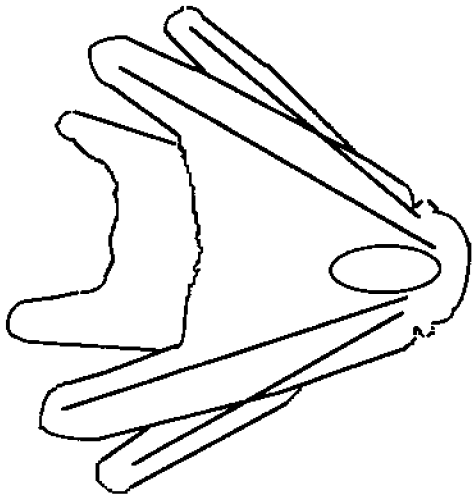
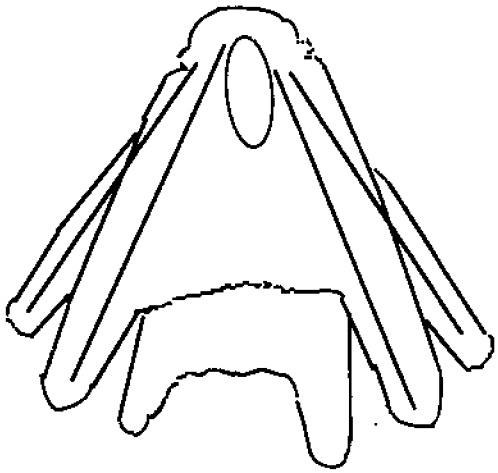
S.A.G.U.

Sustainable Aquaculture of the Green Urchin





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ABSTRACT

Within the past decade, commercial harvesting of the green sea urchin, *Strongylocentrotus droebachiensis,* in Maine and New Hampshire expanded rapidly and became the second largest fishery in the state of Maine, behind lobsters (Harris et al., 1996). Since then the fishery has been declining due to over- fishing. This has led to a great interest in developing a plan for a more sustainable fishery and, thus, a growing interest in the aquaculture of sea urchins.

This experiment was designed to investigate ways of planning a system for the culturing of urchins from larvae through the metamorphosis stage to produce seed stock for outplanting. Three species of algae were cultured to be used as the variable treatments. Urchin larvae were fed four different algal diets: *Nanochloropsis, Isochrysis galbana,* a mixture of *Nanochlorpsis,* and *Isochrysis*, and *Dunaliella,* and a mixture of *Nanochlorpsis, Isochrysis* , and *Dunaliella*. Measurements of the arm spicules in the three different treatments were made weekly and no significant differences were found between treatments.

Results indicate that *Nanochloropsis* may be the most suitable algae for urchin aquaculture. Such a diet led to a greater amount of settlement than that of an *Isochrysis* diet. However, the test sizes of urchins fed *Nanochloropsis* were found to be significantly smaller than those of urchins fed an *Isochrysis* diet. It may then be suggested that a larger ration of *Nanochloropsis* would be the most sustainable diet.

INTRODUCTION

The expansion of the green sea urchin, *Strongylocentrotus droebaciensis,* fishery in the Gulf of Maine began in 1987, after increased populations of urchins were observed in the region. Eventually the sea urchin fishery became the second largest in Maine (Harris and Chester, 1996). This fact has caused an increasing concern that stocks are declining in this region where waters have been over fished during the past decade (Harris et al., 1996). Urchin recruitment, which is vital to an urchin fishery, is variable in the Gulf of Maine. Northern parts of the Gulf of Maine have a recruitment level measured in the 10's/m2, where as the southern parts have been measured in the 1000's/m2. This difference could be due to the current patterns in the Gulf of Maine which may bring more larvae to the southern part of the Gulf (Harris, and Chester, 1996). If this hypothesis proves to be true, a sea urchin hatchery would need to be implemented for a future fishery to exist.

The commercial harvesting of sea urchins has also created a large industry in Japan, where total consumption is about 60,000 tons of whole sea urchins per year, of which 5,000 tons are imported catch (Hagen, 1996). As the demand for urchins continues to rise, fishing enhancement techniques are being developed in Japan to expand the fishery world wide.

In an attempt to meet the ever increasing demand for urchins, many companies have tried to follow Japan's lead to create a sustainable fishery and implement urchin aquaculture. To date, a truly successful aquaculture system in the United States has not been attained. Although, the Japanese were able to sustain a seed stock level of 60 million individuals on a diet of *Chaetocerous*, the harvestable population was still low(Hagen, 1996).

The Maine Aquaculture Innovation Center has also funded an aquaculture study in which the larvae cultured were fed algal diets consisting of *Isochrysis*, *Chaetocerous*, *Rhodamonus*, *Navicula*, and *Nitzschia*. However, only 3300 out of the initial 20 million larvae were able to settle and metamorphose (Dowling 1996).

A possible reason for the lack of a completely successful aquaculture system involves many variable factors. These include temperature, food ration, quality of food, type of algae fed to the larvae, and suitable substrate for settlement. Fortunately, there have been many studies which have dealt with these factors. Hart and Sheilbling (1988) compared the effects of food ration, temperature and algae type (*Dunaliella* and *Chaetocerous)*. Comparisons of these treatments were determined by measurements of the arm spicule lengths and rudiment sizes. They concluded that larvae growth is most rapid at 14°C, but did not statistically differ between different food rations and algal types.

Other experiments have also suggested that the shape and size of the echinoderm larvae change according to food quality and quantity. Fenaux (1994)studied the size and shape of the sea urchin *Paracentrotus lividus*. When a natural diet was compared with one that was enhanced with algae, the food limited form had longer arms, longer mid body length, a larger rudiment, and a delayed formation of the rudiment . Moreover, a study by Boidron-Metairon (1988) found that it took longer for echinoderms(the sea urchin, *Lytechinus variegatus*, and the sand dollar, *Dendraster excentricus)* that were fed a decreased food ration to develop a rudiment and metamorphose .

A study performed by McEdward (1984) examined and compared the lengths of the ciliated band, rudiment, body lengths, arm lengths, as well as protein content and metabolic activity of echinoderm larvae. It was found that as the larvae develops, the body length, arm length, surface area, volume, ciliated band length, protein content and

metabolic activity increase with positive allometric respect to one another. McEdward also determined that most of the growth during larval development is accounted for in the oral arms (1984). Thus, since the measurement of the oral arms may indicate the nutrient availability better than a measurement of the rudiment can, the spicule lengths in the oral arms were measured in this present study.

Other laboratory experiments have confirmed that urchin larvae can settle and metamorphose on settlement plates (Dowling, 1996; Harrold et al., 1991; Pearce and Scheibling, 1991). Plates with well developed microbial films induced more settlement than those plates that had less microbial film on them (Pearce and Scheibling, 1991). Thus, in this experiment, plexiglass plates, with developed microbial film and tub walls were examined for settlement of the sea urchin larvae.

The first objective of this study was to develop a diet which would sustain growth and lead to the greatest settlement of the green sea urchin larvae past the metamorphosis stage. Larvae were cultured at a constant temperature and given equal food ration of three different diet types (*Isochrysis*, *Nanochloropsis*, and a mixture of *Isochrysis*, *Nanochloropsis*, and *Dunaliella*). Since it has been suggested that a better diet leads to smaller arm spicules (Shilling, 1995), the spicules were measured to compare the three diets during larvae growth and development. The urchins that settled were quantified and the test sizes of the settled urchins were measured for comparisons between the two substrates types and the different diets. The results obtained from this study can be used to further develop methods best suited for the aquaculture of the green sea urchin.

MATERIAL AND METHODS

Algal cultures

Three types of algae, *Dunaliella tertiolecta, Isochrysis galbana* and *Nanochloropsis* were cultured in a sea water medium with fritz A and B nutrients, from Aquaculture Supply Co. The algae *Dunaliella* was supplied by Dr. L. Jehnke at UNH and both *Isochrysis* and *Nanochloropsis* were supplied by Mr. King at UNH. To ensure maximum growth rate and surface area, the algae were grown in 250 ml test tubes and supplied with an air bubbler. These three algae were to be used to feed the urchin plutei.

The absorbency reading of each algae was measured by using a Colman 295 spectrophotometer. Cell counts were determined by placing 1-2 drops of algae onto a hemocytometer and enumerating by use of a compound microscope. Graphs of the absorbency versus the number of cells were later used in determining the volume of algae required to feed the larvae 15,000 cells/ml.

Urchin Fertilization

Adult urchins were collected on November 11, 1996, at Nubble Light , Maine, using SCUBA in about eight to ten meters of water. They were transported to the UNH Marine Coastal Laboratory, located at the mouth of Portsmouth Harbor, New Hampshire. The urchins were placed into a sea water table with running water and were fed kelp, *Laminaria saccharina* weekly.

Three trials were performed, however the same process for fertilization was used for each. The adult urchins were induced to spawn gametes by injection of ~5 ml of 0.5 M KCL. The spawning females were inverted to shed their eggs and placed in sea water, so that the eggs would settle. The spawning male urchins were placed in a dry bowl since urchin sperm becomes active on contact with sea water. The eggs from 3-5 female urchins were extracted with a pipette into clean filtered sea water. A small amount of sperm, approximately one drop, was then added to the eggs and gently stirred for 1-2 minutes. The mixture of sperm and eggs was kept at 6°C for 15-20 minutes. The eggs were observed for fertilization of most of the eggs, approximately 90%, and then washed in filtered sea water to remove excess sperm . The fertilized eggs were then placed in finger bowls in a monolayer. After about five days, the urchin larvae developed a gut and were placed into their respective 40 liter tub or 1000ml beaker, as in the case of the test trial of *Isochrysis*. Each tub contained approximately 7,400 urchin larvae.

Larval growth, monitoring and feeding

The source of sea water throughout these experiments was from the UNH Coastal Marine Lab, Newcastle, NH. For each trial, the tub was filled with 30 liters of filtered UV sterilized sea water and was equipped with an air bubble disc to keep the water circulated in a 10°C cold room. Three trials were run during the course of the semester. The first trial was a test trial to determine whether an *Isochrysis* diet would allow the larvae to live and develop. Half of the larvae were split into three 1000 ml beakers (Figure 1). Each beaker was filled with 800 ml of sea water and the larvae were fed 15,000 cells/ml of *Isochrysis* daily. Water changes were made every 3 days using a 4 inch PVC filter with a 110 um mesh. The other half of the larvae were placed into a 40 liter tub and fed 15,000 cells/ml of *Nanochloropsis*.

The second trial was started on February 16th. Approximately 7,400 larvae were each placed into three 40 liter tubs. The larvae were fed 3 different diets of algae daily: Tub A was fed 15,000 cells/ml of *Nanochloropsis*, Tub B was fed 15,000 cells/ml of

Isochrysis and Tub C was fed 5000 cells/ml each of the three algal species (*Nanochloropsis, Isochrysis and Dunaliella*) (Figure 2).

Half of the water in each tub was changed every other day. The larvae and water were filtered through a siphon into a 24 inch PVC pipe with a 110 um mesh. The pipe was placed in a 5 gallon bucket and the water overflowed into a fish bucket (Figure 4). The larvae remained suspended in the water column since they were too large to filter through the mesh. Full water changes and cleaning of each tub was done weekly.

The third trial was performed almost the same as trial 2 ; however four different tubs were used. The larvae were fed diets of *Isochrysis ,Nanochloropsis,* a mixture of *Isochrysis, Dunaliella and Nanochloropsis*, and a mixture of *Nanochloropsis* and *Isochrysis*.

Originally, twenty larvae from each tub were observed and photographed weekly. By overlapping the slides of the larvae with a slide of a stage micrometer, the spicule length of the larvae could be measured(figure 5). Since this method was not as accurate as first thought, the urchins were later measured directly using an ocular micrometer under a compound microscope.

Once the larvae reached the eight arm stage, plexi glass plates were placed in the tubs for the larvae to settle on and metamorphose. These plates were originally placed in a tank of raw sea water for three days so that they could acquire a thin layer of microorganisms, which makes a good settling substrate for urchins (Pearce & Scheilbing 1990).

The plates were checked daily for settlement by examining the plates for white or brown spheres. The "sphere" was then pipetted up onto a slide and looked at under a compound microscope. When the first settled urchin for each tub was found, pictures were taken and the date was recorded. The urchins were still fed for one week after the first settled urchin was found to ensure that any urchins left in the water column would be fed until they settled. About two weeks later, the settled urchins were counted.

In order to count the number of urchins that had settled, magnesium chloride was used to relax the urchins so that they would detach from the surface that they were attached to. The urchins found on the plates and in the tubs were counted separately. The urchins on the plates were placed in a 7 % MgCl solution (made from 70 ml MgCl and 1000 ml sea water) and allowed to sit for 15-20 minutes. The dishes were then examined under the dissecting scope where the urchins were magnified 40x, and were counted and measured using an ocular micrometer.

Concentrated (100%) magnesium chloride (2,000 ml) was also poured into the 30 liter tubs of filtered UV sea water., agitated and left for 15-20 minutes. All of the MgCl

sea water was filtered out of the tub and into the 24 inch PVC pipe. The PVC pipe was inverted and cleaned out into a 5 gallon bucket. The contents in the bucket were allowed to settle and the top layer of water was siphoned out back into the PVC pipe. The contents on the bottom of the bucket was analyzed; 87 urchins were from each tub measured and the rest were counted. The newly settled urchins were transferred to the coastal lab and were put in dish pans with running sea water through the dish pans.

Statistical Analysis

T-tests were performed to determine if there was a difference between the two algal diets on the growth and settlement of the urchins. The tests compared the larval arm spicule lengths, settled urchins' test sizes, and the amount of settlement between the two diets. Bar graphs were then constructed to illustrate any differences between the two diets and substrates.

RESULTS

Trial 1 indicates that *Isochrysis* and *Nanochloropsis* can sustain an urchin larval culture to at least the four arm stage. However, no larval measurements were recorded since both treatments died.

In trial 2, the diets of *Isochrysis* and *Nanochloropsis* (treatment C& D) showed no significant difference in the arm spicule lengths of the urchins during their plutei stage (table 1). However, a pattern appeared of the *Isochrysis* fed urchins having longer arm spicules than the urchins fed *Nanochloropsis* (figure 6, table 1). After settlement, the *Isochrysis* fed urchins had a significantly larger test size ($p= 0.00$, figure 8, table 1). The *Nanochloropsis* diet led to earlier settlement (36 days) than the diet of *Isochrysis* (55 days)(table 2). Both of the diets displayed greater amounts of settlement on the tub walls and bottom than on the plates (figure 9). However, the test diameter was not significantly different between the settlement substrates (tub versus plates)(figure 9). The *Nanochloropsis* diet allowed 631 (8.5%) of the urchins to settle while the *Isochrysis* diet only yielded 93 (1.25%) settled urchins. The diet of *Isochrysis, Nanochloropsis* and *Dunaliella* (treatment E) did not sustain the urchin larvae past the four arm stage.

In trial 3, the treatments F (*Isochrysis)*, G (*Nanochloropsis*), H (a mixture of *Nanochloropsis, Dunaliella* and *Isochrysis*) and I (a mixture of *Isochrysis* and *Nanochloropsis*) did not live past the four arm stage. Furthermore, there was no

significant difference between arm spicule lengths between the tubs of F, G and H during week 2 of their growth (figure 7, table 1).

DISCUSSION

The green sea urchin fishery in the Gulf of Maine has recently been plagued with problems. These include over harvesting, poor roe quality, and low recruitment in the northeastern part of the Gulf (Harris et al., 1996). The need for a sustainable aquaculture system has become increasingly apparent in recent years. A sustainable aquaculture system could not only supply the major, existing commercial industry, but could also supply a seed population for enhancement of natural populations and/or sea ranching in aquaculture lease sites..

Although our results did not indicate a single diet which would result in both sustainable growth and settlement of the urchin larvae, the observations made suggest that a *Nanochloropsis* diet is the most effective, of the diets tested, for a sustainable urchin aquaculture. Such a diet results in earlier settlement rates as well as a greater density of urchin settlement (figure 4). The *Nanochloropsis* diet allowed for 8.5% of the *S. droebachiensis* larvae to metamorphose in the second trial, while only 1.3 % of the larvae fed *Isochrysis* settled (table 2). Furthermore, the larvae fed *Nanochloropsis* showed a slight trend in having smaller oral arms, indicating a better quality diet (figure 5, table 1).

For urchin aquaculture, the ideal condition to achieve would be to have a higher percentage of larger urchins settle in a shorter amount of time and to insure a high quality diet. Thus, perhaps a larger ration diet of *Nanochloropsis* would be the most sustainable diet. A larger ration may lead to longer arm lengths, and, thus, larger urchins. One experiment which has suggested such a trend, was a study by Boidron-Metairon (1988) which measured the post oral arm lengths, as well as the midbody lengths, of the sea urchin ,*Lytechinus variegatus* , and the sand dollar, *Dendraster excentricus*. This study showed that at first the larvae had longer arm lengths with decreased food ration, but eventually the oral arms of the treatment with the higher ration became longer.

Trial 1

Trial 1, which consisted of treatments of *Isochrysis* and *Nanochloropsis*, did not survive past the four arm stage. This may be due to improper filtering of the *Nanochloropsis* tank (a siphon with a 110 mesh on the bottom of the tube was used initially) and infrequent water changes every third day instead of every other day on both the beakers and tub. Ciliates were found in the tub, which indicated contamination of the water. The *Isochrysis* trial was originally run to determine if urchin larvae would be able to live on an *Isochrysis* diet (which is why the treatment consisted of three 1000 ml beakers, instead of a 40 liter tub).

Trial 2

Arm Spicule Length

Comparisons of larval arm spicule length between the two diets suggested a trend (although not significant) that *Nanochloropsis* fed larvae had smaller spicules than *Isochrysis* fed larvae (figure 6, table 1). A possible explanation for the smaller growth of larvae fed *Nanochloropsis* may be due to the amount of nutrients, amino acids, sugars and fatty acids released by the algae (Shilling, 1995). *Nanochloropsis* may release a better quantity and quality of nutrients. The urchins fed *Isochrysis* may need longer arms to get more food by creating more of a surface area to feed with, since the food may not be as nutrient dense. Longer oral arms increase the capacity of the urchin to capture food. This increase may also serve to increase the larvae's defense against predators (Fenaux, 1994).

Settlement

The difference between the settlement rates of the two treatments also seems significant (table 2, figure 10). However, since there was only one replicate of the treatment, a t-test could not be performed and, thus, a p value could not be derived. Our observations also indicated a faster settlement rate for urchins fed *Nanochloropsis* (36 days) than those fed a diet of *Isochrysis* (55 days). There was a much greater amount of settlement by *Nanochloropsis* fed urchins. Both of these results help to further suggest that a diet of *Nanochloropsis* is a better quality diet for aquaculture. *Nanochloropsis* fed urchins may have settled early for the urchins were able to store up the required nutrients earlier and thus, were ready to settle sooner. Since *Isochrysis* may not be as rich a nutrient diet, the larvae required more time to store up the essential nutrients and, thus, settled later. As Boidron-Metaion (1988) speculates," the larvae are adapted to use

energy first for the development of feeding structures to insure a high ingestion rate before using it for development of the adult rudiment....when larvae are in a nutritionally dilute environment" .

Previous studies have recorded settlement rates of three weeks for *Strongylocentrotus droebaciensis* larvae fed a mixed diet (Dowling, 1996). Another experiment using a diet of *Dunaliella tertiolecta* showed a settlement rate of 33 to 51 days (Pearce and Scheibling, 1990).

The observations in this study were subjected to possible factors of human error. Observations for settlement were made daily but the identification and isolation of a settled sea urchin can be difficult. The urchins were easily blended with the white substrate plates, thus it is possible that settlement occurred prior to the date of observation.

Test Size

The test sizes of the settled urchins which were fed *Nanochloropsis* were significantly smaller than those fed *Isochrysis* ($p = 0.00$, table 2, figure 7). The test size of *Isochrysis* fed larvae may be larger due to the fact that they were in the water column longer. This would have allowed them to acquire more food than the *Nanochloropsis* treatment. Since the arm spicule lengths of the *Isochrysis* fed larvae were slightly longer during the larval free swimming stage, which may have lead to an increase in size when they settled. One way to culture larger urchins on a *Nanochloropsis* diet would be to feed the larvae more than 15,000 cells/ml

Settlement Substrates

Settlement rates indicate, unexpectantly, that the majority of urchins, of both diets, settled mostly on the walls of the tubs, rather than on the plexi glass plates. This result could be important for future aquaculture projects because past aquaculture attempts may have overlooked the fact that the urchins may have settled on the vats or tubs, as well as on the settlement plates.

This result also indicates that the plexi glass plates may not be as favorable as other substrates for settlement. It is possible that the plates were subject to agitation and were not able to support the larvae or were knocked off their substrate and into the water column. In addition, three days may not have been enough time for microorganisms to form a sufficient slime layer on the plates, and thus settlement was not induced to its greatest potential. Another possibility is that the plates were not exposed to enough light. A study by Pearce and Scheibling (1991) found that the percent metamorphosis in

response to acrylic plastic plates with marine microbial film was higher when films were developed in light than in dark and higher with age of the film.

Other substrates for settlement to be considered may be wave plates (with contours which increase the surface area that the urchins may settle on), clam or mussel shells, astroturf panels or coralline algae. Previous studies have revealed that coralline red algae induces urchin larvae to settle and metamorphose to the juvenile stage (Pearce and Scheibling, 1990). More than 85% of laboratory reared larvae of *S. droebachiensis* were induced to settle on coralline species of algae in an experiment by Pearce and Scheibling (1991). They also observed a high rate of metamorphoses of the larvae in response to a variety of macroalgaes .

Trial 3

The third trial was not as successful as trial 2. The larvae did not live past the four arm stage. Their death may have resulted from the fact that the urchins were spawned late in the spawning season. This may have resulted in smaller larvae which, consequently, fell through the 110 um mesh. Even though table 1 shows that the urchins in trial 3 were about the same size as in trial 2, thoseurchins may have been the few and only exceptions that had not fit through the mesh. Another explanation may be that perhaps the larvae were not as healthy as larvae cultured earlier in the season. Unfortunately, due to the loss of the larvae, there is no data on how a diet consisting of both Isochrysis and *Nanochloropsis* affects the growth and settlement of urchin larvae.

The mixed diet of *Dunaliella*, *Nanochloropsis* , and *Isochrysis* did not sustain urchin larvae in the second nor the third trial. The spicule length did not differ significantly between the two diets at 2 weeks old. This die off could have been due to improper filtering techniques or human error. Even though *Dunaliella* has been shown to be an effective algae for aquaculture (Hinegardner, 1969 ; McEdward, 1984), it was difficult to culture and maintain a sustainable concentration. Thus, we found it was not a successful algae to culture and use for urchin aquaculture.

Our results suggest that a diet of *Nanochloropsis* and *Isochrysis* might be a better mixture to use for it may lead to larger urchins which settle sooner and have a greater settlement rate. Plus, culturing *Nanochloropsis* and *Isochrysis* was very successful because both algae were able to sustain high concentration of cells/ml.

Further Studies

Additional studies are needed to gain a further understanding of urchin aquaculture. Such studies may include a larger ration of food, different types of diets

(including *Rhodemonas* and *Chaetocerous*), greater replications of treatments, and the testing of different settlement substrates. Also, both oral arm spicule lengths should be measured instead of only one.

Conclusion

In conclusion, this study suggests that to improve a sustainable urchin aquaculture population, a diet of *Nanochloropsis* should be used. Such a diet leads to earlier and greater settlement than diets consisting of *Isochrysis* and *Dunaliella* algae. *Nanochloropsis* may also provide a greater quantity and quality of the essential nutrients needed for settlement into and past the metamorphosis stage. Lastly, when performing urchin aquaculture, the whole tub/vat should be examined for settled urchins because the settlement substrate being used may not be as effective for settlement as the walls of the tub/vat.

ACKNOWLEDGEMENTS

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Trial 1

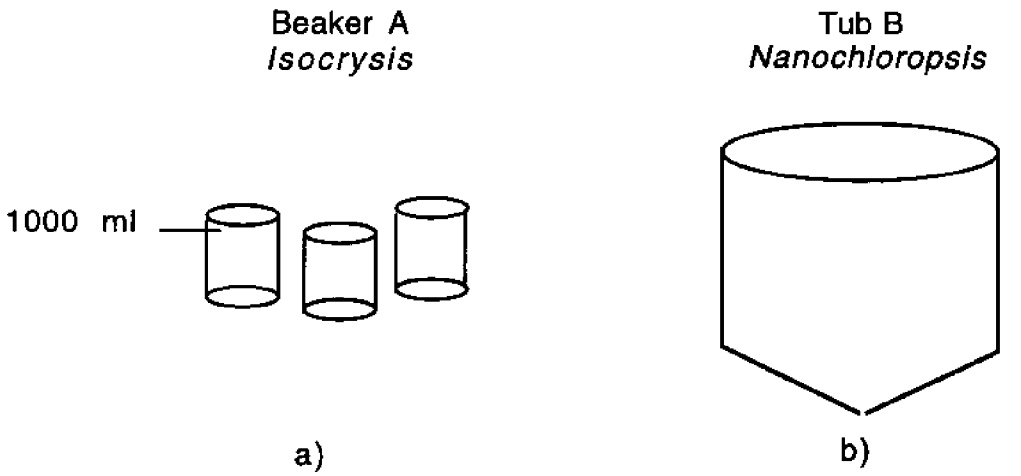

Figure 1. a) Small batch of *Isocrysis* (800 ml) b) 1st big tub of larvae.

Trial 2

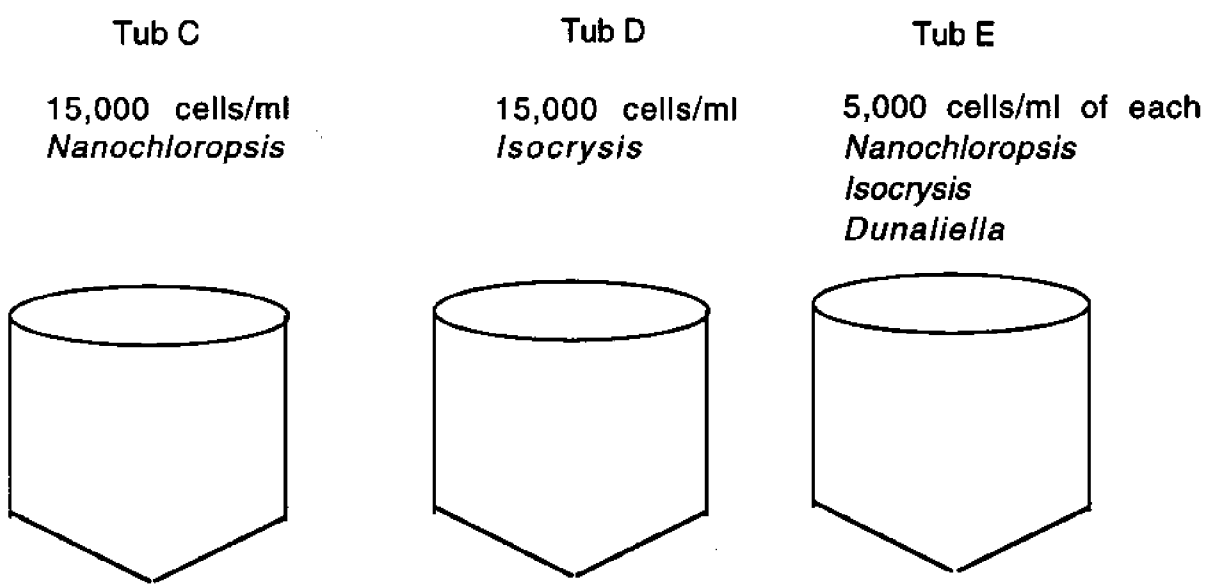

Figure 2. Three treatments of different diets

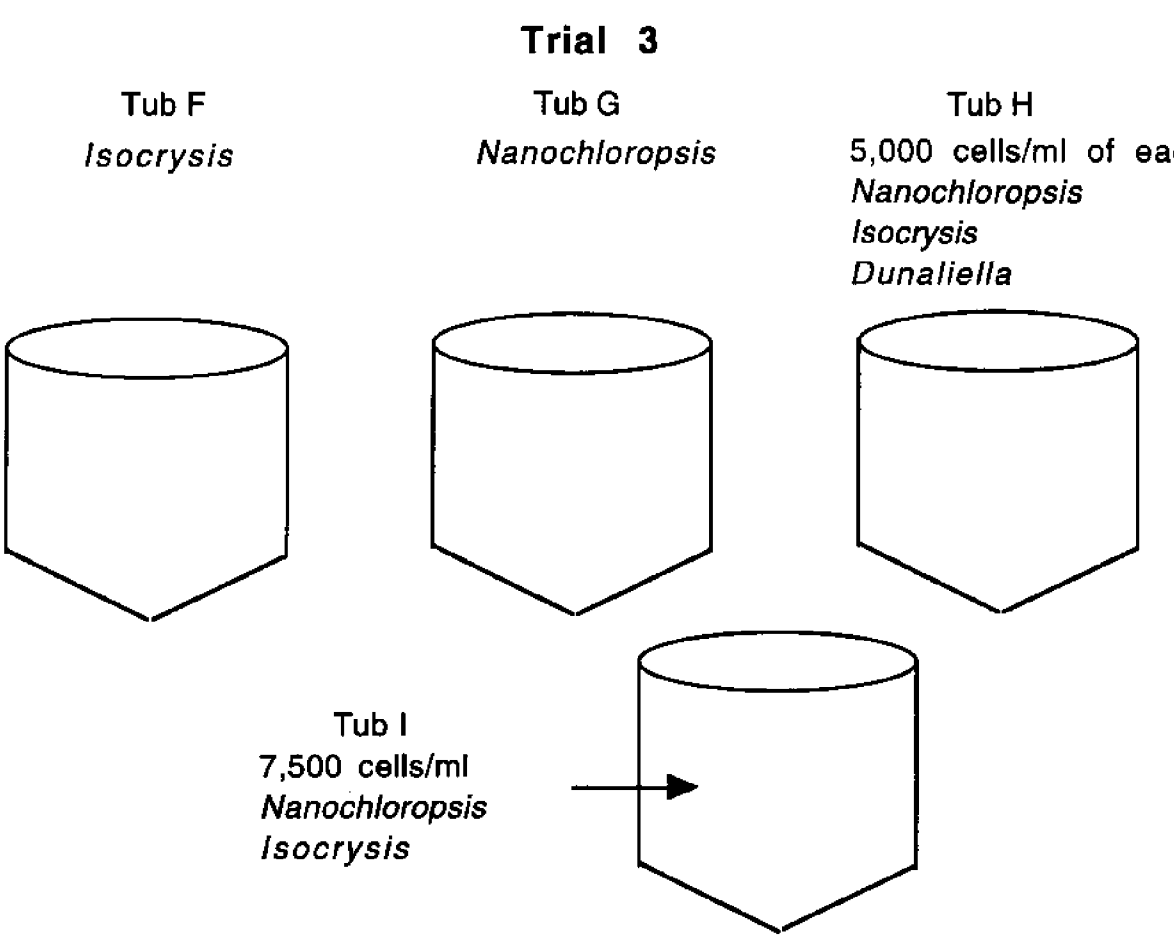

Figure 3. Cultured for more replicates and different diets.

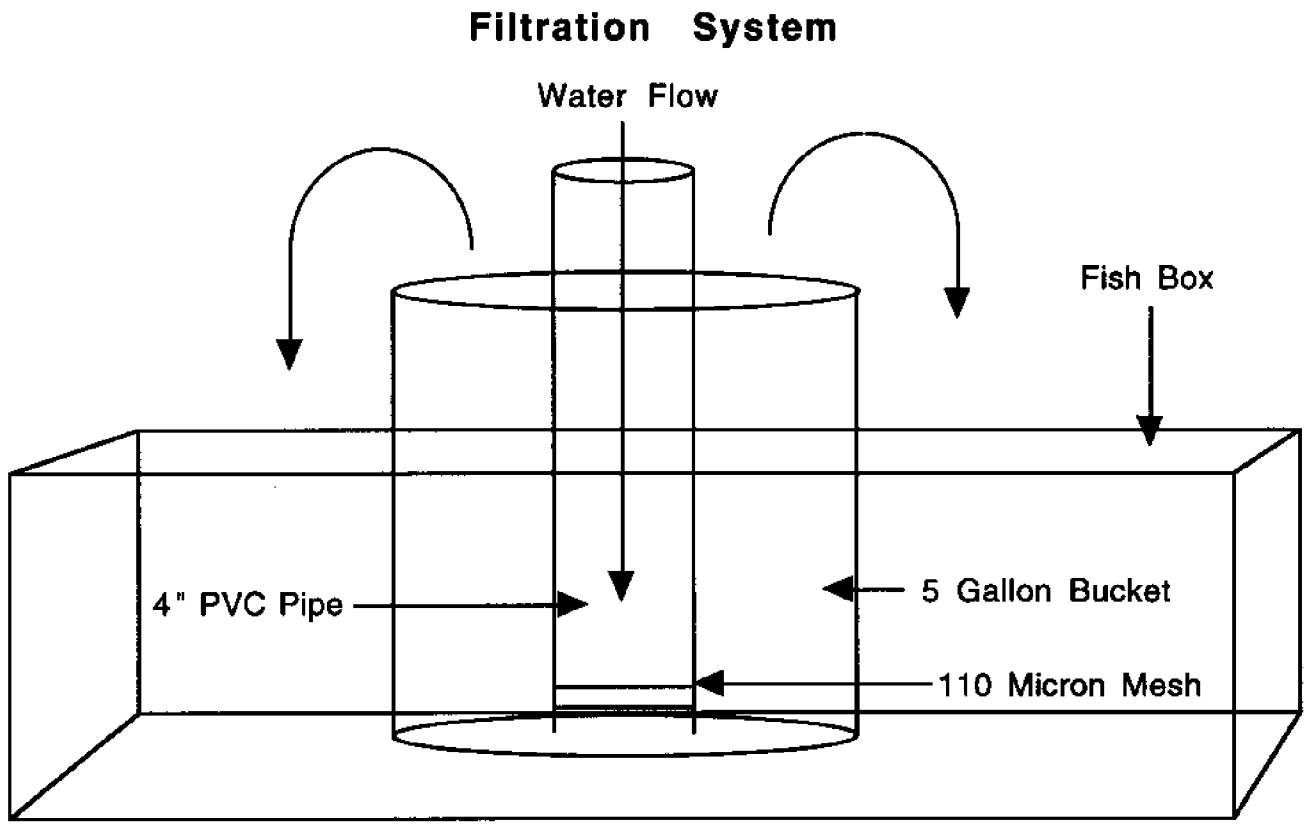

Figure 4. Filtration system used to keep larvae in the tubs.

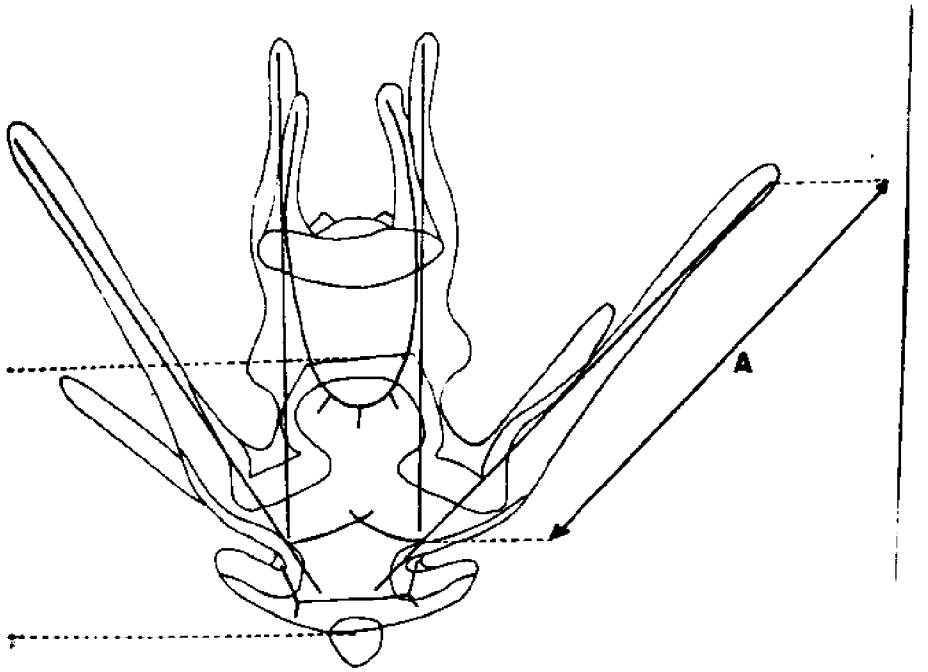

Figure 5 The spicule (a) was measured

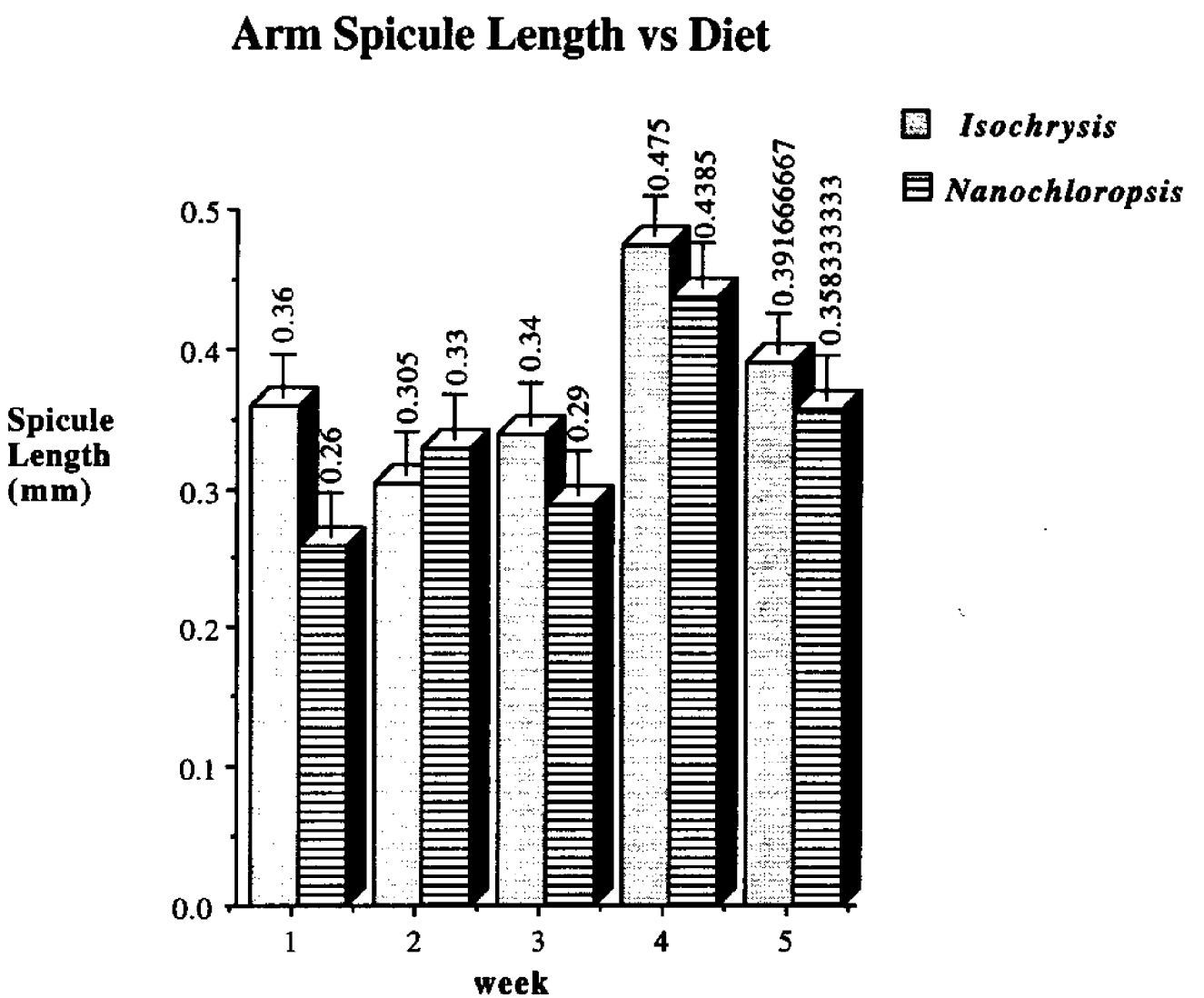

Figure 6

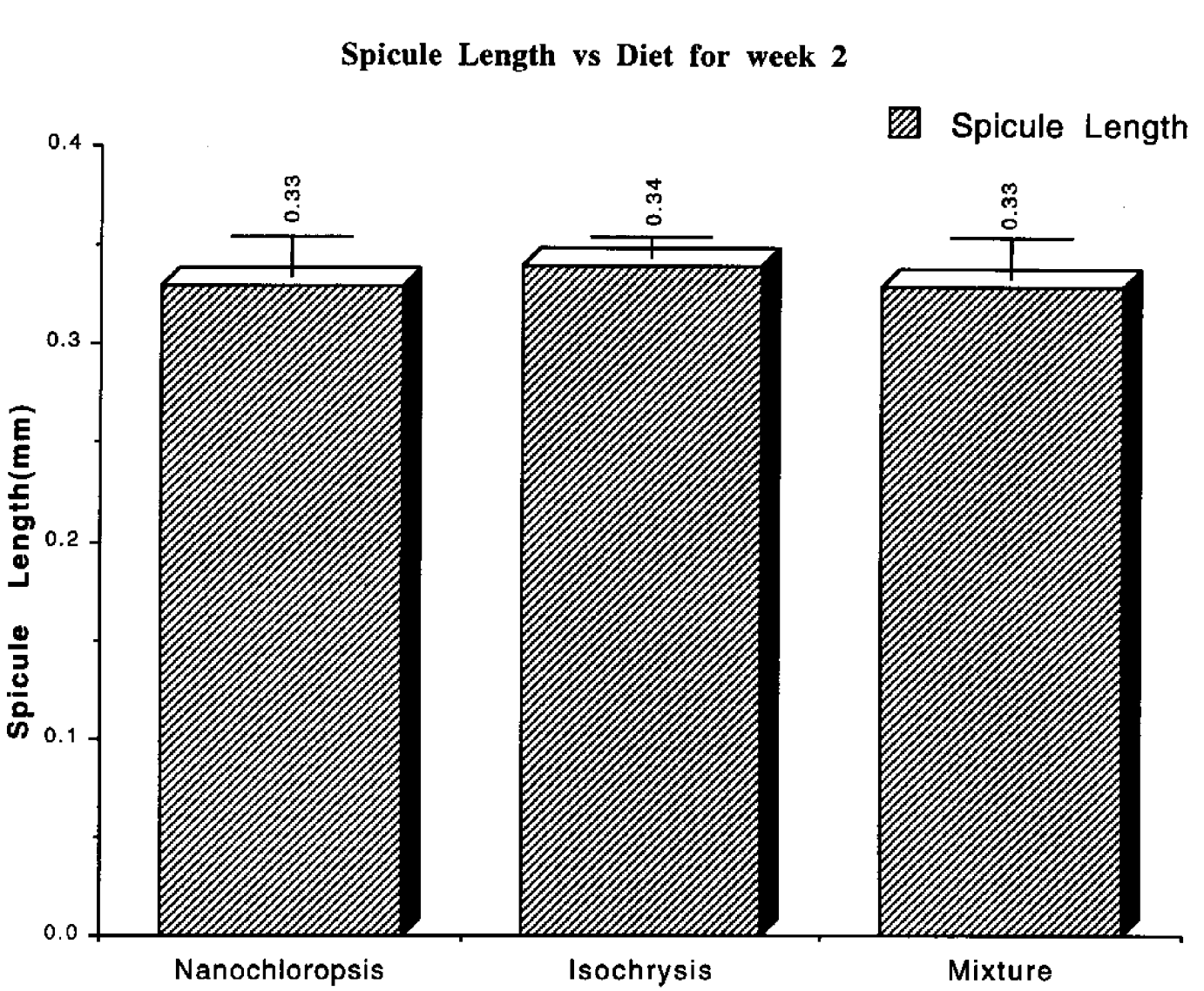
Spicule Length vs Diet for week 2
Spicule Length
0.4
0.3
0.2
0.1
0.0
Spicule Length(mm)
0.33
0.34
0.33
Nanochloropsis
Isochrysis
Mixture

Figure 7

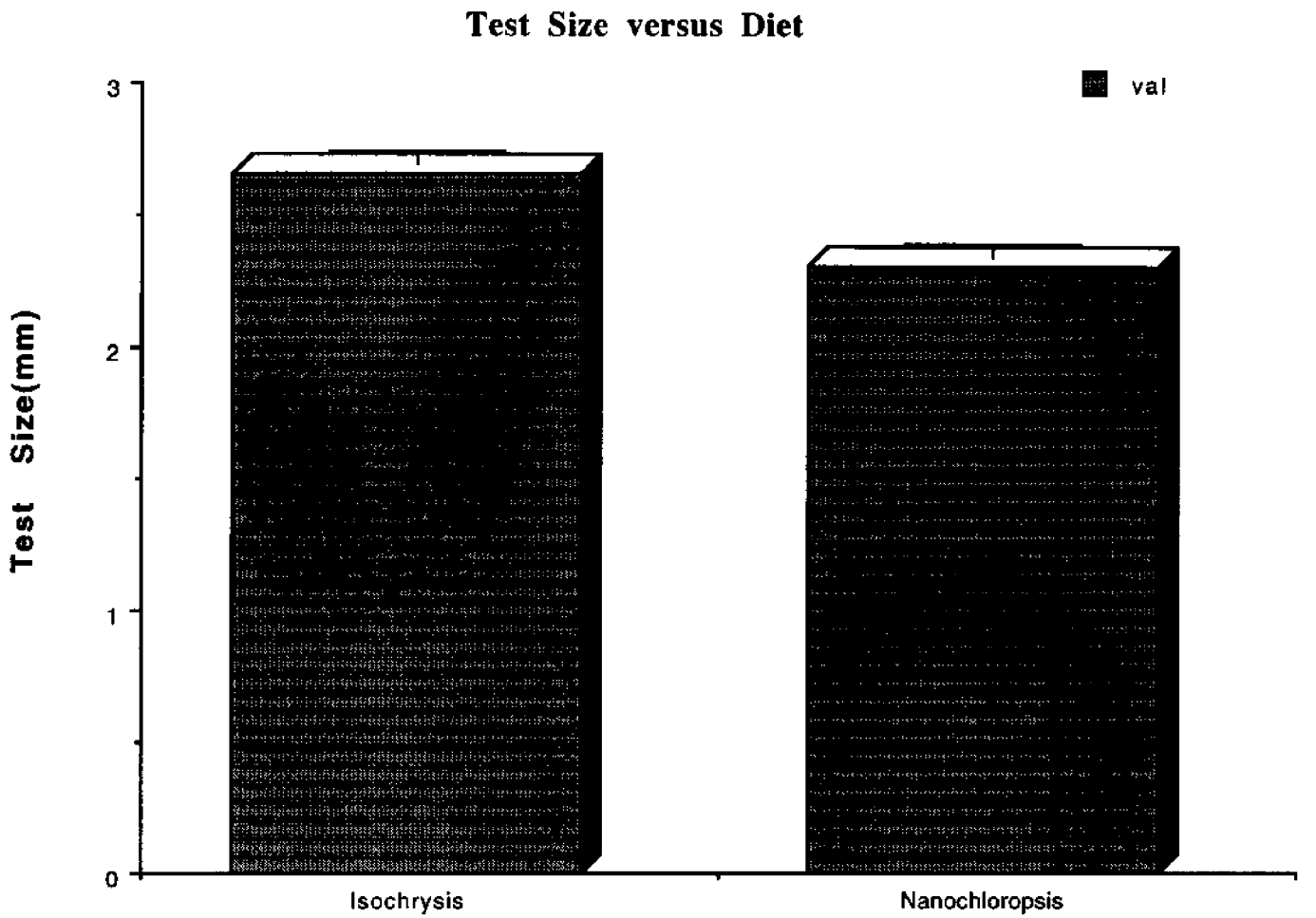
Test Size versus Diet
val
Test Size(mm)
3
2
1
0
Isochrysis
Nanochloropsis

Figure 8

Urchin Test Size vs Diet

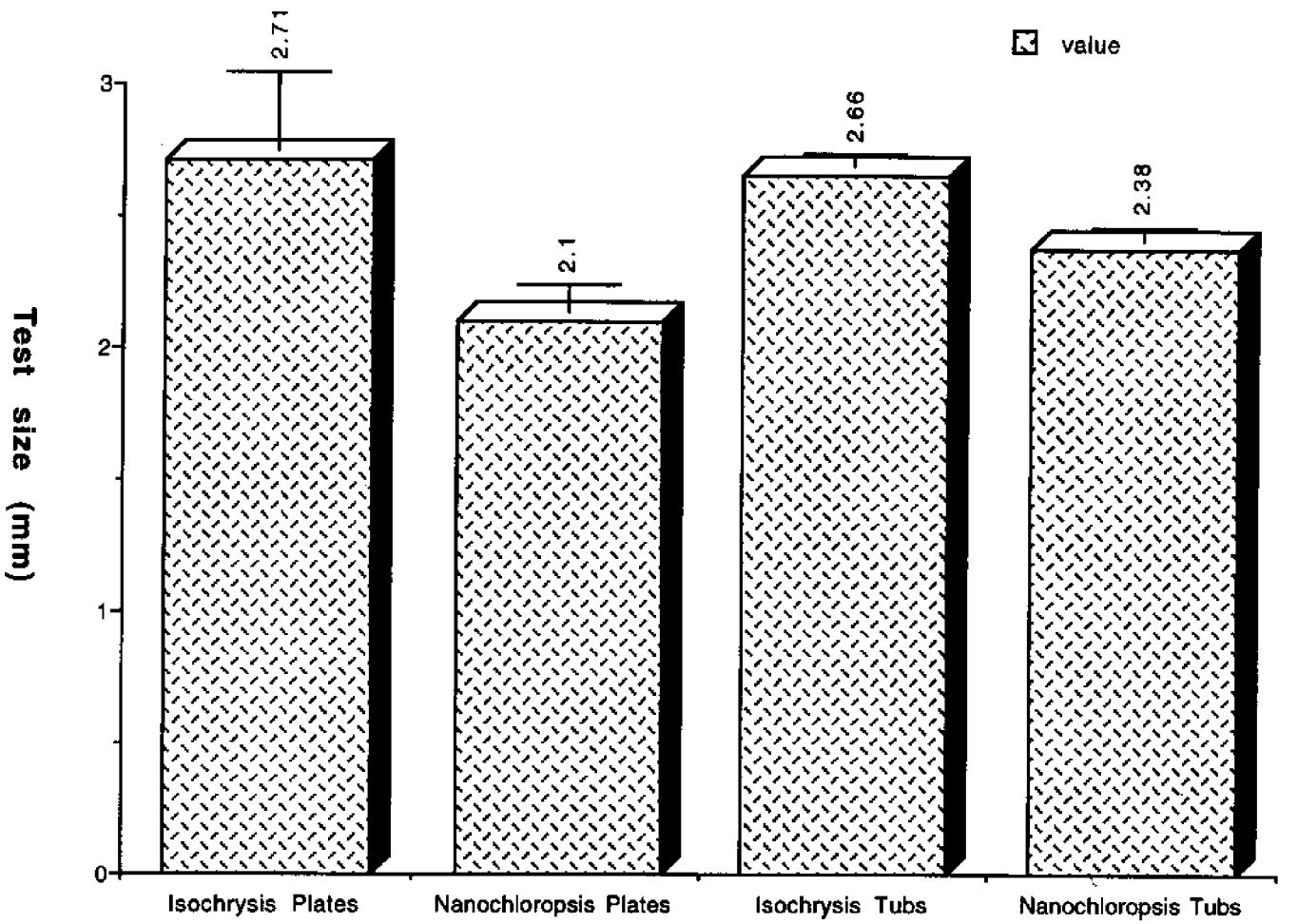

Figure 9

Trial 2

Urchin Settlement versus Surface Area

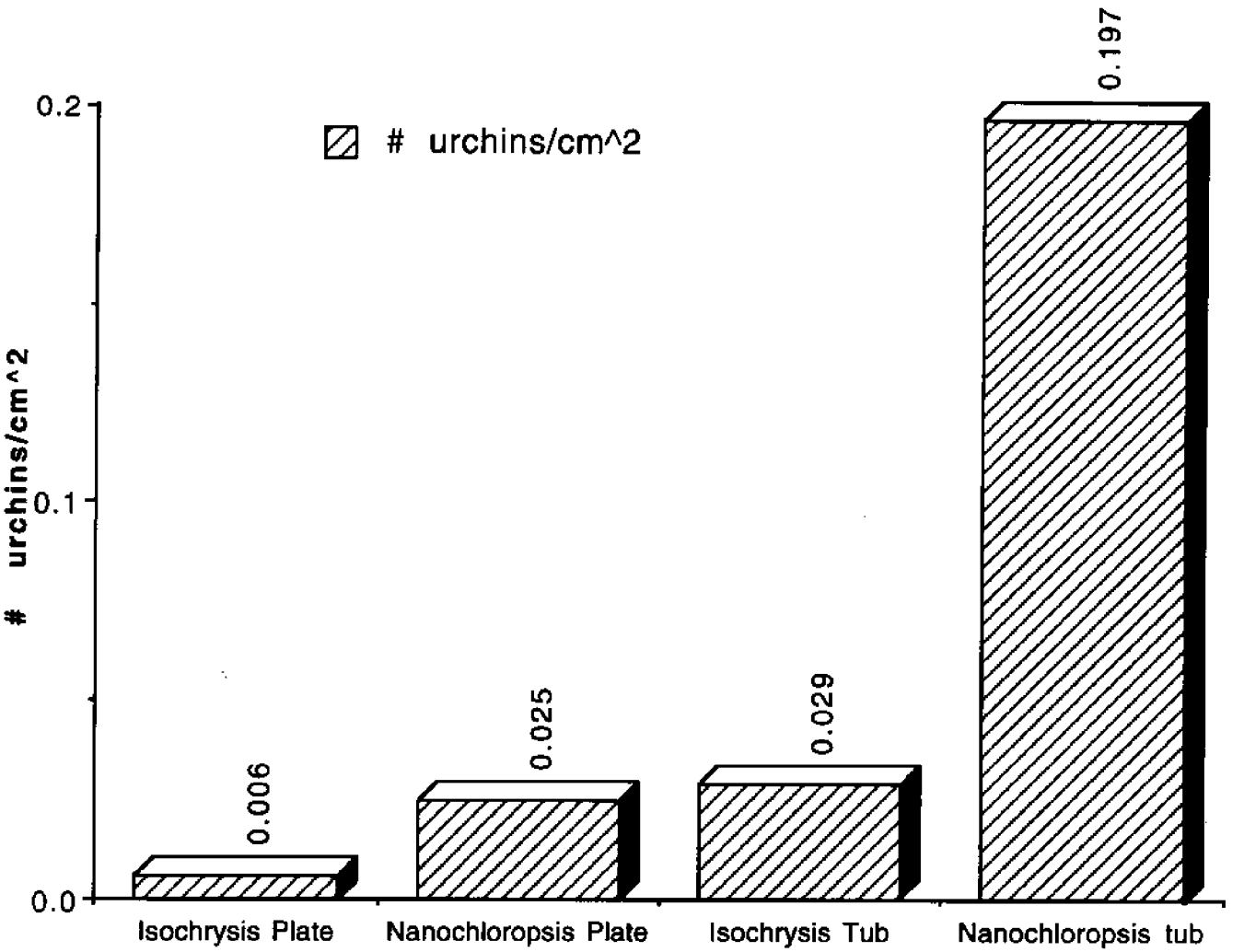

Figure 10

Trial 2				
Average Spicule Length (mm)				
	Isochrysis	*Nanochloropsis*	T-test value	P-value
week1	0.36	0.26		
week2	0.31	0.33	0.7873	> 0.4611
week3	0.34	0.29	1.6984	> 0.1647
week4	0.48	0.44	2.4238	> 0.0202
week5	0.39	0.36	1.1509	> 0.2766

Trial 2				
Substrate	Average Test Size (mm)			
	Isochrysis	*Nanochloropsis*	T-value	P- value
Tub	2.66	2.38	2.27	> 0.03
Plates	2.71	2.1	4.63	> 0.00
Combined	2.66	2.31	5.82	> 0.00

Trial 3				
Average Spicule Length (mm)			T-value	P-value
Isochrysis	*Nanochloropsis*	Mixture		
0.34	0.33	0.33	0.2988	> 0.7439

Table 1. Size of urchins from 1 week after fertilization through settlement based on different diets.

Diet	Days from fertilization to settlement	# urchins/cm^2		Total # Urchins		Percent Settlement
		plates	tub	plates	tub	
Nanochloropsis	36	0.025	0.197	30	601	8.50%
Isochrysis	55	0.006	0.029	6	87	1.25%

Table 2. Settlement rates based on different diets and substrates