

**The symbiotic relationship between green algae  
and freshwater sponges: quantification of  
archaeocyte-associated algae.**

Submitted in Partial Fulfillment of the Requirements for  
Graduation with Honors to the Department of Biology and Chemistry at  
Carroll College, Helena, Montana


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April 11, 1998

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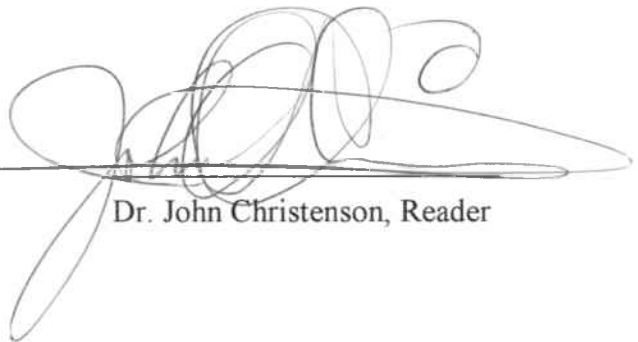
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This thesis for honors recognition has been approved for the  
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## ABSTRACT

Freshwater sponges often contain symbiotic algae, genus *Chlorella*, within their cells, yet there is no quantitative data available on either the number of algae present in sponge host cells or the proportion of the host cell occupied by algae. The objectives of my study were (1) to determine the number of algae in archaeocytes, the sponge cell type containing most of the symbiotic algae, and the ratio of algal volume both to total host cell volume and to host cytoplasmic volume and (2) to determine the effect of light deprivation on the number of algae per host cell and the ratio of algal volume to host cell volume. Samples of the freshwater sponge, *Ephydatia muelleri*, were collected during the summer from Salmon Lake in Western Montana. The samples were dissociated and observed using brightfield and epifluorescence microscopy. There was an average of  $20.5 \pm 16.5$  algae per host cell, which occupy on average  $3.8\% \pm 6.5\%$  of the host cell volume and  $4.0\% \pm 7.0\%$  of the total cytoplasmic volume. The effect of light deprivation on the algae was a continuous decrease in algae numbers per host cell over time (numbers remain almost constant over time when sponges are kept in alternating light and dark). Unexpectedly, the ratio of algal volume to host cell volume in light deprived sponges remained almost constant over time. Further study is needed to reconcile the decrease in the number of algae with the constancy of the volume ratio.

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## INTRODUCTION

The green algae, *Chlorella*, exists in symbiotic relationships with freshwater protozoa and invertebrate animals, including hydra and freshwater sponges (Reisser, 1984). In these relationships, the algae exist inside the host cells, avoiding intracellular digestion. These unusual relationships are of interest because they could give insight both into how pathogens escape degradation within the cells they infect (Eissenberg, 1993) and how organelles, such as mitochondria and chloroplasts, originated (Kite, 1986).

Baseline data, such as the number of algae per host cell and the proportion of the host cell volume occupied by algae, exist for hydra (Smith, 1987), and this data has served as a foundation for investigations of symbiosis in this animal (e.g. McAuley, 1990; Douglas and Smith, 1984). The objectives of my study were (1) to provide such baseline data for a freshwater sponge by determining the number of algae per host cell and the ratios of algal volume to total host cell volume and to host cytoplasmic volume and (2) to investigate the effect of light deprivation on algal numbers and algae per host cell ratios. Since the majority of symbiotic algae in freshwater sponges are housed in archaeocytes, my studies focused on this cell type.

## LITERATURE REVIEW

### Freshwater Sponges

Sponges (phylum Porifera) belong to the simplest of the multicellular animal phyla. Although sponges lack organs and true tissues, they have many specialized cells that accomplish basic biological functions such as food gathering, digestion, and reproduction. Sponges are abundant in marine environments and include some freshwater representatives. Of 5,000 extant species, less than 300 are freshwater sponges (Frost, 1991). Many species of freshwater sponges harbor algal symbionts, the most common of which is *Chlorella* spp. (Reisser, 1984).

Frost (1991) states that the gross structure of freshwater sponges derives from an interplay between two fundamentally different components, a mineral skeleton made of siliceous structures termed spicules and an organic skeleton made up of collagen. The basic organization of a sponge consists of epithelium made of pinacocytes and choanocytes, surrounding an organic matrix, termed the mesohyl, which contains a variety of specialized cells.

Hickman et al. (1993) describe pinacocytes as thin, flat, epithelial-type cells. These cells cover the exterior surface and line the internal canal systems of the sponge. Choanocytes, which line the flagellated canals and chambers, are ovoid cells with one end embedded in the mesohyl and the other exposed. The exposed end bears a single flagellum, which beats to circulate water through the sponge for respiratory gas exchange and food gathering. The exposed end also bears a collar of microvilli, which surrounds the flagellum. Within the collar, microvilli are connected by delicate

microfibrils, forming a fine filtering device for straining food particles (including algae) from the water.

The principal cells of the mesohyl are archaeocytes. Hickman et al. (1993) describe archaeocytes as ameboid cells that move through the mesohyl and carry out a number of functions. They can phagocytose particles at the pinacoderm and receive particles for digestion from the choanocytes. Archaeocytes also can differentiate into any of the other types of more specialized cells in the sponge.

The particles phagocytosed by the archaeocytes were originally suspended in water. According to Frost (1991), water carrying suspended particles enters a sponge through ostia (openings) spread across its surface epithelium. Water next enters a large subdermal cavity and then flows into incurrent canals that lead to choanocyte chambers. Each chamber consists of numerous choanocytes, which serve as the final filters in the feeding system. After passing through the choanocyte chambers, water enters the excurrent portion of the water processing system. Exiting the chambers, the canals join together forming larger and larger diameter vessels, finally entering a broad chamber termed an atrium. From the atria, water exits the sponge through specialized structures termed oscula, which channel water from large portions of a sponge.

### ***Chlorella***

*Chlorella* is a unicellular green alga (phylum Chlorophyta). Algae are eukaryotic and phototrophic. Typically, freshwater invertebrates bearing algal symbionts host species of *Chlorella*, and marine symbiotic invertebrates host dinoflagellates (McAuley, 1993). According to Reisser (1984), hosts of symbiotic systems usually are well known whereas the green algal partners of most freshwater

hosts are less well characterized and are usually described simply as "zoochlorellae". Since most symbiotic green algae have a coccoid (spherical) morphology, their classification by light microscopy alone is not easy. Therefore, the term "zoochlorella" has been arbitrarily extended to every green algal cell that lives in association with a heterotrophic organism.

According to Reisser (1984), endosymbiotic cells of *Chlorella* differ from free-living ones principally by the release of mono- and disaccharides, such as glucose, fructose, xylose, and maltose. There are no consistent differences in other physiological or morphological features between symbiotic and free-living *Chlorella*.

Algae contain chloroplasts which are organelles that carry out photosynthesis. The fixed carbon and oxygen produced during photosynthesis are released and used by the host. The green chlorophyll of the chloroplasts often results in the misidentification of their sponge hosts as plants rather than animals.

### ***Chlorella*-Freshwater Sponge Symbiosis**

Symbiosis implies mutualism in a close association between two dissimilar kinds of organisms, interacting spatially and metabolically so that, under certain natural conditions, each benefits from the association (Lewin, 1995). The mutual profit in the companionship has been reported to be protection of the algae inside the sponge and the receiving of products (i.e., glucose and maltose) of algae photosynthesis by the sponge (Saller, 1991). The costs to the host include both the provision of nutrients to symbionts and the allocation of energy and other resources to the accommodation and maintenance of symbionts (Smith, 1987).

*Chlorella*-freshwater sponge symbioses have not been studied extensively as other hosts with which *Chlorella* forms symbiotic relationships. Much more detailed information has been obtained from *Chlorella*-hydra symbioses studies.

### ***Chlorella*-Hydra Symbiosis**

According to McAuley (1993), intracellular algal symbionts in hydra are almost always within enclosed individual vacuoles of host origin. These vacuoles, termed perialgal vacuoles (PAV), are derived from modified phagosomal vacuoles. The inner faces of PAV membranes are rich in clusters of transmembrane proteins, which transport sugars and amino acids between symbionts and their hosts. In hydra digestive cells, phagocytosis of potential symbionts is stimulated by a high negative charge at the surface of the symbiotic *Chlorella* cells. After entering host cells, symbionts inhibit fusion of lysosomal vacuoles with the phagosome.

Smith (1987), in his study of the *Chlorella*-green hydra symbionts, found that each digestive cell in the host has 15-30 unicellular *Chlorella* symbionts, located at the basal end of the cells. McAuley(1990), in general agreement with Smith (1987), found that each green hydra host cell contains on average between 10 and 25 algae and that individual cells may contain as few as one and as many as 80 algae.

Douglas and Smith (1984) studied the relationship between algal and digestive cell volume in green hydra. They found that the algae occupied approximately 10% of the total volume and 18% of the volume of the basal region of the digestive cells. They determined that the number of algae per digestive cell varies with digestive cell volume.

Smith (1987) found that under constant conditions the proportion of symbiont to host is similar for hydra strains of widely differing sizes. Cell size analysis showed

that, in addition to numerical constancy within a cell, the symbiont population under normal conditions tends to maintain a fixed cell size distribution (Pardy, 1981; McAuley, 1993).

Smith (1987) states that changes in the proportion of symbiont to host volume are usually induced by alterations in the illumination and/or feeding regimes in which the hydra are maintained. Under normal conditions, changes in the biomass of symbiont are not achieved by either digestion or expulsion of algae. Pardy (1981) found that because symbiont reproduction is reduced in the dark, the number of symbionts per hydra declines as the hydra population continues to grow and expand.

Douglas and Smith (1984) studied factors involved in the regulation of the amount of algae per host. They determined that the host can regulate the algal population to different sizes depending on external conditions (food and illumination). The space available for the algae may be one factor that determines the population size of the algal symbionts. They proposed that the regulation of algal cell division in the natural symbiosis is principally mediated through relatively small and temporary changes in the pH of the perialgal vacuole. At more acidic values, photosynthetically fixed carbon is primarily directed towards maltose release and little or no algal growth occurs. At higher pH values, maltose release declines sharply and the carbon becomes primarily directed towards symbiont growth. Douglas and Smith (1984) believe that this hypothetical pH model can explain the observed responses to change in environmental conditions, as well as the relation between the timing of symbiont and host cell division.

McAuley (1985, 1986) suggested that algal division is dependent upon host supply of 'division factor', an unknown metabolite derived from host digestion of food. In cells with large numbers of algae, competition for division factor would be more limiting with regard to algal division than in cells with few algae. Since large digestive cells may possess a large pool of division factor, the size of host cells may have an indirect rather than a direct influence on numbers of algae, because of their greater phagocytic capacity. This theory would mean that large cells would contain more algae due to the larger supply of division factor.

According to McAuley (1993), the wide variation in numbers of algae may be explained by two factors. First, since almost all algal division is associated with host cell mitosis and, in fed hydra, algal division may not be completed until after host cell telophase, any effect of host cell size on numbers of algae would be manifested in the daughter cells, which may themselves be of unequal size. Second, the way in which algae are distributed between daughter cells would inherit only the variation of the parent population. Conversely, if the distribution tends to be random, as preliminary results suggested, then a new element of variation would be introduced at each round of cell division.

Although similarities between hydra-*Chlorella* and sponge-*Chlorella* symbiotic relationships exist, there are also differences. Cook (1983), for example, found the photosynthate of sponge-*Chlorella* symbioses differs from the products of other *Chlorella* associations in its composition, as well as in the quantity of photosynthate that is transferred to the host. Glucose rather than maltose seems to be the primary constituent of photosynthate in the sponge-*Chlorella* relationship. Cook (1983) also

found that the symbionts of the freshwater sponge, *Ephydatia fluviatilis*, only transfer 9-17% of the total fixed carbon, in contrast to 25-30% of the total fixed carbon released by hydra hosts.

## MATERIALS AND METHODS

### Sponge Sample Collection

Samples of the freshwater sponge, *Ephydatia muelleri*, were collected during June and July from Salmon Lake (47°06'N/113°24'W) located in the Upper Clark Fork Basin in Western Montana. Samples (approximately 2.5 x 2.5 x 1 cm) were scraped from rocks, placed in containers with lake water, and maintained at close to lake temperature in a cooler during transportation to the laboratory. At the laboratory, samples were transferred into two glass tanks. A small piece of the sponge tissue was cut off each sample and observed for confirmation of the sponge species. Microscopic observation of spicule morphology confirmed the samples were *Ephydatia muelleri*. Each tank contained 2.35 L of lake water, which had been filtered through two layers of Whatman #2 filter paper. The sponges were submerged in the water 9 cm below the surface. An aerator was used to keep the water oxygenated. Tap water of about 20°C was circulated around the tanks in order to prevent large temperature fluctuations. The tanks were kept in either continuous darkness or on a 14-hr light/10-hr dark(14L/10D) cycle. A Gro Light (Sylvania) was positioned 50 cm above the water surface.

### Cell Dissociation

Samples were analyzed on the day collected, at 24 hr, and at 48 hr. After a sample was removed from its tank, the sample was weighed in a tared weighing dish containing enough calcium-magnesium free medium (CMF) to keep the sample moist. CMF contained the following adjusted to pH 7-8: 6 mM Tris, 34.0 mM NaCl, 1.34 mM KCl, 1.38 mM glucose, 1.07 mM NaHCO<sub>3</sub>, 0.7 mM K<sub>2</sub>HPO<sub>4</sub>, 0.25 mM EDTA

(ethylenediaminetetraacetate, disodium salt). After the wet weight of the sponge sample was measured, additional CMF was added to establish a ratio of 1 g of wet sponge tissue to 10 mL of CMF. CMF was used to help break down the connections between sponge cells, allowing for dissociation of the sample into single cells and small clumps of cells.

The sponge tissue submerged in CMF was finely minced with an oil-free razor blade, transferred to a 15-mL test tube, and vortexed five times for 5 s each time using a VortexGenie 2 at a setting of 5. The sample was then allowed to sit for approximately 5 min. The sample was vortexed again for three pulses lasting 5 s each at the same setting. The suspension was filtered through a Swinnex filter unit containing Nitex 48- $\mu\text{m}$  mesh. The filter allowed single cells and small clumps of cells through, while holding back spicules and larger particles.

### **Cell Microscopic Examination**

Dissociated cells were examined with a Nikon Ophiphot 2 microscope adapted for epifluorescence. A Nikon B-2A filter was used to view the autofluorescence of the chlorophyll contained in algae associated with the sponge cells. Brightfield microscopy was used to confirm observations of the number of algae per cell and for measurements of cell and nuclear diameters. Archaeocytes, which were the sponge cells studied in this project, were identified by their single prominent nucleolus.

### **Volume Determinations**

Archaeocyte, nuclear, and algal volumes were determined by assuming that the cells were spherical and using the equation  $\frac{4}{3} \pi r^3$ , where  $r$  is the radius. Radii were obtained from the diameters measured microscopically.

## RESULTS

*Ephydatia muelleri* grow abundantly as mossy, green, 1-2 cm thick growths on sun-exposed rocks at depths as little as 0.2 m near the outlet of Salmon Lake (Fig. 1). The archaeocytes obtained from these sponges were identifiable after dissociation by their prominent nucleoli (Fig. 2A). Archaeocytes were present in dissociated cell preparations as isolated cells or in small cell clusters. Algae were recognizable by their green color and small size (Fig. 2A) or by their red autofluorescence (Fig. 2B).

The number of algae per archaeocyte ranged from 0 to 43 (Table 1). The mean number of algae symbionts per host cell was  $20.5 \pm 16.5$ . The total symbiont volume of  $151.5 \mu\text{m}^3$  occupied  $3.8\% \pm 6.5\%$  of the  $4000.2 \mu\text{m}^3$  total host volume and  $4.0\% \pm 7.0\%$  of the  $3820.0 \mu\text{m}^3$  host cytoplasmic volume (Table 1). The distribution of algae within the cells did not appear to be restricted to any part of the cytoplasm.

The number of algae per sponge host cell kept on 14L/10D cycle remained almost constant over the 48-hr period, whereas the number of algae per host cell dropped daily in samples kept in continuous dark (Fig. 3). In a 2-factor ANOVA, the interaction between light regime and time was significant ( $P < 0.002$ ;  $\alpha = 0.017$ , Bonferroni-corrected for three response variables). In contrast, neither the ratio of algal volume to total host cell volume nor the ratio of algal volume to cytoplasmic volume showed different patterns over time for light treated and light deprived samples (Figs. 4, 5). The interaction between light regime and time furthermore was not significant ( $P > 0.017$ , Bonferroni-corrected for three response variables).

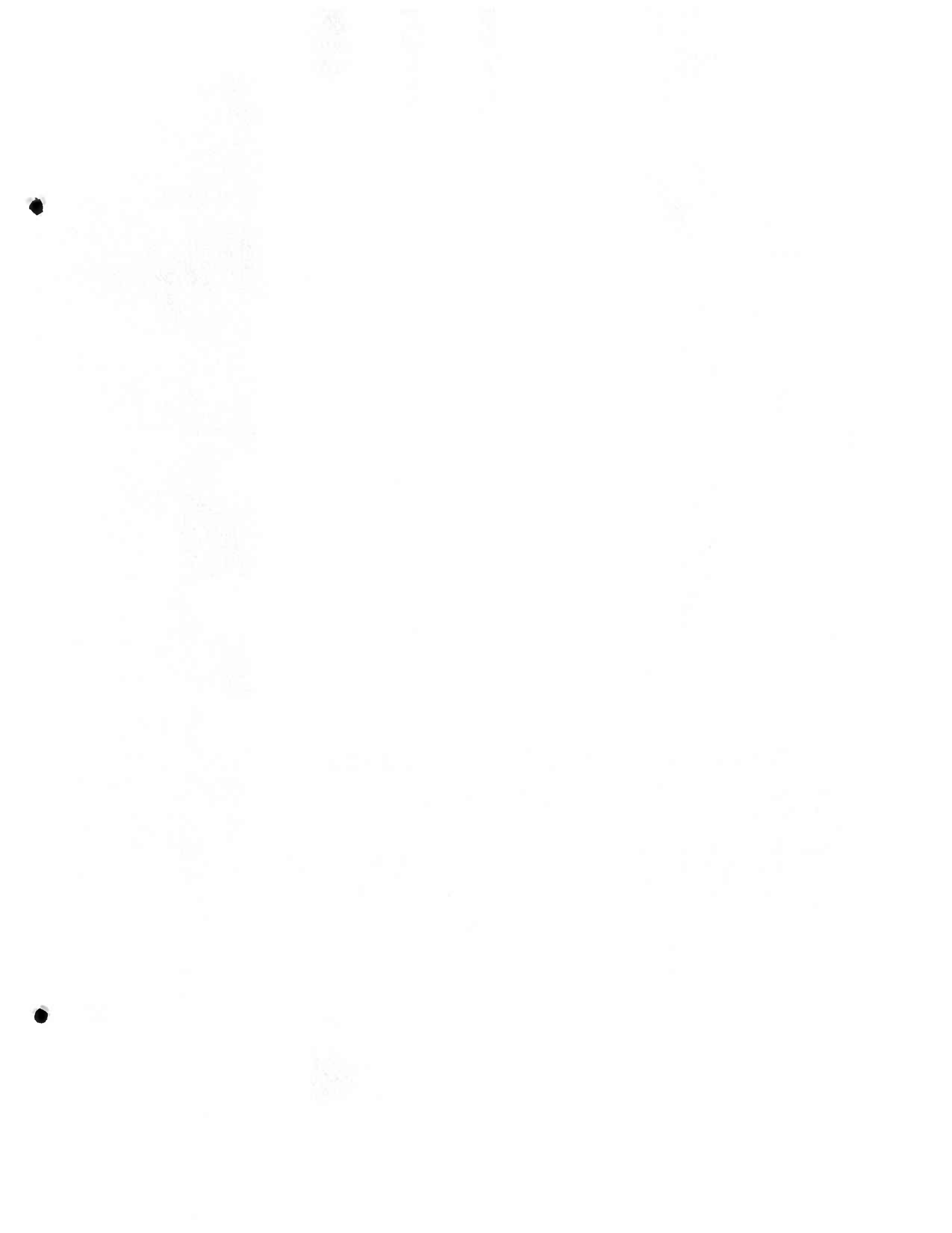
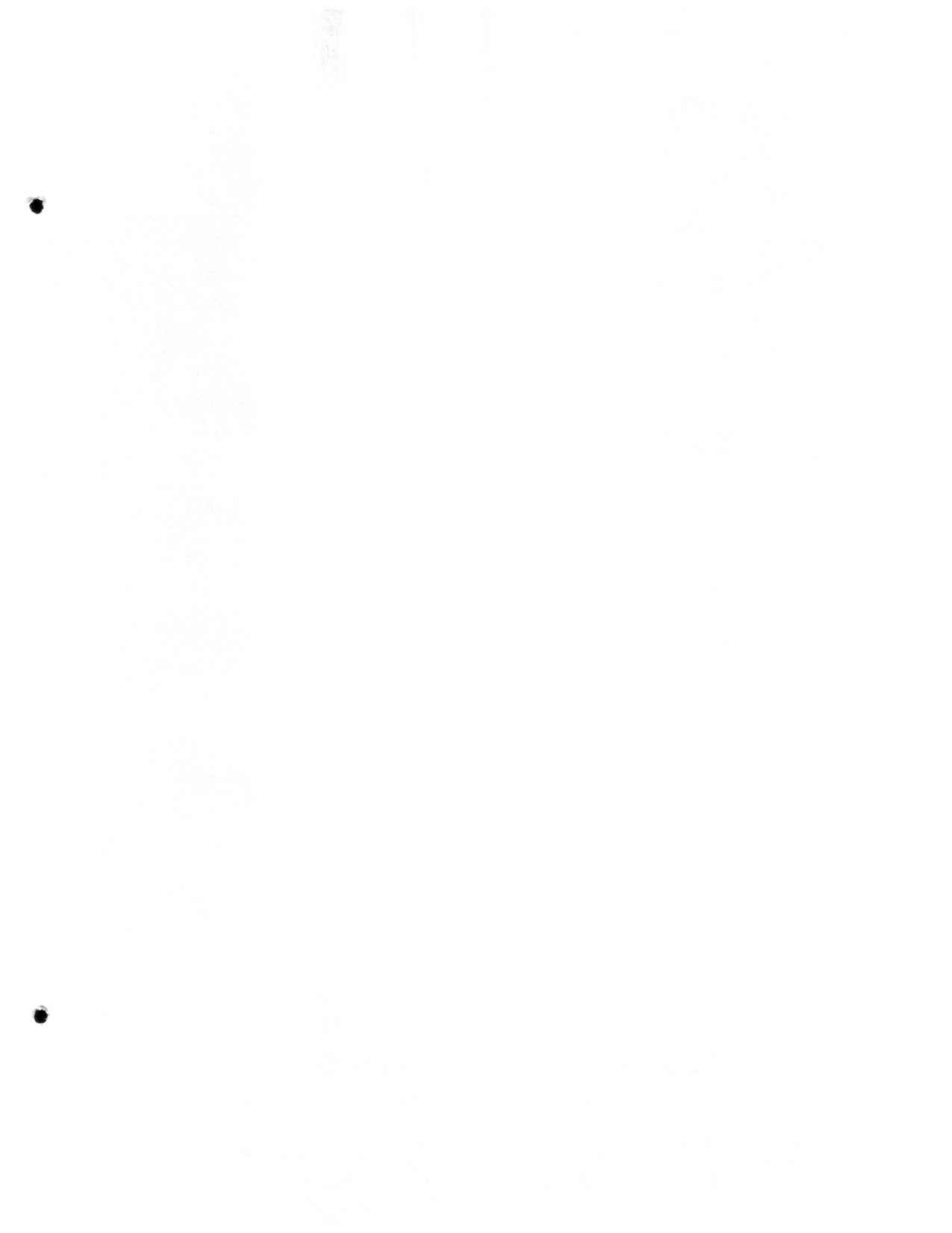
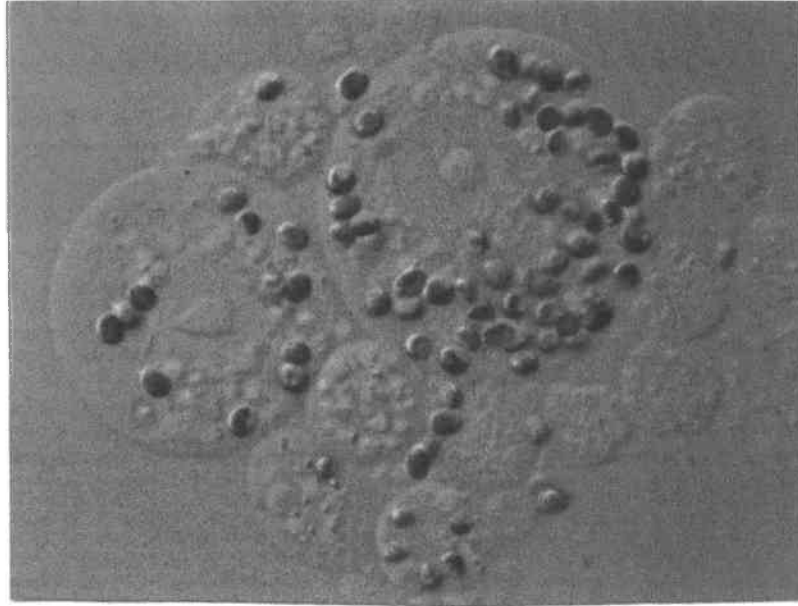




Fig. 1. *Ephydatia muelleri* forming an encrusting growth on a rock surface. The rock was originally located approximately 0.6 m below water surface.



A



B

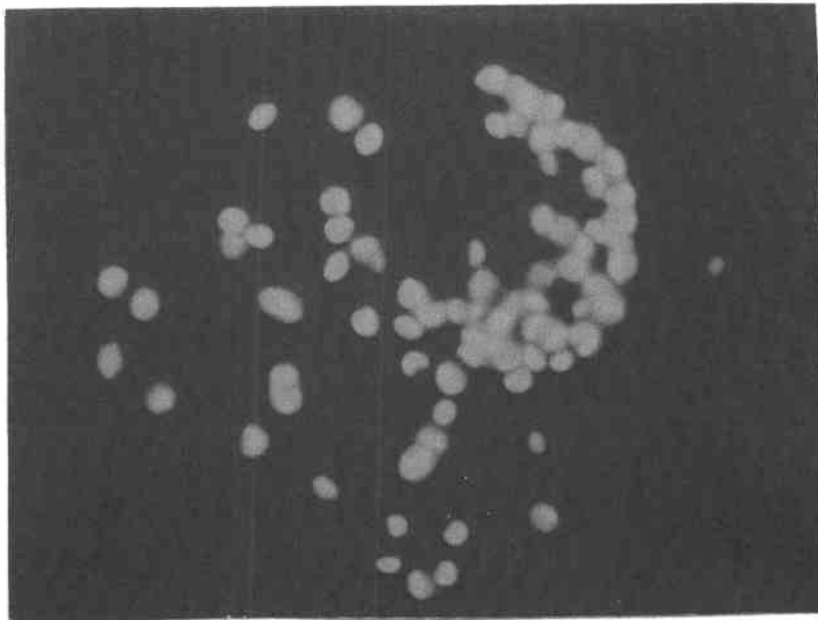
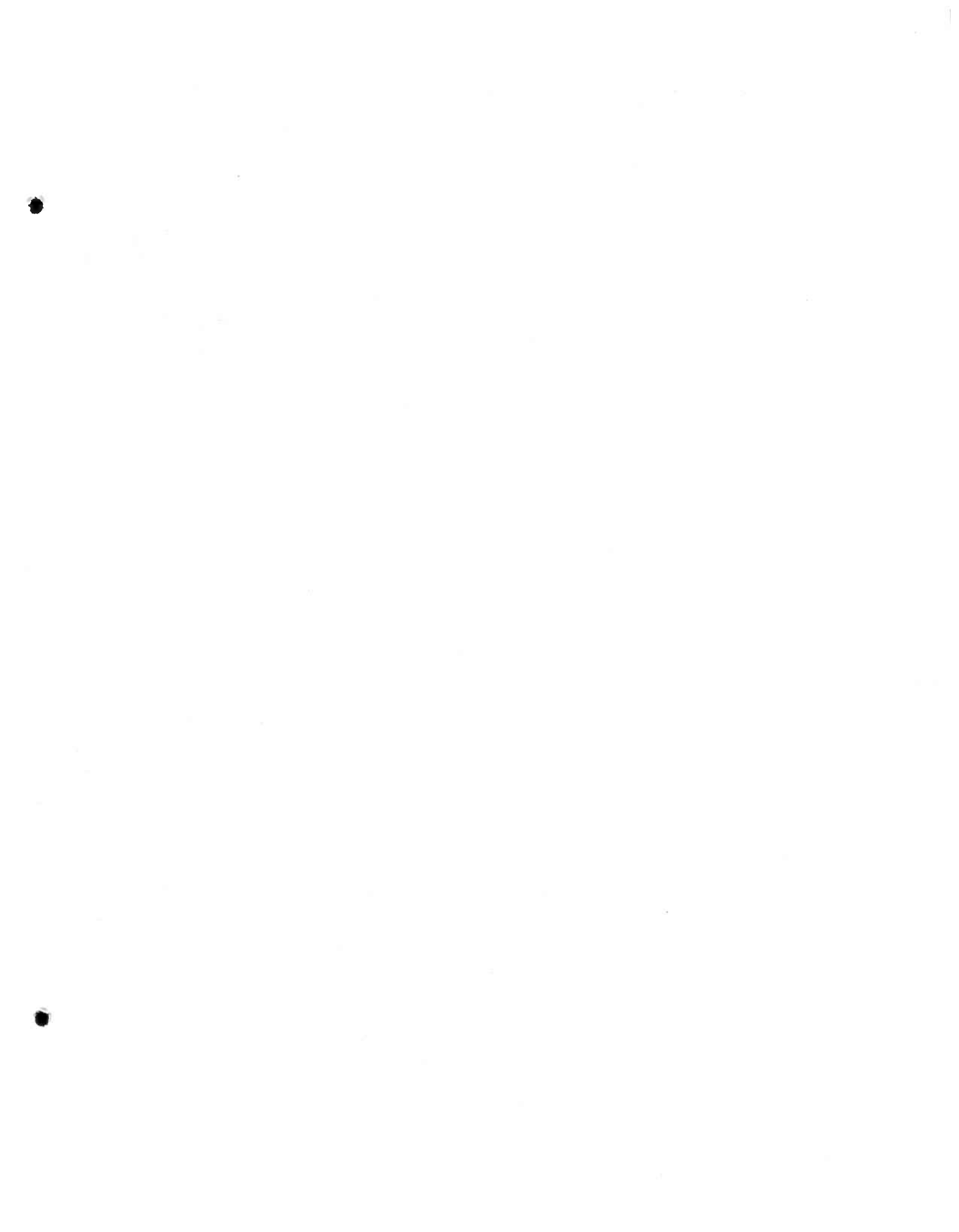


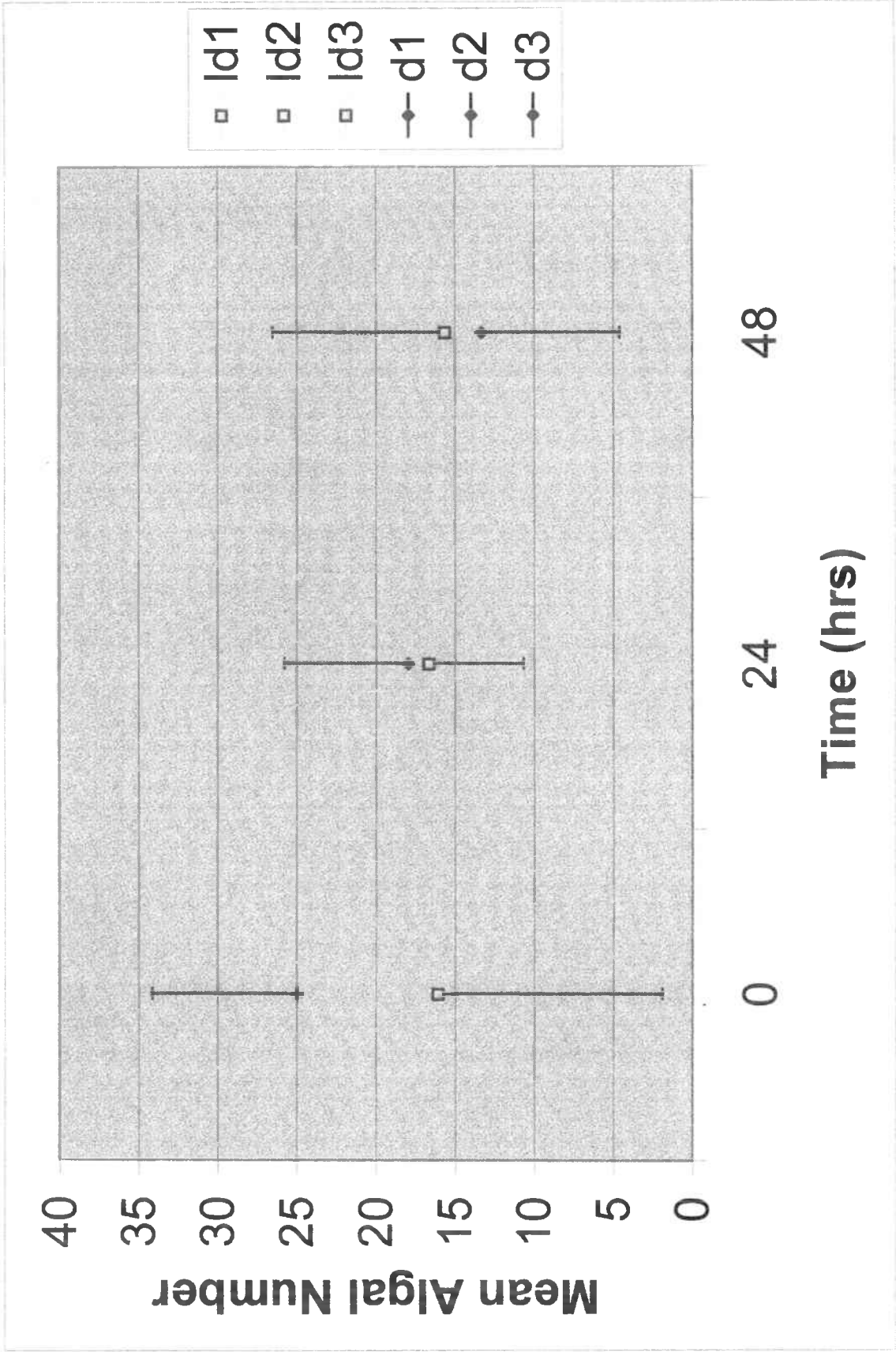
Fig. 2. *Chlorella* within archaeocytes and other sponge cells. A, green chlorophyll of *Chlorella* in archaeocytes, identified by prominent nucleoli (differential interference contrast microscopy, 1000X); B, autofluorescence (shown with red dots) of *Chlorella* in archaeocyte (epifluorescence, 1000X).

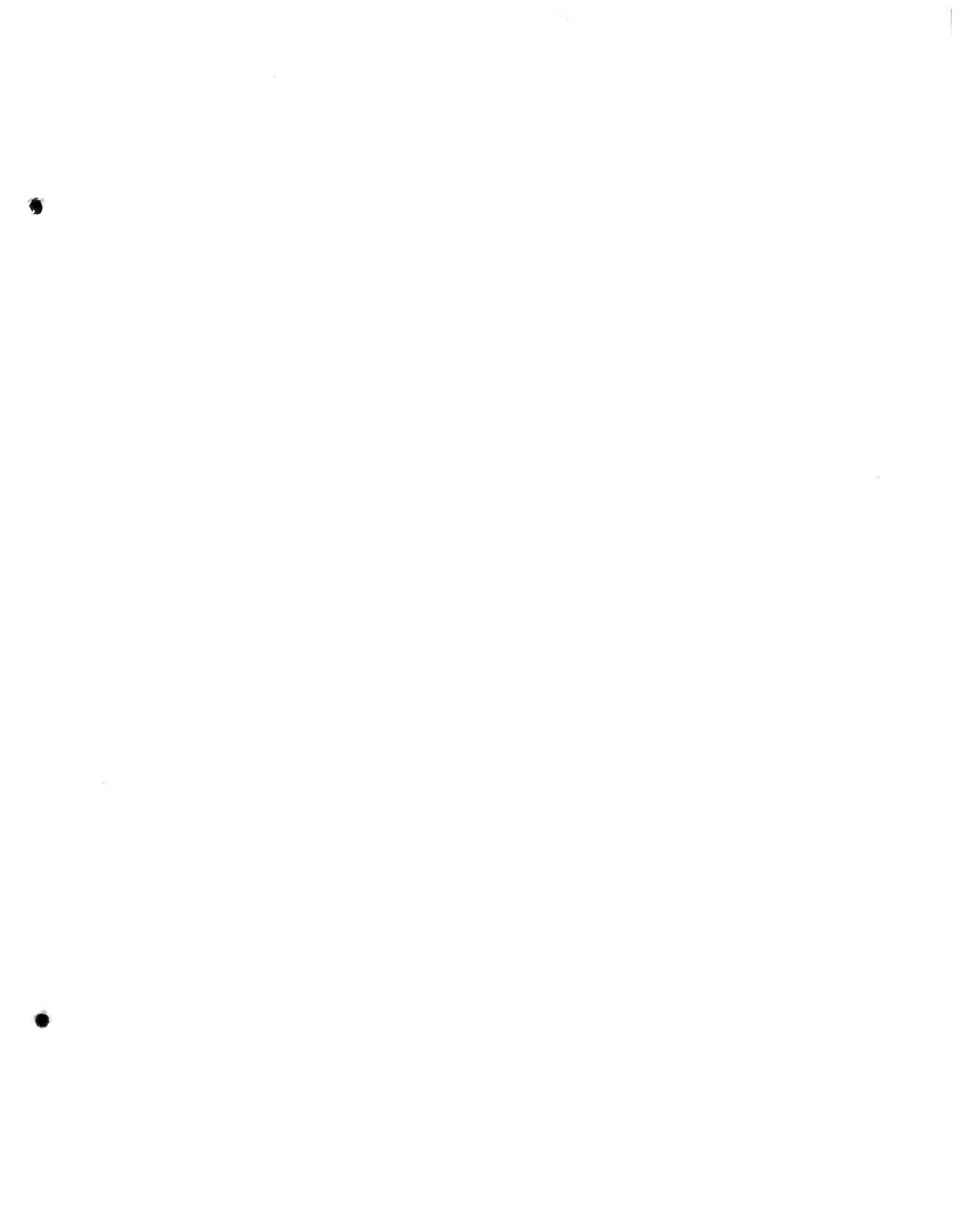
Table 1. Number, volume, and volume ratios determined for symbiotic *Chlorella* within archaeocytes of *Ephydatia muelleri*. Determined from specimen dissociation on day of collection. N=40.

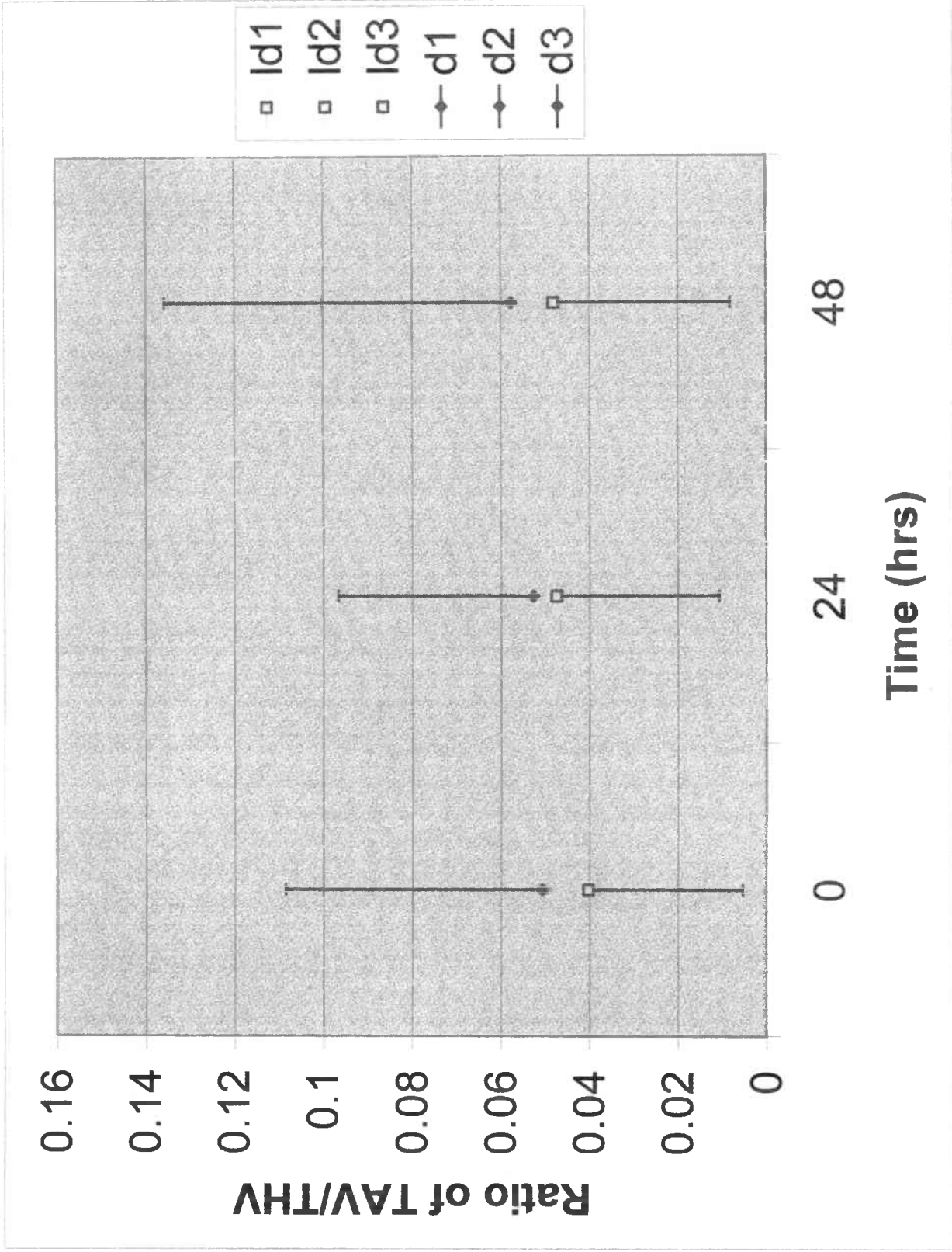
VARIABLES	MEAN $\pm$ S.E.
Total cellular volume of archaeocytes	4000.2 $\pm$ 4423.0*
Total cytoplasmic volume of archaeocytes	3820.0 $\pm$ 4167.0*
Number of algae/archaeocyte	20.5 $\pm$ 16.5
Total algal volume	151.5 $\pm$ 180.9*
Total algal volume/total host cell volume	3.8% $\pm$ 6.5%
Total algal volume/host cytoplasmic volume	4.0% $\pm$ 7.0%

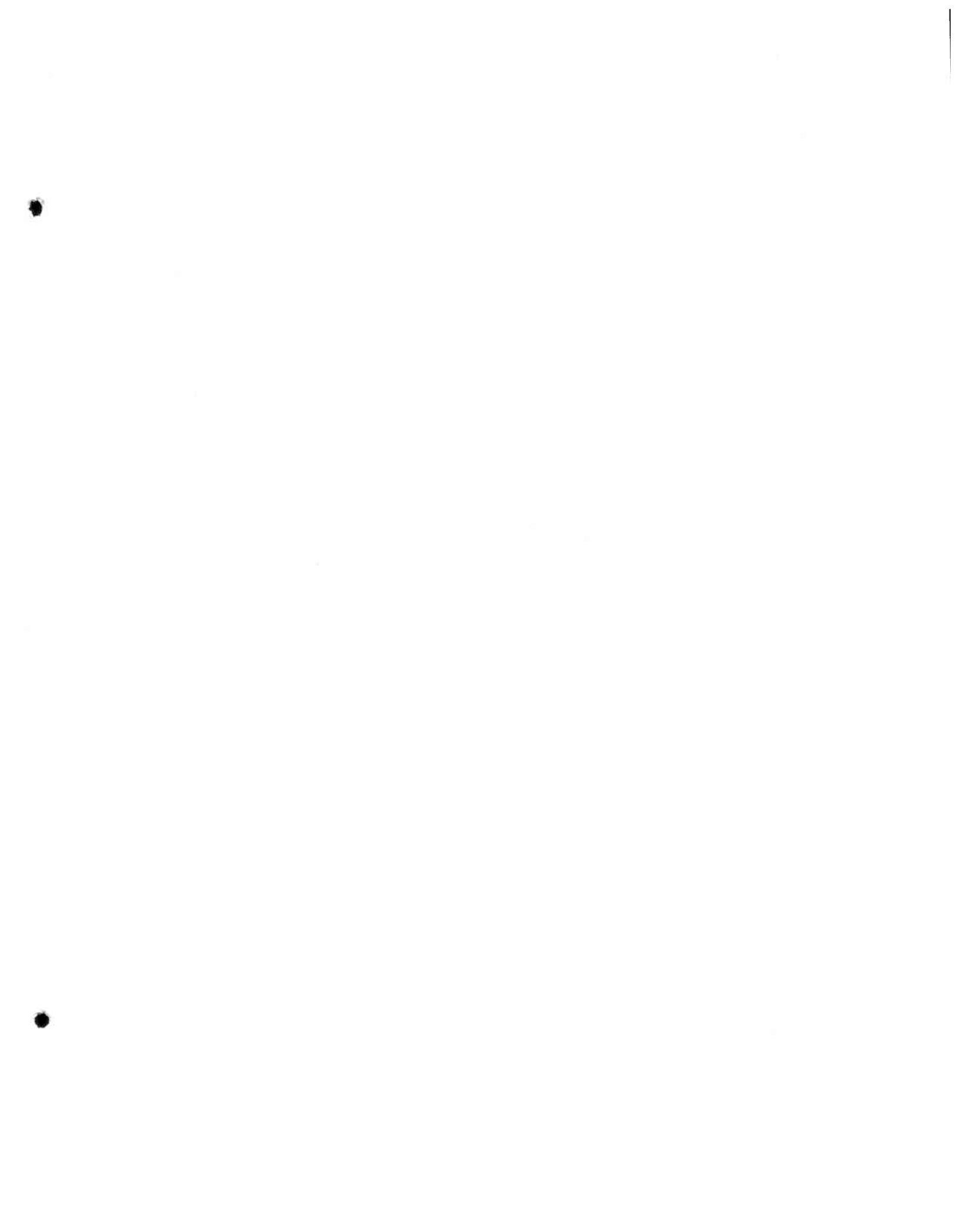
\*  $\mu\text{m}^3$

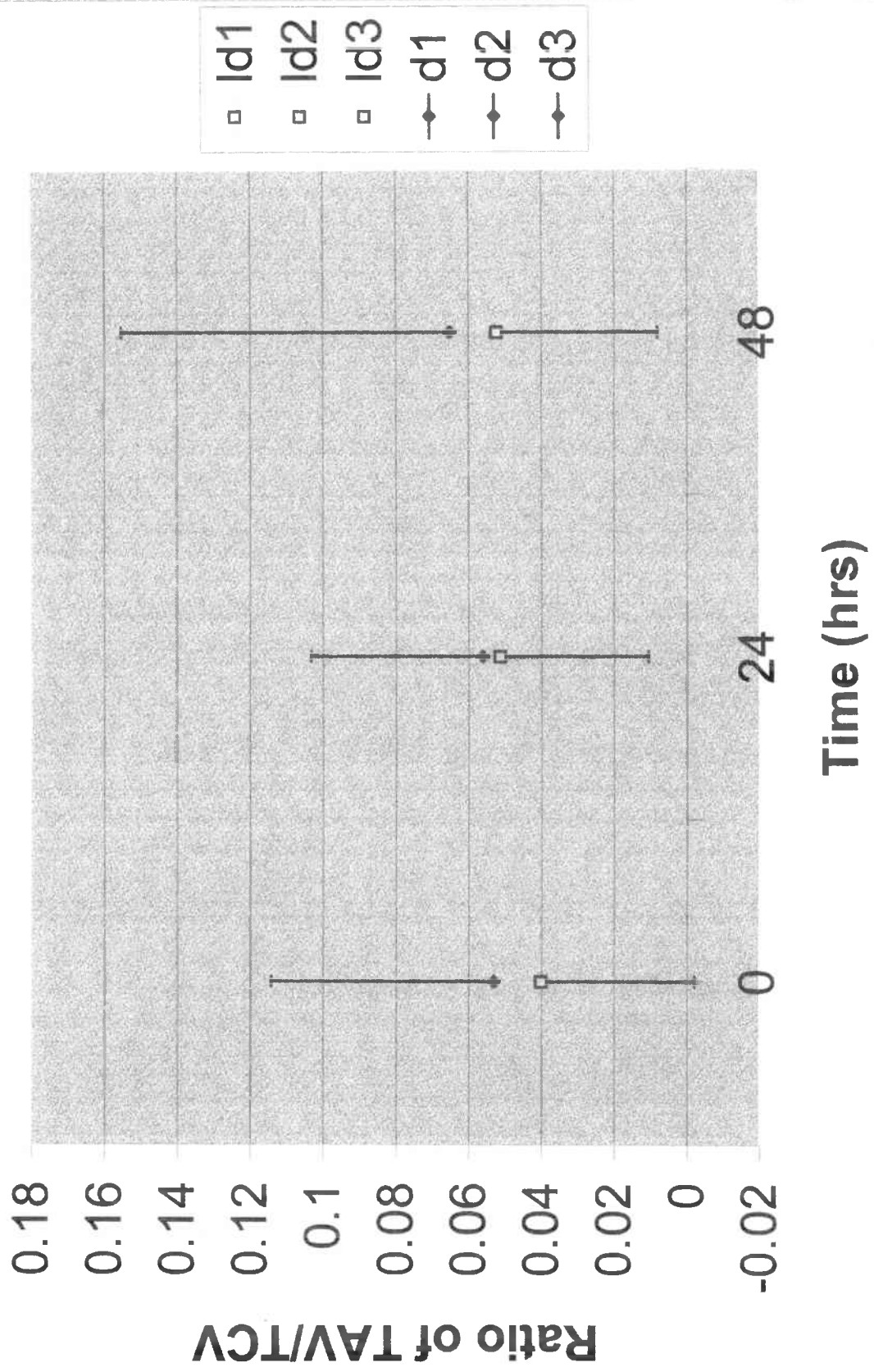












## DISCUSSION

This project had two major goals. The first was to determine the number of algae in archaeocytes and the ratio of algal volume to total host cell volume and to host cytoplasmic volume and the second was to determine the effect of depriving the symbionts of light for 48 hr.

The average number of algae per host cell in the sponge ( $20.5 \pm 16.5$ ) is similar to the number found in studies of green hydra hosts conducted by Smith (1987) and McAuley (1990). Since counts and measurements were performed on the same day sponge samples were collected, it was possible that not all of the intracellular algae were symbiotic. Some may have been engulfed as food and been in the process of being degraded. The virtual absence of algae from the filtered lake water, precluding replacement of degraded algae, and the constancy of algal numbers over 48 hr in sponges kept on a 14 L/10D cycle, however, suggest that the algae were in fact symbiotic.

The algae occupied a mean of approximately 3.8% of the total archaeocyte volume. This percentage is lower than the 10% Douglas and Smith (1984) found in green hydra. This could represent a real difference between sponge and hydra. There are, however, other explanations. A small error in the estimation of the algae diameter will result in a large change in calculated algal volume and therefore in the percentage of the host volume occupied by algae. For example, the ratio of algal volume to host volume is 3.03% if an average algae diameter of 2.4  $\mu\text{m}$  is used, but increases to a ratio of 5.92% if an average diameter of 3.0  $\mu\text{m}$  is used. It is also possible that calculated host cell volumes were overestimated due to flattening of the sponge cells on the

microscope slide. Since flattening of cells in the method I used was a concern, the diameters of large sponge cells were checked in a preliminary experiment by placing dissociated cells in a hemacytometer chamber, which allowed the cells to move freely. The average diameter found using the hemacytometer was slightly larger (15.2  $\mu\text{m}$  for 20 samples) than the average diameter found using a standard microscope slide and coverslip (14.5  $\mu\text{m}$  for 30 samples). Therefore, cell flattening did not seem to be a concern.

The light/dark study showed that samples kept in 14-hr light/10-hr dark maintained an almost constant number of algae per host cell, while samples kept in continuous dark showed a continuous decrease in algae number. These results are consistent with the results obtained on green hydra by McAuley (1993), Smith (1987), Douglas and Smith (1984), and Pardy (1981). The reason for the decrease in algae numbers in continuous dark is probably host digestion. Since symbionts cannot conduct photosynthesis in the dark and therefore cannot produce nutrients for the host, the algae may be used as nutrients. If this were the case, then the ratio of algae volume to host cell volume would be expected to decrease. Rather than decreasing as expected, however, the ratio of algae volume to host volume remained almost constant. This was due to the fact that the host volume decreased over time along with the number of algae. The decrease in host volume may be due merely to a sampling effect, or it could reflect a compensatory mechanism at work.

In conclusion, I have fulfilled the two main objectives of this study in an attempt to provide baseline data on the relationship between *Chlorella* and the freshwater sponge *Ephydatia muelleri*. However, further experimentation is needed to confirm the

number of algae and volume ratios. As well, more data on changes in algae deprived of light needs to be obtained. Specifically, the decrease in algal number needs to be reconciled with the lack of change in ratios, which might be detectable with a larger sample size.

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