris* agglutinin (WGA) bind to the digestive vacuole membrane and digestive vacuole contents of *Trichosphaerium* respectively (unpublished). Lectins conjugated with FITC, were purchased from Vector Laboratories (Burlingame, CA). For staining, amoebae were fixed in 8% formaldehyde, washed in PES medium and treated for 30 min with 40 µg of lectin in 1 ml of medium. After three further washes, cells were examined in a Zeiss Photomicroscope III with epifluorescence.

Algal divisions within Trichosphaerium and estimated retention time

Individual *Trichosphaerium* packed with algae were compressed under a cover glass and examined at 1000× magnification. The number of dividing and non-dividing algae in 20 amoebae were counted directly as were an equivalent number of algae free in the medium. Thin sections were examined by TEM for evidence of dividing intracellular dinoflagellates. Procedures for specimen preparation are given below.

To estimate the rate of flux of algae through amoebae, several complementary methods were used. Individual amoebae in cultures containing free dinoflagellates were examined in an inverted microscope with low illumination for 30 min intervals ($n=20$). Over this period, the number of algae ingested and egested by *Trichosphaerium* was counted. Amoebae were also observed for longer time intervals using time-lapse photography. These experiments lasted 12–48 hr and photographs were recorded every 2 min. No external free dinoflagellates were present in these experiments. Because of visual limitation due to the orientation of large numbers of algae inside an amoeba, only amoebae with 10 or less sequestered algae were examined; higher numbers of intracellular algae were impossible to monitor automatically. The final method to investigate flux rate used dinoflagellates labelled with the electron dense marker, cationized ferritin (Sigma Chemical Co., St. Louis, MO). This label readily bound to the cell surface of *Symbiodinium* when used at a concentration of $50 \mu\text{g ml}^{-1}$. After 24 hr of exposure to labelled algae, amoebae were fixed, thin sectioned and examined by TEM. The percentage of intracellularly labelled algae (i.e. those ingested within 24 hr) to non-labelled algae was calculated.

Electron microscopy

Amoebae were fixed simultaneously in 2% glutaraldehyde and 1% osmium tetroxide in a 1:1 mixture of C75S medium and 0.1 M sodium cacodylate buffer (pH 7.2), at 4°C for 30 min. After 4 washes in cold distilled water, cells were dehydrated through an acetone series and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 TEM. Sections containing ferritin labelled cells were left unstained.

Transfer of photosynthetic product from algae to amoebae

Amoebae packed with algae were incubated for 1 hr in the light in 5 ml C75S medium containing ^{14}C sodium bicarbonate (total activity, $5 \mu\text{C}$). The number of amoebae was determined in a parallel sample by subsampling and counting the cells in $10 \mu\text{l}$ drops ($n=10$). After the 1 hr incubation, amoebae were washed 8 times in PES medium. One sample was concentrated by centrifugation, added to 5 ml of Hydrofluor (National Diagnostics, Somerville, NJ) and counted by liquid scintillation spectrometry. This gave a measure of the total incorporation of label by dinoflagellates. A second sample was washed in PES and placed in the dark for 18 hr. When *Trichosphaerium*

is washed in inorganic seawater medium (PES), the cells undergo multiple fission typically dividing into a large number of small (20 μm) cells. During the process of fission cells eject all their vacuolar content thereby providing a convenient method for separating the algal and amoebal components. After 18 hr, released algae were decanted leaving the amoebae adhering to the surface of the culture vessel. Both samples (amoebae and algae) were collected separately, rinsed in fresh media, pelleted by centrifugation and counted by scintillation counter. Controls used amoebae incubated with label in the dark and free dinoflagellates in labelled medium in the light. All translocation experiments were repeated three times.

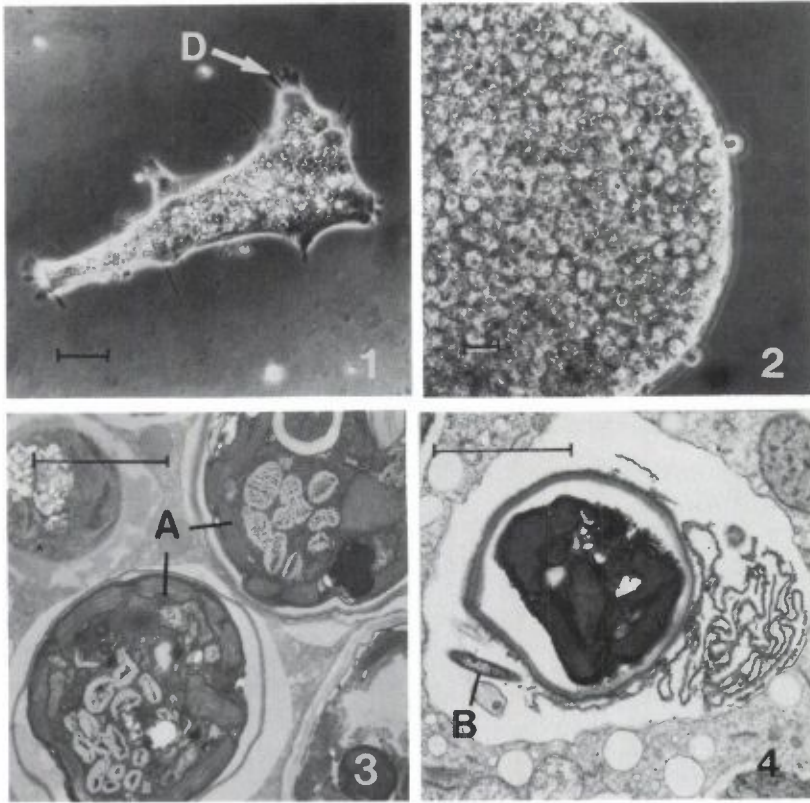
The transfer of labelled photosynthetic product to amoebae was verified by autoradiography. Procedures were as described but instead of scintillation counting, amoebae were air-dried on slides and coated with Kodak NTB-2 nuclear track emulsion. After 10 days, the emulsion was developed for 2 min (20°C) in undiluted Kodak D 19 and the number of silver grains overlying whole cells and background areas compared.

3. Results

The laboratory association between the amoeba, *Trichosphaerium* AM-I-7 (without algae, Fig. 1) and the dinoflagellate, *Symbiodinium* sp. #8 survived for over 2 years in bacteria free PES with no change of medium. In C75S medium dense populations of bacteria took over the cultures killing the amoebae.

In a mature association single amoebae sequestered over 100 algal cells and commonly 260 ± 49 S.D. ($n=20$) dinoflagellates per amoeba (Fig. 2, and front cover picture). The establishment of this intracellular algal population was as follows: after an initial lag phase (up to 100 hr) in which uptake rates of algae were low (typically 5 cells in total) the rate of incorporation of algae increased exponentially. The semilogarithmic plot describing this phase had the equation $y = 0.12x - 0.418$ ($r = 0.987$). Thus amoebae sequestered algae at a rate of about 1 cell h^{-1} up to a saturation level of about 260 algae. This rate is similar to the rate of incorporation of "inert" plastic beads (15 μm diameter). Here the slope describing the exponential phase was 0.011 ($r = 0.901$). [Note: "inert" = A mutant of *Trichosphaerium* Am.-I-7 (presently under investigation), is capable of degrading and digesting plastic polymers such as polyethelenes and polyvinyls. Therefore "inert" plastic beads may be recognized by *Trichosphaerium* as potential food source.]

The vast majority of sequestered dinoflagellates were maintained intracellularly as viable cells. Examination of algae by TEM showed that the



Figures 1-4. (1) LM of *Trichosphaerium* fed bacteria. Note the dactylopodia (D) which protrude through pores in the amoeba test. Bar = 10 μ m. (2) LM of *Trichosphaerium* partially compressed to show numerous intracellular dinoflagellates. Bar = 10 μ m. (3) TEM of *Trichosphaerium* showing intact intracellular algae (A). Bar = 5 μ m. (4) TEM of *Trichosphaerium* with dinoflagellate in food vacuole showing evidence of digestion. Bacteria (B) also in vacuole. Bar = 5 μ m.

majority were structurally intact (Fig. 3) although occasionally partially digested or autolysed cells in vacuoles were observed (Fig. 4). This result was supported by staining with FITC-conjugated lectins specific for digestive vacuoles. Vacuoles full of digestible cells (yeast, *Chlamydomonas*, ground macroalgae, etc.) stained brightly by the lectins RCA¹²⁰ and GWA while lectin treatments on amoebae packed with *Symbiodinium* #8 failed to stain the membranes or the contents of vacuoles.

The proportion of both free and sequestered algae undergoing digestion or

Table 1. Percentage of dinoflagellates accumulating neutral red stain (i.e. non-viable cells) and percentage of algae undergoing cell division. Observations made 20 days after inoculation into PES media.

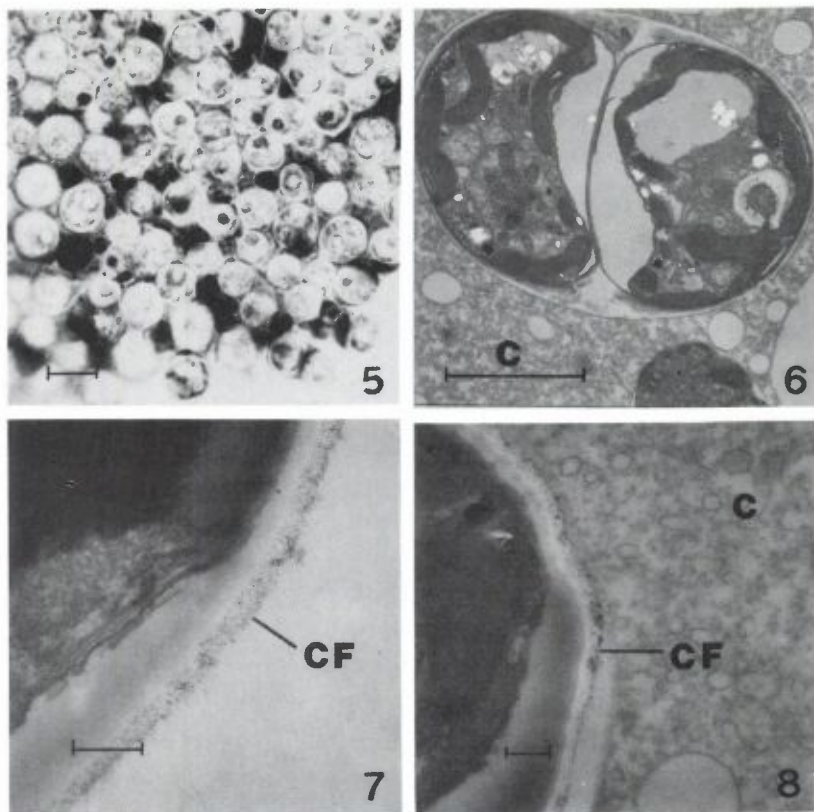
Experimental condition	Light/Dark	Percentage ¹	
		Staining	Dividing
<i>Symbiodinium</i> free	L	0.1	6.5
No amoebae	D	0.2	2.6
<i>Symbiodinium</i> free	L	0.5	4.9
With amoebae	D	7.2	0.1
<i>Symbiodinium</i> sequestered	L	2.2	5.2
Within amoebae	D	17.8	0.4

¹ Percentages based on at least 1000 observations

possibly autolysis was estimated by staining with neutral red (Table 1 and Fig. 5). *Symbiodinium* free in the medium were generally intact with only 0.1–0.5% taking up stain when cultures were kept in the light. However, in the dark 7.2% of free dinoflagellates showed staining. The majority of algae sequestered by amoebae in the light were viable (only 2.2% stained). In the dark, however, many more intracellular algae showed evidence of digestion or autolysis with 17.8% taking up neutral red.

Sequestered algae showed some evidence of replication within amoebae. From examination of thin sections by TEM, dividing cells were occasionally observed over a 24 hr period (Fig. 6). However, comparisons between the number of dividing intracellular algae and dividing free algae (Table 1) did not show any significant differences.

The observed low rate of intracellular replication can be related to the flux rate, and low residence time, of most of the algae within the host amoebae. Using cationized ferritin as a marker to identify freshly sequestered dinoflagellates (Figs. 7 and 8), it was found that 90% of the algae were ingested within a 24 hr period when amoebae were in culture with ample free dinoflagellates. Direct observations support this result. During a series of 30 min observations (n=20) the mean rate of uptake was 10 cells hr⁻¹ with the release of a similar number. This is higher than the rate of uptake by cells during establishment of the association. This rate, however, implies a significant daily turnover in line with that indicated by ferritin labeling. When similar observations were made on cells in media with no free dinoflagellates, much lower flux rates were found. Here amoebae retained a stable resident population of algae over the series of 12 hr observational periods. However, these amoebae contained on average, only 15 dinoflagellates.



Figures 5-8. (5) Sequestered dinoflagellates of *Trichosphaerium* treated with neutral red to stain partially digested vacuoles (in black). Bar = 10 μm . (6) TEM of *Trichosphaerium* showing recently divided intracellular alga. Amoebal cytoplasm (C). Bar = 5 μm . (7) TEM (unstained section) showing part of a wall of a free dinoflagellate to which cationized ferritin was bound (CF). Bar = 0.2 μm . (8) TEM (unstained section) showing edge of recently ingested dinoflagellate identified by its cationized ferritin (CF) labeled wall. Amoeba cytoplasm (C). Bar = 0.2 μm .

Growth rates of amoebae under different experimental conditions were computed from semilogarithmic plots (base 10) of the exponential phase describing cell counts with time. These rates, and corresponding generation times are given in Table 2. Amoebae packed with algae and cultured in PES with ample free dinoflagellates in the surrounding medium divided after 49.8 hr in the light and 54.6 hr in the dark. The generation time of amoebae in PES medium without free dinoflagellates was 327 hr in the light; cell division was completely suppressed in the dark. *Trichosphaerium* grown

Table 2. Comparison of growth rates and calculated generation times of *Trichosphaerium* both with and without intracellular *Symbiodinium*. The effects of different media, light, dark and whether free dinoflagellates were added to the culture system were examined.

Pre-experimental condition	Culture medium	Light/dark	With/without algae	Specific ¹ growth rate (hr ⁻¹)	generation time (hr)
Amoebae full of sequestered dinoflagellates	PES	L	+	6.0 (1.8)	49.8
	PES	D	+	5.5 (1.7)	54.6
	PES	L	-	0.9 (0.4)	327
	PES	D	-	no growth	-
Amoebae full of sequestered dinoflagellates	C75S	L	-	15.5 (4.0)	19.3
	C75S	D	-	8.9 (2.0)	22.7
Amoebae without dinoflagellates (bacterial diet)	C75S	L	-	11.9 (2.5)	25.2

¹ Specific growth rates ($\times 10^{-3}$); all regressions with correlation coefficients (r) 0.9; standard deviations in parenthesis

with ample bacteria in C75S medium either with or without sequestered dinoflagellates grew rapidly and divided in 24 hr or less.

The percentage of total ¹⁴C labelled product transferred from algae to amoebae was 9.8% (+5.0 S.D.). It is unlikely that any of this labelled product was due to digestion of dinoflagellates since amoebae induced to undergo multiple fission rapidly expel their vacuolar contents within the first few hours. Moreover, the physiological changes which accompany this phenomenon are not conducive to digestion and assimilation of ingesta. Algae released from amoebae harbored 25.9% (+9.9 S.D.) of C⁻¹⁴ label, the remainder 64.1% was unaccounted for. It could have been rinsed away during the multiple fission as decomposed but not yet assimilated digestive products. Autoradiography on small amoebae (after fission) supported the translocation of photosynthetic product. Counts of silver grains in an area overlying amoebae ($\approx 400 \mu\text{m}^2$) averaged $140.8 + 39.6$ S.D. ($n=15$) which was significantly above that of background ($15.8 + 3.8$ S.D.).

4. Discussion

Many symbiotic associations involving protists and algae have been described (reviewed in Smith and Douglas, 1988) with the vast majority involving dinoflagellates of the genus *Symbiodinium*. This is also true for sarcodines

where planktonic foraminiferans, radiolarians and acantharians frequently associate with dinoflagellates (Lee et al., 1985). Despite the fact that research on marine protozoa has increased markedly, largely because of an increased awareness about the ecological importance of symbiosis, no algal symbionts have been reported from any species of *Trichosphaerium* nor from any of the morphologically similar gymnameobae. This suggests that the association between *Trichosphaerium* and *Symbiodinium* described in this paper is a laboratory phenomenon and is either rare or does not occur in the marine environment. In support of this, all attempts by the authors to isolate dinoflagellate-laden cells from the field were unsuccessful.

In view of what we understand about protist/algal symbioses, this is not surprising. The majority of associations described in sarcodines and ciliates occur in the euphotic zone of oligotrophic waters. In this nutrient depleted environment it is beneficial for the host to harbor endosymbiotic algae and profit from the retention and recycling of nitrogen and phosphorous compounds within an essentially closed system. *Trichosphaerium*, on the other hand, is common in littoral habitats where it grazes on the bacteria and microalgae associated with the surfaces of seaweeds and on the algal tissue itself (Polne-Fuller, 1987). In this nutrient-rich microhabitat the retention of symbionts is not favored from an energetic standpoint.

Our laboratory results demonstrate the importance of nutrient status on the stability of the algal association. When amoebae containing algae were cultured in organically rich bacterized media they egested over 95% of their dinoflagellates within 24 hr and preyed on bacteria. Under these conditions, *Trichosphaerium* reproduced rapidly, dividing every 20 hr or less. At the other extreme, when amoebae with algae were cultured in inorganic medium with few bacteria and no free algae they retained their full complement of dinoflagellates. Their growth rates were low and they divided only once every 2 weeks or longer. A similar retention of symbionts in inorganic media was observed by time-lapse photomicroscopy where amoebae in cultures without free dinoflagellates retained a stable complement of symbionts. Under these conditions, algae were retained intracellularly without flux and the association approached that of a true symbiosis.

The stability and duration of the association was different when dinoflagellates were abundant in the inorganic medium. Here, *Trichosphaerium* turned over the majority of its algal population in about 30 hr. For these reasons we prefer, at this time, to term the relationship an "induced association" rather than a symbiosis even though other features of the relationship may imply mutualism. For example, amoebae retained a full complement

of intracellular algae, albeit a fluxing population, for over 2 years in culture. Furthermore, although *Trichosphaerium* was not nutritionally dependent upon its sequestered algae, feeding instead on bacteria and perhaps a small percentage of dinoflagellates, they did assimilate 10% of the photosynthate derived from their associated algae. This supplementary source of nutrition may have accounted for the slightly improved growth of amoebae with algae in the light compared to those in the dark. There is little information on the nutritional relationships of other sarcodine/algal associations, but there are indications that photosynthetic products alone rarely support rapid growth. For example, the foraminiferan *Globigerinoide sacculifer* grows slowly but fails to reproduce when starved in the light (Lee and McEnergy, 1983). It appears, therefore, that one of the major benefits of retaining symbionts is to ensure survival of the host in habitats which are subject to periodic fluctuations in prey and/or nutrients.

The laboratory induced association described here has several advantages for the study of algal/invertebrate symbioses. Aside from the fact that both partners can be cultured with ease, *Trichosphaerium* can readily be induced to egest its intracellular algae upon transfer to unenriched seawater where amoebae undergo multiple fission. While this sensitivity on the part of the amoebae can be problematic during routine handling, the benefits outweigh the disadvantages as intracellular algae can easily be separated from host amoebae. Moreover, *Trichosphaerium* has the ability to discriminate between different dinoflagellate species. In a separate study (manuscript in prep.) it was shown that the incorporation of *Symbiodinium* sp. #8 is favored by this amoeba over other species of symbiotic dinoflagellates and thus supports the presently established fact that symbiosis between microalgae and invertebrates demonstrate specificity (Trench, 1987, 1988). In light of the above, perhaps the greatest promise for this system is as a tool to investigate the mechanisms of cell recognition. These are the processes by which algae first gain entrance into the cell and the subsequent processes that determine whether or not these algae will be retained by the host. We hope that further studies with the *Trichosphaerium/Symbiodinium* association may solve some of the many unanswered questions surrounding these processes.

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