THE APPLICATION OF SODIUM ALGINATE MICROPARTICULATE DIETS FOR NUTRITION RESEARCH ON JUVENILE AND LARVAL BIVALVE MOLLUSCS

by

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
CANADA
May 1994

ISBN 0-315-98877-0

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Abstract

The expanding molluscan culture industry and the concomitant increasing reliance on hatchery produced spat have created a demand for detailed fundamental knowledge on the nutritional requirements of bivalve molluscs. Our knowledge in this field has lagged behind that of fish and crustacean nutrition simply because it is more difficult to do. Involved is the production of synthetic diets of defined nutrient composition and the development of protocols for testing and evaluating these diets.

This thesis has focused on a sodium alginate microparticulate diet which was tested in nutrition studies on larval and juvenile bivalve molluscs. New methods of diet presentation, quantifying ingestion and measuring digestibility were used. The microparticulate diet was ingested and assimilated by juvenile sea scallops (*Placopecten magellanicus*) and bay scallops (*Argopecten irradians*). However, growth and survival were greater among scallops fed algae. In contrast, short term tests with bivalve larvae and spat (*Crassostrea gigas* and *Tapes philippinarum*) show that the microparticulate diets support growth and survival at least as well as the algae diets.

The effect of current velocity and food concentration on the ingestion rates of young sea scallops was tested in a flow tank using a factorial design with fifteen treatment combinations to evaluate ingestion at three current velocities (5, 10, and 15 cm sec^{-1}) and five food concentrations (5, 10, 20, 40, and 80 mg l^{-1}) of the microparticulate diet, labeled with Fluoresbrite™ beads. Digestive tracts were excised, homogenized and the number of beads enumerated to quantify ingestion. Ingestion of the microparticulate diet was greatest when fed at 20 mg l^{-1} 10 cm sec^{-1}. This direct method for quantifying ingestion is more accurate than indirect methods, and it can be also be used to investigate other variables which impact on feeding. Based on this work recirculating units were designed for long term nutrition experiments with juvenile scallops, which kept 2 l of seawater in motion at a flow rate of 8-9 cm. sec^{-1}, and the ration of microparticulate diet in suspension. These recirculating units were also successfully designed to be inexpensive, and easily serviced and maintained.

To measure digestion, the sodium alginate diet was labeled with a Procion dye and was subsequently incubated in vitro with digestive enzymes from scallop crystalline styles and diverticula. Dye release was monitored and compared to treatments of pure amylase and protease enzymes. This *in vitro* digestion method is useful for testing the digestive capabilities of various bivalves prior to *in vivo* testing with the microparticles. Microparticles labeled with either the Procion dye or the Fluoresbrite™ beads were easily seen (at a low magnification) inside the larvae of three species tested allowing ingestion and digestion of microparticles to be confirmed and monitored.

In conclusion, the sodium alginate microparticulate diet shows great promise as a tool in nutrition studies with larval and juvenile bivalve molluscs. Equipment and protocols designed and tested in this thesis research will allow researchers to focus on specific nutrient requirements of bivalve molluscs and to develop practical, economical diets.

Acknowledgments

This work was completed under the sponsorship of the Department of Fisheries and Oceans. I thank Dr. John Castell for encouraging me to enter a PhD. program and other DFO employees, especially Ms. Shirley Parker, Dr. Robert Cook, Dr. René Lavoie, Dr. Jim Stewart, and Dr. John Pringle for their encouragement and patience.

I thank the members of my supervisory committee (Dr. Ron O'Dor, Dr. John Castell, Dr. Bob Ackman, Dr. Gary Newkirk) for their many contributions which improved this work and Dr. Judith McDowell Capuzzo (National Marine Fisheries Services, Wood's Hole) for her role as external examiner.

It was Dr. John Castell who initially provided much of the inspiration for this research and continued to provide not only constructive criticism, but also an objective philosophical viewpoint. I thank my supervisor Dr. Ron O'Dor for his consistent encouragement, endless enthusiasm, and frequent flashes of brilliance. His unique skills for problem solving are greatly admired by his students and I trust that I have acquired a few new tools. I thank Dr. Robert Ackman for his generosity with his laboratory facilities, his extensive reference files, and most importantly, his challenging questions.

I also thank my friend and colleague, Ms. Linda Boston, for her assistance and enduring encouragement. My thanks to Dr. Ellen Kenchington and Mr. Ken Freeman for their continued confidence in me. I thank my fellow graduate students, who supported my work in many individual and special ways: Mr. John Shears, Mr. Danny Jackson, Ms. Yonghong Guan, Mr. Andrew Harris, Mr. Cyr Couturier and my dear friend Ms. Angelica Silva. I thank Mr. Andrew Hebda and Dr. Gwyneth Jones for their many diverse contributions. I am grateful for the superb technical assistance provided by Ms. Nancy McAllister Irwin, Ms. Donna Krailo, and Ms. Becky Field.

I thank Drs. Mindy Goldstein and Jim Hayward of Collaborative Labs, Long Island for their willingness to co-operate and support this program. My sincere thanks to Dr. Chris Langdon (Hatfield Marine Science Research Laboratory, Oregon) for the knowledge and experience gained while working in his lab.

My thanks to Dr. Mike Dadswell, Mr. Paul Neima and Mr. Mike Helm for providing experimental animals. Dr. Dadswell was also a constant source of information and stimulating discussion. My thanks to Mr. Wayne Rogers for his encouragement and advice. I thank Dr. Jon Grant and for his valuable comments throughout the course of this program and also Dr. Bob Rodger for his direction in the statistical analyses of the data.

My deepest thanks and blessings go to Dr. Kim Harrison, who provided countless hours of constructive criticism, guidance, moral support, and the devotion of a lifelong friend

Heartfelt thanks to my husband, Mr. Les Howie, for his endurance, technical assistance, and infinite patience. My thanks and blessings to my mother-in-law, Mrs. Joyce Howie, and my two sons, Masters Andrew and Adrian Howie, who, in countless ways, have made this possible.

Part I - Introduction

1. Tools of Nutrition Research

Fundamental to our understanding of bivalve nutrition are the development and utilization of formulated microparticulate diets. This permits the manipulation of dietary composition and the subsequent identification of specific nutrient requirements. To be useful as a research tool, the diet must be readily ingested, digested, and assimilated. As such, the diet also gives information on feeding mechanisms, digestive processes, and physiological requirements. The optimal formula for a synthetic diet depends on species, age differences, and numerous environmental parameters. These variations in nutrient requirements will provide yet more knowledge on physiological processes. The synthetic diet, therefore, is a tool which is essential for the examination and comprehension of bivalve nutritional physiology.

This thesis explores the use of a sodium alginate microparticulate diet which has been formulated for nutrition studies with larval and juvenile bivalve molluscs.

1.1. History of Methods

In order to identify an animal's dietary requirements and produce a balanced dietary ration, an extensive research program is required. This is costly, time consuming, and labour intensive, but leads to the possibility of nutrient supplementation which allows animals to do well on a diet that otherwise would be of low quality. This approach can have dramatic effects on animal culture, first by improving growth and survival, but more importantly by improving feeding economics. It is especially

important that nutrient deficiencies, which contribute to poor growth and survival, be minimized.

Symptoms of nutrient deficiencies have been recognized for many centuries. For example, one of the earliest identified diseases caused by vitamin deficiencies in humans was beriberi. Although recognition of this was traced by Vedder to the Chinese as early as 2697 BC., it was not until 1912 when Funk wrote a paper on deficiency diseases that the word 'vitamine' was used (Halver 1989). Scientific investigation on components of human nutrition therefore had its start in this century. This work on human nutrition together with similar research on other terrestrial mammals was used later in this century as a basis for studying the nutrition of aquatic animals.

The best example of aquatic nutrition, where most work has focused, is in finfish culture. Although fish have been cultured for thousands of years, one of the first scientific reports of a specific vitamin deficiency in fish only appeared as recently as 1941. When rainbow trout were given carp as food, paralysis resulted. This was remedied by injections of thiamine. Later, Wolf reported that the enzyme thiaminase present in fresh fish could hydrolyze thiamine in the commercial feed mixtures used in fish hatcheries (Halver 1989).

One excellent description of the history of fish diets is given by Hardy (1989). Some of the highlights follow.

A primary goal in developing feeds is to produce a diet which contains feed ingredients that will meet production demands. With fish diets the goals are often practical; i.e. to produce rapid growth and successful reproduction at low cost. These so called practical diets must

meet several criteria: economy of manufacture, shipping and storage; palatability to fish; stability in water and a low leaching rate.

Early fish diet formulations were based on investigations of the natural diets of fish. For example, gut analysis of trout revealed the prey species consumed and their relative proportions. This was then used as a guide to determine the composition of artificial feeds. Another source of information was the proximate composition of the wild trout. These early results for fish (Embody and Gordon 1924, cited in Halver 1989) were: 49% crude protein, 15-16% fat, 8% crude fiber, and 10% ash on a dry weight basis. This is very close to the composition of the diets fed today to young trout and salmon.

During the 1920's and 1930's salmonids were fed a variety of feeds, often based on mixtures of ingredients available locally. These included: fresh, canned or frozen fish; oilseed meals; brewer's yeast; beef liver; horse meat; and cottage cheese. In the 1940's increased hatchery production placed greater demands on feed production. At that time meat-meal mixtures were fed. One of the first, Cortland dry feed mixture no. 6, consisted of 24% each of dry skim milk, cottonseed meal, white fish meal and wheat middlings. The remaining 4% was salt. Such diets are still widely used in some parts of the world.

The development of semi-moist feeds occurred during the 1940's and 1950's. This was accomplished during studies at the Oregon Fish Commission and the Seafoods Laboratory of Oregon State University. Included in this development were not only the testing of various feed ingredients, but also methods for forming the pellets. The latter was especially difficult when appropriately sized particles were needed for smaller fish. For larger fish, the chunks of food could be formed by hand,

although this was labour intensive. Smaller pieces were produced from rice mixers, meat grinders, and eventually noodle machines. The Oregon moist pellet formulation continues to be refined and modified, and today there are three formulations in use (Table 1).

Table 1 Oregon Moist Pellet Specification. (From Halver 1989)

Ingredient	Percentage in Diet		
	Oregon mash (OM3)	OP-4	OP-2 (1/8 or 0.32 cm and larger pellets)
Herring meal (or anchovy or hake meals up to one-half of fish meals) except mash	49.9	47.5	28.0
Wheat germ meal	10.0	Remainder	Remainder
Cottonseed meal (48.5% protein)	-	-	15.0
Dry whey product or dried whey	8.0	4.0	5.0
Corn distillers' dried solids	-	-	4.0
Sodium bentonite	-	3.0	-
Trace mineral premix	0.1	0.1	0.1
Vitamin premix	1.5	1.5	1.5
Choline chloride (70%)	0.5	0.5	0.5
Wet fish	20.0	30.0	30.0
Fish oil	10.0	6.5-7.0	6.0-6.75

Table 2 Semipurified Diet Formulations for Salmonids (From Halver 1989)

Ingredient	H-440	Oregon Test Diet
Vitamin-free casein	38.0	49.5
Gelatin	12.0	8.7
Dextrin	28.0	15.6
α-Cellulose	-	7.7
Carboxy-methylcellulose	-	1.3
Salmon oil	-	10.0
Vitamin E	-	0.2
Choline chloride	-	1.0
Mineral mix	4[a]	4.0[b]
Vitamin premix	9[c]	2.0[d]
Oil premix	9[e]	-

a Mineral premix contains the following (g/kg premix): calcium biphosphate, 137.5; calcium lactate, 326.9; ferric citrate, 29.7; magnesium sulfate, 132; potassium phosphate (dibasic), 239.8; sodium biphosphate, 87.2; sodium chloride, 43.5; $AlCl_3$ $6H_2O$, 1.0; $ZnSO_4$ H_20, 3.0

b Mineral mix contains the following (g/kg premix): $CaCO_3$, 21; $CaHPO_4$ $2H_2O$, 735; K_2HPO_4, 81; K_2SO_4, 68; NaCl, 30.6l; Na_2HPO_4 $6H_2O$, 21.4; MnO, 25.0; $FeC_6H_5O_7$ $3H_2O$, 5.58; $MnCO_3$, 4.18; $2CuCO_2Cu(OH)_2$, 0.34; $ZnCO_3$, 0.81; KI, 0.01; NaF, 0.02; $CoCl_2$, 0.2; citric acid, 6.88

c Vitamin premix contains the following (g/kg premix): α-cellulose, 893; choline chloride, 56; inositol, 22; ascorbic acid, 11; niacin, 8.4; calcium pantothenate, 5.6; riboflavin, 2.2; menadione, 0.45; pyroxine HCl, 0.56; thiamin HCl, 0.56; folic acid 0.17; biotin, 0.06; vitamin B_{12}, 10 μg

d Vitamin premix contains the following (g/kg premix): thiamin HCl, 3.2; riboflavin, 7.2; niacinamide 25.6; biotin, 0.08; calcium pantothenate, 14.4; pyridoxine HCl, 2.4; folic acid, 0.96; menadione, 0.8; vitamin B_{12}, 2.67; inositol, 125; ascorbic acid, 60.0; *p*-aminobenzoic acid, 20; vitamin D_2, 0.4; BHA, 0.75; BHT, 0.75; Celite, 735.8

e Oil premix contains the following (g/kg premix): corn oil, 664.7; cod liver oil, 331.9; DL-α-tocopheryl acetate, 4.4.

During the 1950s and 1960s the development of dry compressed pellets progressed. These proved to have several advantages over moist pellets: they do not require frozen storage, are less expensive, and can be used in some types of automated feeding systems. The disadvantages are: they are not as readily accepted by young salmon as moist feed; there is a limit to the amount of fat that can be included; and they do not float in the water.

A significant step in the understanding of fish nutrition came in the 1950's and 1960's with the development of semi-purified diets, which made possible the determination of nutritional requirements for many species of cultivated fish. Semi-purified diets contain highly refined ingredients of known composition. For example, casein (refined from cow's milk) may be used as a protein source instead of fish meals, which can vary significantly. Examples of such diets are shown in Table 2. By systematically eliminating and adding specific nutrients, the species' nutrient requirements can be identified. As information on nutrient requirements accumulates, production diets can be modified in a manner which improves cost effectiveness and ensures product quality. These semi-purified diets therefore, proved to be essential tools in probing the nutritional physiology of finfish.

The use of semi-purified diets has made it possible to gather information on many areas of fish nutrition including requirements for energy, protein, lipids, vitamins, and minerals. For example, all 15 recognized vitamins have been shown to be essential in the diets of several fish species (D'Abramo and Lovell 1991).

Other variables like food consumption rate, digestibility and assimilation have been examined to some degree, however conducting such

studies with aquatic animals poses many problems which do not exist with terrestrial animals, including:

1. Direct measurement of the amount of feed ingested may not be possible because of the difficulty in removing uneaten and possibly decomposing food from the water.
2. Foods often leach in water, thereby reducing the level of some water soluble nutrients. This is critical since there could be a big difference between the amount included in the feed and the amount ingested by the animal.
3. Collecting and measuring fecal material for analyzing unassimilated nutrients may be difficult.
4. Often feeds must be adjusted to accommodate the feeding habits of the fish. For example, a sinking pellet would not suit a fish which is a surface feeder.
5. Leaching and decomposition of feeds can also cause problems with water quality which could in turn affect the health and appetite of the fish.

Because of these problems, new approaches and methodologies had to be developed.

Researchers have identified strategies to deal successfully with many of these problems. For example, a pre-weighed ration of food pellets can be fed slowly to a group of fish to minimize the amount of uneaten food which could sink to the bottom. Leaching can be reduced in diets by using a lipid coating on the pellets. To separate uneaten food and fecal production, fish can be fed in one tank and moved to another before fecal production and collection.

1.2. Molluscan Nutrition

Many of the approaches used to investigate fish nutrition can also be applied to molluscs. For example, the determination of a diet formulation for bivalves can be based on proximate analyses of bivalve tissue or of live food items normally eaten. These would give a good starting point for formulating a semi-purified test diet.

Early experience with fish feeds showed the importance of having a diet acceptable both in terms of particle size and of palatability. For fish, size determinations can be approximated by mouth gape width. This is not the case for bivalves. Since bivalves feed by filtering, a wide range of planktonic food items are available. Depending on the age and species of the bivalve, an acceptable food size varies greatly. Bivalves usually require small food particles (less that 20 µm; Langdon 1981) although gut analysis of sea scallops has revealed particles ranging from 10-350 µm (Shumway *et al.* 1987). Size of food, then, is very specific.

Palatability as a criterion for fish feed has been addressed by many researchers. Some cultured fish prefer a moist to a dry pellet and will more readily initiate feeding on the former. Also, some factors (usually called attractants) may enhance palatability. Mohsen and Lovell (1990) found that channel catfish consumed more of a soybean meal based feed when a small amount of the soybean meal was replaced by animal protein. When cottonseed was used to replace soybean meal in channel catfish production diets, palatability appeared to be improved (D'Abramo and Lovell 1991).

Defining palatability for bivalve molluscs is more problematical. What are the criteria which make a diet more attractive for a bivalve? First we must identify how food selection occurs, the mechanism whereby

one food is selected over another. It is well known that bivalves will cease to feed if food concentration reaches a maximum level (Bayne and Newell 1983). Ward and Targett(1989) has shown that mussels will preferentially select beads coated with some algal exudates (excretory products) over inert micro-beads with other coatings. Thus some algal components appear to stimulate feeding, but this alone gives little information for defining 'feed palatability' for bivalves. The question of acceptability is further complicated in molluscs which produce pseudofeces. This occurs when food concentration becomes high or unpalatable foods are presented. At any one time therefore, food, feces and pseudofeces can be present together in the same chamber, making the experiment difficult to both control and interpret.

Feed application or food delivery is relatively straightforward for fish. For example, floating pellets are best presented to fish which feed at the surface and a sinking feed pellet to fish which feed in the water column. Most bivalves filter their food from water flowing around them. What features of the flow and the food it contains are critical to successful diet delivery?

With fish, a diet is often presented over a short feeding period and an examination of the surrounding water can reveal how much of the feed has been accepted. With bivalves, the food is microscopic and estimates of the food ingested are usually determined by monitoring the disappearance of algae from the water. This is an indirect measurement and sampling and counting errors (e.g. counts due to pseudofeces) can lead to incorrect estimates of ingestion.

Digestibility of fish feeds is usually conducted with an inert (not altered during digestion) marker, frequently chromic oxide. The

concentration of chromic oxide in the diet is predetermined and the concentration in the collected fecal material can be measured. With bivalves, minute amounts of pseudofeces and feces can be present, making collection of discrete fecal samples very difficult. Additionally, we know very little of what constitutes 'inert matter' in the digestive systems of bivalves.

Fish nutritionists have tested a variety of ways to produce fish diets (pelleting machines, extruders, etc.) to make the diets more acceptable. For marine bivalves, methods for the production of formulated synthetic diets must be identified and tested to determine one which can produce a diet which is both accepted and effective. Microencapsulated or microparticulate feeds would permit control of the diet formulation and composition. Indices of response to the diet (e.g. growth and survival) can then be identified and evaluated.

At a 1981 World Aquaculture Society meeting on Aquaculture Nutrition, Dr. C. Epifano concluded: "It is difficult to conceive of rapid development in the field in the absence of a defined dietary formula that will support growth of bivalves. Without such an experimental diet whose detailed composition can be easily manipulated I foresee slow progress in our understanding of bivalve nutrition at the biochemical level." (Epifano 1981).

This project addresses the applicability of a sodium alginate microparticulate diet as a tool for research in nutrition studies with bivalve molluscs.

1.3. Usefulness of Synthetic Diets

Traditionally, bivalve hatcheries have relied on live feeds, specifically unicellular algae, production of which is costly, time consuming, and labour intensive. There is great variability in the quality and quantity of nutrients supplied by different algal species, as algal composition is affected by factors such as photoperiod, light quality and intensity, nutrient supply and time of harvest (Enright *et al.* 1986b, Fabregas *et al.* 1985, Whyte 1987). Such variations make provision of food for bivalve culture difficult and indicate the need for alternative diets. Some researchers have altered the algal growth medium in order to manipulate the algal composition (Enright *et al.* 1986a, Wikfors *et al.* 1984). This has met with some success but it is not an ideal tool. There is considerable variability in the resultant changes in the biochemical composition of the algae. Also, this approach often leads to systematic starving of the algal cultures (e.g. no silica in media) which, in turn, can cause crashes in the algal populations and may lead to clumping.

Some attempts have been made to increase or alter specific nutrient composition of algal populations, such as the levels of essential fatty acids. The importance of 20:5ω3 and 22:6ω3 for bivalves was demonstrated by Trider and Castell (1980) and Waldock and Holland (1984). In order to improve the fatty acid composition of phytoplankton, various levels of irradiance or photon flux density (PFD) have been tested. The work of Thompson *et al.* (1990) has shown that fatty acid composition changed considerably at different PFD's. The specific light conditions which optimized the fatty acid composition of various algal species were species specific. The authors concluded that fatty acid composition is variable and

the dynamics are greatly influenced by changes in PFD. This approach is effective in changing the nutrient composition of algae but it can be costly and time consuming. Ackman *et al.* (1968) found that lower temperatures can increase the degree of unsaturation in fatty acids of phytoplankton; lowering the culture temperature, however, would cause lower overall growth rates and result in reduced algal production.

In recent years, much work has been done on the development of artificial diets suitable for filter feeding bivalves. Excellent reviews on this topic have been prepared by Langdon (1983) and Langdon and Levine (1984). Some of the microencapsulation procedures are discussed in Part II, Section 3 of this document. These artificial diets could permit experimental manipulation of the ration. Furthermore, the delivery of specific nutrients incorporated with radiotracer labels would be greatly facilitated.

Table 3 summarizes the benefits of synthetic diets. Live feeds, in this case, refers to cultures of phytoplankton, unicellular algae. This requires a large investment of time, labour cost, and space. Algal culture technicians spend much time maintaining cultures and transferring them through various production stages in test tubes, flasks, carboys, tubes, etc., at all times carefully using methodology intended to reduce the risk of contamination. The cost of algal production is high. Langdon *et al.* (1985) estimated the cost of algal food production to be from $18 to $200 per kg dry weight. The calculations from DePauw *et al.* (1984) are similar with estimates indicating a cost from $4 to $23 per kg dry weight . In monoculture systems, however, the cost can go as high as $120-200 per kg dry weight (DePauw *et al.* 1983). In terms of bivalve hatchery production, therefore, the cost of algal food is a major expense. By

comparison, commercially available feed for adult fish and crustaceans is a fraction (1/100) of this cost (Langdon *et al.* 1985).

Survival, growth and production rates, nutrient composition, and purity of algal cultures are difficult to control. Therefore the algal supply for a hatchery can be unpredictable both in terms of availability and nutrient quality. Phytoplankton cultures are known to crash, often for no apparent reason. In one bivalve hatchery a sharp decline in the condition of phytoplankton cultures was observed a few days after local storms (personal communication, Dr. Andre Mallet).

Algae can have a negative impact on the water quality in bivalve production. Any toxins or bacterial contamination in the algal growing chamber can be carried into the bivalve rearing facility. There are also inherent problems with some algal species. It was noted in the DFO Halifax Laboratory that *Tetraselmis* sp. often formed a green slime in culture and great care had to be taken not to cross-contaminate the other cultures (personal communication, Brenda Bradford).

Some efforts have been made to resolve the above problems by producing processed (for example, spray dried) microalgae which could be more readily available, more convenient, and much cheaper than live algae. Walne (1979) tested spray dried *Chorella* sp., vacuum dried *Monochrysis lutheri* and freeze dried *Isochrysis* aff. *galbana* (clone T-ISO) fed to oyster (*Ostrea edulis*) larvae. While these species have been shown to be effective as live feeds, he found that none of these processed forms supported significant grov th in oyster (*Ostrea edulis*) larvae. In contrast Masson (1977) found that preserved *Monochrysis lutheri, Tetraselmis suecica* and *Chorella* sp. gave results comparable to live algae in the mussel, *Mytilus galloprovincialis.* A review of the progress in the use of processed

microalgae for aquaculture is given by Sommer *et al.* 1990. A spray dried *Tetraselmis* was tested in the research program of this thesis and is discussed in Part III, section 2.

Several researchers have used encapsulated diets to supplement algal foods for bivalves and examine the requirements for specific nutrients (e.g. essential fatty acids). For example, gelatin-acacia capsules containing dietary lipids have been fed to juvenile oysters *Crassostrea gigas* in combination with algal foods (Langdon and Waldock 1981). This approach does have inherent problems. Relative proportions of live and inert fractions must be determined. Interactions may exist between the food sources (for example bacteria resident in algal culture may feed on the microparticle.) Also, deficiencies in the algal fraction of the diet could inhibit efficient utilization of the nutrients in the capsule fraction. Finally, data may be difficult to interpret if it is not clear which nutrient source is responsible for a specific result.

Table 3 Comparison of Live vs. Synthetic Feeds

Comparison of Live versus Synthetic Feeds		
Factor	Live Feeds	Synthetic Feeds
Time	Intensive	Minimal
Labor	Intensive	Minimal
Cost	High	Low
Space	Extensive	Minimal
Availability	Not always	When needed
Water Quality	Variable	Variable
Nutrition	Variable but sometimes unpredictable	Controlled, but needs research

Synthetic feeds have many benefits and in principal can be produced and utilized with no great demands on time, labour, money or space. They can have a long shelf life and can be tailored to meet the nutrient requirements of the cultivated species. Such feeds have been used for many years with fish (Hardy 1989) and in recent years considerable success has been achieved with crustaceans (AQUACOP *et al.* 1989, Galgani and AQUACOP 1988).

A negative impact on water quality can occur for many reasons. First, some diets can clump or settle out of the water column. In either case they become unavailable to the bivalves and decompose. Agitation of the culture medium is required to keep the microparticles in suspension. Also, synthetic diets are excellent surfaces for bacteria growth and can lead to bacterial contamination of the bivalve culture chamber. Bacteria are not necessarily a negative feature as they are known to be ingested by bivalves (Chu *et al.* 1987), they can be detrimental because they can be difficult to control and can interfere with determining nutrient requirements. As a last resort, one solution to bacterial contamination is the addition of antibiotics to the culture chamber.

Synthetic diets can also be prone to leakage of nutrients. Leaching can eliminate many of the essential nutrients from the diets, for example: free amino acids, free sugars, and water soluble vitamins. The levels of nutrients contained in the diets may be greatly reduced by the time the diet is ingested. The results, however are presented and interpreted by researchers based on the level of dietary inclusion, not the actual amount of (a) nutrient(s) consumed. This makes it difficult to quantify specific nutrient requirements.

Leaching is not necessarily a negative feature, especially if the culture system is not a flow-through one. Marine bivalves readily remove dissolved substances from solution both as larvae and as adults (Manahan and Crisp 1982). The uptake of free amino acids (FAAs) can contribute to meeting energy requirements and can be available for protein synthesis. Furthermore, the bivalves can compete effectively with bacteria for those FAAs (Stephens 1983). Dissolved nutrients may in fact be of benefit to bivalves. The typical metamorphosis of a bivalve larva involves switching feeding apparatus from the velum to the gill. During this time particulate food is not used and the dissolved nutrients can be used. The diet of marine bivalves is therefore biphasic with both particulate and dissolved components (Stephens 1983). For a controlled nutrition experiment leaching is therefore not necessarily a totally negative factor.

The major advantage of synthetic diets is that they carry specific nutrient formulations. These can be manipulated and the effects of various nutrient levels and combinations be can tested. Once specific nutrient requirements are identified in purified experimental diets, it will be possible to use these data in producing low cost practical diets using inexpensive ingredients.

It is apparent, then, that the best approach to examine bivalve nutrition physiology is to use a capsule which will carry a complete diet. Only then can energy budgets, specific nutrient requirements and optimal diet formulations be identified and quantified.

1.4 Overall Objectives

A complete scientific understanding of bivalve physiology is impossible without knowledge of their nutrient requirements and subsequent nutrient utilization. In addition, the development of the

molluscan aquaculture industry and the concomitant increasing reliance on hatchery produced spat have created a demand for detailed knowledge on the nutritional requirements of bivalves. One of the most useful tools will be a synthetic diet of known nutrient composition. Modifications in the diet formulation can be tested to identify specific nutrient requirements, energy demands or other aspects of nutritional physiology. The goals of this program are:

1. Select suitable methodology for microparticle preparation.
2. Test the formulated diet to ensure that it is readily ingested by bivalves.
3. Establish a method for testing digestibility.
4. Develop food presentation procedures.
5. Conduct feeding trials to evaluate responses.
6. Evaluate results.
7. Summarize conclusions and propose new directions.

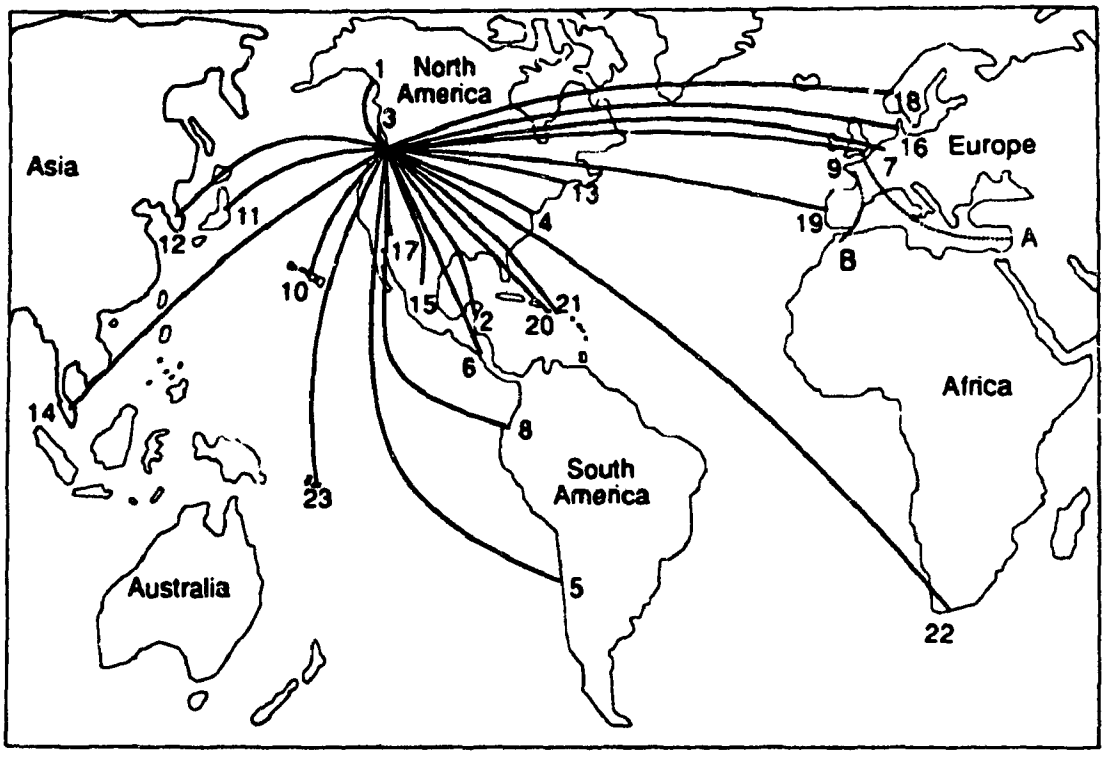
2. Global Importance of Mollusc Culture

Clearly, worldwide commercial demand for spat production requires first and foremost a successful hatchery technology. Specifically, the nutritional requirements of the broodstock, larvae, and spat must be met to ensure their growth and survival.

The pacific oyster (*Crassostrea gigas*) is a hardy species which is well known throughout the world and accounts for approximately 80% of the world oyster production. Chew (1990) discusses the *C. gigas* industry, with emphasis on the west coast of North America. One important development was the establishment of hatcheries on the west coast because, although some natural spatfall occurred in this area, it was not sufficient to meet the demands of commercial production. It is the mandate of these hatcheries, therefore, to produce sufficient seed to meet the demands of oyster farmers. In recent years, a technique called "remote setting" of eyed larvae has been used. The farmers construct seed catching tanks on their sites and the hatcheries send eyed larvae for settlement. The eyed larvae ship well and some hatcheries provide an algal paste for feeding.

These hatcheries on the west coast of North America provide eyed larvae for experimental and commercial purposes to various sites around the world as well as for meeting the local demands (Figure 1). One Oregon hatchery visited by this author produced over 5 billion eyed larvae annually with a staff of only two employees. The gross worth of these larvae is over half a million dollars.

Figure 1 Recent transfers of eyed larvae, seed or adult Pacific oysters (*Crassostrea gigas*) for experimental or commercial purposes to various global sites from the Northwestern United States (From Chew 1990).



1-Alaska (US);2-Belize; 3-British Columbia (Canada); 4-Chesapeake Bay(US); 5-Chile; 6-Costa Rica; 7-Denmark; 8-Ecuador; 9-Great Britain; 9-Hawaii (US); 11-Japan; 12-Korea; 13-Maine (US); 14-Malaysia; 15-Mexico; 16-Netherlands; 17-New Mexico (US); 18-Norway; 19-Portugal; 20-Puerto Rico; 21-Saint Croix (V.I.); 22-South Africa; 23-West Samoa.
From other sites: A-Israel from United Kingdom; B-Morocco from France.

Another commercially grown bivalve, the bay scallop *Argopecten irradians*, can grow to a 5-6 cm height in a short time and is iteroparous, living only 18-24 months. An interesting introduction took place in 1982 when bay scallops were brought to China. Only 26 bay scallops survived the trip from the United States to China, but these were spawned successfully in January, 1983. When Dr. K. Chew, who participated in this transfer, visited China in 1987, he found that during the intervening 5 years the 26 scallops had led to an annual production of over 50,000 tonnes, with estimates for 1990 of 70,000 tonnes. Furthermore, bay scallop farming has expanded, and operations exist in five other Chinese provinces. It is important to note that spat are hatchery produced and that Chinese scientists have carefully developed hatchery operations for conditioning and spawning the broodstock, and growing the larvae. In fact, more hatcheries are being established to meet the demand for bay scallop seed (Chew 1990).

The sea scallop, *Placopecten magellanicus,* is an important commercial species in Atlantic Canada with a landed value exceeding $100 million in 1985. Scallops command a high market price and are prime candidates for aquaculture operations. Another scallop species, *Patinopecten yessoensis*, has been intensively cultured in Japan, and the Japanese methodology is currently being tested in Newfoundland. The Japanese industry depends upon wild spat but in Atlantic Canada large supplies of wild spat cannot be guaranteed. Hatchery production of spat would ensure a supply for growout in aquaculture operations, in addition to restocking overfished areas and seeding new locations.

It is notable that it is the hatcheries which provide spat for growout of shellfish. Even in areas where natural spat collection is possible, hatcheries make it possible for growers to experiment with spat collection, although not to rely on it. Furthermore, hatcheries also make spat available in years when natural spat collection is poor or extends over a long period.

As stated, hatchery production of quality spat also provides the opportunity to reseed overfished areas. In some places historic shellfish areas have become depleted due to overfishing, disease, climatic change, or even pollution. Not only is the economic benefit of restocking very clear, but it is also welcomed as perpetuating a seafaring tradition or maritime culture in many areas.

The scallop industry in Japan has been of prime commercial importance since the seventeenth century. It was so important as an export item that, earlier in this century, harvesting of the scallop for purposes other than export was forbidden (Ventilla 1982). Some areas including Matsu Bay have excellent spat collection while others, including Iwate and Tohoku, have no major collection and must import seed from the north. Methods for artificial propagation are being actively sought because of depletion of wild stocks and the difficulty in collecting enough spat. Furthermore, even when spatfall is plentiful, poor quality seed can be a problem.

Ventilla (1982) also discusses the problems associated with collecting wild spat. Data collected in Mutsu Bay show 5 days on either side of peak settlement is the opportune time for collecting spat. If collectors are set outside this narrow margin, the settlement will be missed. Furthermore, neither spawning nor settlement are synchronized throughout the bay and

there is great variability between sites. The larval behavior is therefore an important component of successful spat collection.

One interesting anecdote in terms of forecasting spat settlement is that a Japanese cherry tree, the Sakura blossom, situated in Gappo Park, Aomori city, blooms annually at the same time as spat settle in the nearby Mutsu Bay. The Japanese meteorological department issues the Sakura blooming forecast one month beforehand. Spatfall can therefore be predicted based on the blooming of the cherry tree. Spatfall in recent years has been good; nevertheless, there are still problems with inconsistency of numbers and quality of spat.

Over 90% of the shellfish grown worldwide are based on collection of wild spat (G. Newkirk, personal communication). The FAO data for 1989 (see Table 4) show the aquaculture production for various species, including molluscs. The production of scallops doubled from 164×10^3 tonnes in 1986 to 311×10^3 in 1989. The total in 1989 for scallops, including both wild catches and those from aquaculture, was 840×10^3 tonnes (from Table B-55, FAO Yearbook 1989). The cultivated scallops are therefore almost 35% of the total.

The total for cultured scallop production is comparable to other important aquatic species. Tilapia production was 326×10^3 tonnes and milkfish production was 344×10^3 tonnes. The figures for other molluscs, cockles and clams, were even higher at 466×10^3 tonnes. If these production levels are possible based solely on a wild spatfall, the effective utilization of hatchery produced spat could greatly increase global mollusc production.

A major problem in the utilization of hatchery produced spat is the high cost of production. For example hatchery production of the scallops,

Chlamys nobilis and *Pectin a.oinicans*, has been successful in Japan but at a seed cost of 7-10 ¥ per shell, while the cost of collecting wild spat was only 1¥ (about $0.0125 Canadian) per shell. The high overhead associated with hatchery production is due mainly to the high cost of producing unicellular algae. This point is discussed in detail in I-1:3.

Hatchery produced spat have many advantages over wild spat:

1. Reliable production numbers are possible once the facility is operating well.
2. Spat can be produced at a predetermined time. This means an operator can, on a given date, distribute a known number of spat in his growout sites.
3. There can be more quality control over hatchery produced spat.
4. Hatchery production means that spat of non-indigenous species, commanding a high market price, can be produced for grow-out.
5. Hatcheries have the capacity, through management of broodstock genetics, to produce spat which are superior (for example, faster growers).
6. Hatchery production is less risky to the grower since many environmental factors can be monitored and to a great extent, controlled. Wild populations and their spatfalls can fall victim to many problems including temperature extremes, toxic algal blooms, and fatal diseases.

In many ways, hatchery produced seed are preferable; however, the costs of hatchery production must be decreased. A better understanding of nutritional physiology and detailed nutrient requirements for bivalve species would greatly simplify the feeding protocol in hatcheries for broodstock and larvae and ensure consistent production of healthy animals.

Table 4 Total Production from Aquaculture x10^3 metric tons. (Taken from FAO yearbook 1989, vol. 68)

	1986	1987	1988	1989
Fish				
Common Carp	800	1012	1101	988
Grass Carp	435	536	599	947
Silver Carp	1280	1431	1598	1433
Bighead Carp	595	631	705	641
Tilapias	213	254	266	326
Channel Catfish	149	170	165	184
Eels	77	87	99	90
Atlantic Salmon	60	68	112	168
Pacific Salmon	44	74	73	108
Rainbow Trout	198	223	240	245
Milkfish	311	333	345	344
Amberjacks	149	160	167	155
Crustacea				
Freshwater Shrimp	14	25	27	29
Red Swamp Crawfish	44	46	33	31
Banana Shrimp	27	29	31	32
Vannamei	36	80	82	79
Giant Tiger Prawn	89	148	149	143
Fleshy Prawn	83	99	110	108
Unclassified Shrimp	89	99	110	108
Total Shrimp	324	509	572	537
Molluscs				
Oysters	929	975	1009	940
Mussels	798	940	1047	1050
Scallops	164	197	306	311
Cockles and Clams	439	444	457	466
Seaweeds				
Brown	2270	2002	2077	1915
Red	736	645	850	863

3. Feeding in Bivalves

3.1 Larvae

Feeding in bivalves follows their characteristic mixed life histories. Following fertilization of the eggs, the yolk-lobe and polar body form. A ciliated gastrula appears at about 12-14 hours of age. An apical tuft of cilia appears at the trocophore stage and the planktonic larvae swim around with the tufts pointing forward. This trocophore stage is present in many protosomes and food particles are collected with a girdle of cilia called the prototroch. Shell material is produced by the shell glands and in sea scallops the straight-hinged veliger stage develops at day 3 or 4.

The feeding mechanisms and evolution of invertebrate larvae are rigorously reviewed by Strathmann 1978. He categorizes larval feeding mechanisms into two groups; the first has a single ciliary band system as found in the oligomera. Particles are retained on the upstream side of the cilia, probably due to a local reversal of beat. The second, the spiralia (including the molluscs) possess a double band of cilia: there is a protoconch comprising a band of long, compound cilia parallel to a band of shorter cilia in the metraconch. Between these two is a food groove with smaller cilia. Some support does exist (Jägersten 1972) for the double band being a primitive trochophore feeding mechanism. For detailed descriptions of these structures and their functions, see Nielsen (1987). A discussion of the evolution of feeding mechanisms is contained in Appendix 1.

Bivalve larvae may develop completely within an egg mass or while brooded by an adult. This direct development requires a good energy reserve and eliminates the need for a free living feeding phase. Lecithotrophic larvae can be of two types. In primary lecithotrophy, the

early stages depend on yolk from the egg (lekithos is Greek for egg yolk). In secondary lecithotrophy, non-feeding larval stages develop on energy reserves accumulated by preceding planktotrophic larval stages.

Planktotrophy has its advantages: a given amount of energy can be apportioned among numerous eggs giving greater opportunity for dispersal, more time to select settlement site, and a large size at settling. The disadvantages are a greater susceptibility to predation and abiotic perturbations. Lecithotrophy, by contrast, avoids direct competition for food between larvae and adults and does not leave the larvae subject to variable food supplies. Egg numbers tend to be smaller, however, and a larger amount of nutrient is invested per egg. (Compiled from Barrington 1967; Vance 1973 a,b; Jackson 1974; Spight,1979; Strathmann 1978,1980,1985; Jablonski and Lutz 1980, 1983; Kempt and Ha.field 1985; Scheltema 1986.)

Few detailed studies have been done on feeding modes during the early stages of larval development. The work of Lucas (1986) on the blue mussel, *Mytilus edulis,* has shown three phases: endotrophy for the first few days followed by mixotrophy from the second to eighth day by which point the animal becomes exotrophic.

3.2 Adults

Feeding by adult bivalves encompasses a broad spectrum of morphological differences in feeding structures and has been the focus of much debate (Benniger 1991). As indicated by Beninger and Le Pennec (1990), the two approaches commonly used to study bivalve feeding are:

1. Direct observation of structures and behavior of particles on them.
2. Indirect techniques such as observations on particle clearance, gut contents and flow cytometry.

In general bivalves can be grouped into two categories: active and passive suspension feeders. The former actively move water over their gills while the latter depend on ambient water movements. Aspects of filter feeding in molluscs are summarized by Jorgensen (1983), Jorgensen *et al.* (1984) and Jorgensen (1990) with emphasis on fluid mechanics. Very little is known about food selection processes in bivalves. For example, does selection occur both before and after ingestion? Some factors which affect food selection have been examined (Shumway *et al.* 1985; Kean-Howie *et al.* 1990, Cranford and Grant 1990).

It is generally accepted that feeding in bivalves involves first the pumping of water through the ctenidia. The food particles are removed from suspension and transported to the mouth. Traditionally it was believed that particle removal could be explained by the mucociliary concept. That means that food particles are mechanically trapped by rows of laterofrontal cilia or cirri and then passed on to the frontal ciliary tracts of the filaments (Ward *et al.* 1993). The three main tracts of cilia on the filaments are known as the frontal cilia, laterofrontal cirri and lateral cilia (Jorgenson 1990). The traditional theory goes on to explain that food particles are then carried by the frontal cilia, in mucus, to the dorsal or ventral ctenidia margins. These are then incorporated into mucus strings before being ingested.

In recent years, a new theory, called the hydrodynamic theory, has been suggested by Owen (1966) and Jorgenson (1981, 1990). It suggests that binding of particles in mucus, as described in the traditional theory, is not an important part of suspension feeding. Instead, food is captured and transported from the pallial cavity to the stomach by fluid mechanical mechanisms which are not yet completely understood. Captured particles

never touch the cilia or cirri and mucus is secreted only to clean the ctenidia. The hydromechanical model is supported also by the work of Kiorboe and Mohlenberg (1981) who reported that particles in the oesophagus of the mussel were in suspension. The absence of mucus indicated that mucus was not a critical component.

A novel methodology was recently developed which enabled direct observation of feeding in bivalves. It involves using an endoscope to examine feeding and subsequent video image analysis of the videotapes. This method (Ward *et. al.* 1991; Benniger *et. al.* 1991; Benniger *et. al.* 1992) has many advantages and the superior feature is that it permits observations of intact whole animals *in vivo*. Earlier work on suspension feeding was open to criticism since it was often based on isolated ctenidial fragments, animals with severed adductors, ablated mantle edges or dissected cavities (Ward *et al.* 1993). More importantly, dissection and surgical procedures could destroy some of the hydrodynamic interactions between the feeding mechanisms and the food.

Using the new techniques, Ward *et al.* (1993) have concluded that both mucociliary and hydrodynamic mechanisms are involved in suspension feeding by functioning concurrently at different sites on the ctenidia. Their data indicate that there are also species differences. The blue mussel, *Mytilus edulis,* carries mucus bound food in the ventral grooves but the sea scallop, *Placopecten magellanicus,* moves material in the dorsal tracts in a slurry. The American oyster, *Crassostrea virginica* , combines both these methods and uses both ventral strings with mucus and dorsal slurries.

This is still a hotly debated topic and certainly some issues will be made clearer as more data become available. For now, both sides agree that analysis of stomach contents of feeding bivalves will be useful.

The endoscopy technique (unavailable at the time present studies were begun) would be a useful tool in evaluating how different food sources are manipulated. For example, are live algal cells and microparticulate diets treated in the same way by the bivalve? Are there species differences in removal and ingestion of microparticulate diets?

It is apparent that a complete understanding of particle capture requires a detailed description of the participating organ's anatomical structure. The structure of bivalve pallial organs has been examined by Atkins (1938), Owen (1966), Morton (1983), and more recently by Beninger and Le Pennec (1990). The latter suggest that if sea scallops can exercise selection in a mixed suspension, the site or sites of this selection have yet to be identified. Beninger *et al.* (Beninger *et al.* 1988, 1990; Beninger and Le Pennec 1990) have searched for the necessary anatomical detectors and effectors on the gills and peribuccal organs of scallops. To date, no chemo-sensory cells have been revealed (Beninger *et al.* 1988, 1990; Beninger and Pennec 1990).

The bivalves are represented in a review paper on molluscan diets by Graham (1955). Various feeding habits including microphagy (detritus and algae); macrophagy (by both carnivores and herbivores) and even parasitism are discussed. There are, however, few data on what bivalves feed on. Although unicellular algae are a major food item, other sources have been investigated including: macrophyte detritus (Stuart *et al.* 1982); sediment (Grant and Cranford 1989); bacteria (Berg and Alatalo 1984); dissolved organics (Manahan 1983) and artificial diets (Laing 1987).

Detritus particles can contribute to energy gain in sea scallops when phytoplankton are less abundant (Cranford and Grant 1990) but may be inadequate to produce tissue and gonad growth. Very little is known about specific nutrient requirements or how larvae, juveniles and adults partition their energy.

The various digestive enzymes found in molluscs have been reviewed by Reid (1983). Other factors must not be neglected. For example, a lectin recently found in sea scallops (*Placopecten magellanicus*) may play an important role in feeding (Gill 1987).

Part II Establishment of Experimental Techniques and Procedures

1. Introduction

As discussed in the introduction (I.1) finfish nutritionists over the years have experimented with diets: composition, formulation, productio formulation, delivery, and performance. Our knowledge of bivalve nutrition is, by comparison, extremely limited.

The application of synthetic diets in bivalve nutrition studies necessitates the development of several techniques and procedures. First a suitable diet formulation has to be determined. Next it was important to identify a suitable microencapsulation procedure and a means of determining whether or not diet so encapsulated could be digested. Finally, a feed application method, based in knowledge of feeding behavior, had to be identified.

2. Diet Formulation

2.1. Sources of Information

From our knowledge of fish nutrition research, two types of information were considered in formulating the test diet: biochemical composition of the algal food and biochemical composition of the scallops. Researchers have attempted to correlate nutritional value of a variety of marine algae as food items for bivalves. Neither Carefoot (1967) nor Walne (1979) found a relationship between the amounts of ash, protein, fat and carbohydrate found in algae and the nutritional value of those algae.

Palatability, digestive processes (and the associated enzymes), and nutrient absorption must also be considered for diet formulation. Reid

(1983) has completed a comprehensive review of bivalve feeding and digestion, including a review of digestive enzymes. When conducting nutrition studies with microparticulate diets, it is important that the bivalve can digest not only the dietary ingredients but also the encapsulating material.

2.2. Proximate Analysis

Much work has been done on adult scallop tissue composition (Taylor and Venn 1979; Ansell 1974; Robinson *et al.* 1981). Seasonal variations in composition related to reproductive cycle have been demonstrated. Little has been done on non-reproductive scallops.

In one case, Manning (1985), analyzed the biochemical composition of samples of juvenile scallops, *Placopecten magellanicus*. Based on the content of protein, lipid and carbohydrate in wild animals, Manning concluded that juvenile scallops at 9 months of age have a lipid-based metabolism and by 23 months, metabolism is based on carbohydrate utilization. There appears, therefore, to be a biochemical metamorphosis inherent within the life cycle of *P. magellanicus*. Manning concludes that this implies concomitant changes in the animals' nutrient requirements as they mature.

The dependence of bivalve larvae on stored lipid is well documented (Gallagher *et al.* 1986; Holland 1978). In postmetamorphic marine invertebrates there appears to be four major patterns of lipid storage: storage for periods of increased energy requirements; storage in case of a reduction in food availability; storage in response to variations in physical parameters; and storage which affects buoyancy (Lawrence 1976). Any one, or a combination of these could explain the variation in lipid levels found by Manning (1985). *P. magellanicus* juveniles, unlike many other

bivalves, are very motile and, no doubt, one of the requirements for these lipid stores is to improve buoyancy. In fact, since stored lipid may serve a variety of roles in the same organism, Lawrence (1976) recommended that a primary goal of future work should be to identify and discriminate among them.

Very little published data were found on proximate composition of wild stocks of juvenile scallops. A study was designed to add to our information base and is discussed in Section 3 'Proximate Analysis of Field Specimens'.

2.3. Formulation Tested

Information on algal composition (Whyte 1987) and the work done by Dr. John Castell (DFO Halifax) on bivalve nutrition (Castel and Trider 1974) and crustacean nutrition (Castel *et. al.* 1989) were used as a basis for the first formulation tested.

Earlier work had been based on diets with large amounts (30-40%) of both protein and carbohydrate. The work of Castell on lobster nutrition was based on a crab protein product (Castell *et al* 1989) which appeared to be readily digested and assimilated. Carbohydrate sources for crustaceans were often based on corn starch. Another major component was lipid and the type of lipid source used was important since omega-3 fatty acids have been shown to be critical (Ackman *et. al.* 1976, Ackman 1981, Ackman and Kean-Howie 1994). Lecithin may not be an essential nutrient but was added since it may have other benefits, e.g. reduces leaching (Kean *et al* 1985).

Little was known about specific vitamin and nutrient requirements in bivalves. Work on crustaceans had included complex vitamin and mineral mixes (Kean *et al* 1985). These had their origin in finfish nutrition and were adopted for this work with very few changes.

The first formulation is shown in Table 5. These ingredients were taken to the Collaborative Labs laboratories, Long Island, New York. Their procedure was comparable to that developed by Levine and Sulkin (1984) using sodium alginate. A modification to the methodology made by Collaborative Labs is patent pending. This author signed a confidentiality agreement and some details of the procedure cannot be disclosed at this time.

Laboratory staff, in preparation for encapsulating the diet, reviewed the formulation and recommended changes. For example, the mineral mix used in finfish diets contained large amounts of calcium carbonate. Calcium, however, in large amounts could replace the sodium (in the sodium alginate) and cause premature cross-linking of the sodium alginate. The mineral mix was therefore modified. Also, the level of menhaden oil was reduced on the advice of Collaborative Labs researchers. Large amounts of oil could make the diet difficult to sieve and sieving is essential in making the diet particles sufficiently small for ingestion.

Table 5 Diet Formulation

Ingredient	Proposed % Dry Weight	Final % Dry Weight
Corn Starch	24.0	20.5
Dextrin	1.0	1.0
Cholesterol Acetate	1.0	1.0
Lecithin	7.0	7.0
Crab Protein	30.0	30.0
Vitamin Mix	2.0	2.0
Mineral Mix	5.0	5.0
Menhaden Fatty Acids	17.0	10.0
Carboxymethyl Cellulose (CMC)	13.0	3.5
Capsule Material (sodium alginate)	20.0	20.0
TOTAL	120.0	100.0

The diet was prepared and then freeze dried, but did not grind well in a centrifugal mill. This was attributed to the 10% menhaden fatty acid content. The particles congealed and clogged the 0.08 mm screen and a small sample was then ground with a mortar and pestle. The diet remained as individual capsules and did not become paste-like. This led us to believe that the diet would grind well in a ball mill, which later proved successful.

Collaborative Labs researchers also prepared microparticles containing one micron latex beads stained with Fluoresbrite™ (Polyscience Inc). The freeze-dried microparticles proved difficult to screen down to the required 20 µm size. Instead, a small sample ground with a mortar and pestle was used in the first test for ingestion (Part II section 5.1.1) The fluorescent particles were checked under a microscope after grinding and again after rehydrating to ensure that dye had not migrated out of the particle (see Plate 1). These diets were stored at -40°C until used in experiments.

3. Changes In Proximate Composition Of Juvenile Sea Scallops (*Placopecten magellanicus*) During Their First Two Years

3.1. Introduction

The proximate composition of an animal is a valuable source of information for establishing diet formulations for aquaculture and is a method which has frequently been used for formulating diets in finfish nutrition research (Hardy 1989). There has been considerable work on biochemical composition of bivalve molluscs but most has been associated with reproductive activity and reflects the cycles of energy storage and utilization which occur with gametogenesis (e.g. Giese and Pearse,1974; Ansell 1974; Bayne *et al.* 1976; Barber and Blake 1981; Bayne and Newell 1983; MacDonald and Thompson 1986).

In mussels, as with many bivalve molluscs, glycogen is stored in various body tissues and is subsequently used as an energy substrate or is converted to lipid to promote gametogenesis. Bayne and Newell (1983) have shown that, in *M. edulis*, the storage sites for glycogen are in the mantle in two cell types. These are the adipogranular (ADG) cells and the vesicular (VCT) cells. As gametogenesis progresses the volume of the gametes increases. There is a concomitant decline in the volume of ADG tissue. These authors reported that the decline in glycogen was due to lysosomally mediated hydrolysis of the ADG cells. This is then followed by a decrease in the mantle glycogen. Their morphological data indicate that the ADG cells undergo a process of regression involving an autophagic mechanism. This glycogen sequestration within the autophagosomes could therefore provide a mechanism for controlled hydrolysis of glycogen.

Although most published work on the reproductive physiology of molluscs has been done on mussels, there is an increasing body of literature on gametogenesis in pectinids. Studies on pectinids have shown that the manner in which nutrient reserves are utilized for gametogenesis is variable and species differences do occur. In the sea scallops (*Placopecten magellanicus* Gmelin 1791) energy from the storage sites and ingested food is used to promote gametogenesis. For *Pectin maximus* (Faveris 1987), *Chlamys opercularis,* (Taylor and Venn 1979) and *Argopecten irradians concentricus* (Barber and Blake 1981) maturation of gametes is dependent upon reserves of glycogen and protein stored in the adductor muscle. In *Argopecten irradians irradians*, gametogenesis utilizes the protein and lipid reserves of the adductor muscle. (Epp *et al.* 1988). For *Argopecten purpuratus,* Martinez (1991) showed that carbohydrate is utilized as the metabolic substrate for gametogenesis. Ansell (1974) has shown that in *Chlamys septemradiata* the energy for gametogenesis is derived from ingested food. Thus, many energy sources are used in support of reproductive activity and proximate composition was used to evaluate these seasonal events.

It is notable that there can also be intraspecific variation for these seasonal changes. MacDonald and Thompson (1986) investigated the role of environmental conditions on the annual cycle of energy utilization in sea scallops. A comparison was made between scallops at two sites, at different depths, one at 31 m and another at 10 m. The shallower group had a higher energy content per unit weight and high carbohydrate and lipid levels in the somatic tissues. A comparison of the gonad tissue however, showed no differences in carbohydrate or lipid between the two sites. These authors concluded that even though the scallops living in deeper

water had fewer somatic reserves, they were able to maintain energy levels in the gonad tissue. In addition, Napolitano (1991) estimated the total lipid and triglyceride reserves of the eggs produced at both the above sites. There was no significant difference. His findings were important since the quality of the lipid reserves in the eggs is probably critical for ensuring larval survival and settlement.

Although seasonal variations in proximate composition of pectinids have been related to the annual reproductive cycle or to environmental parameters, the composition of juvenile scallops has been analyzed in only a few studies. Martinez (1991), in her work on *Argopecten purpuratus* , analyzed specimens which were non-reproductive. Results on the mantle tissue showed that the smallest group (not yet reproductive) had higher carbohydrate and protein levels in the autumn and winter. She concluded that these smaller scallops store nutrients in the mantle.

Manning (1985) analyzed samples of sea scallop juveniles taken from the wild. Her data indicate that lipid is the primary constituent (next to protein) of young scallops until they reach nine months of age. At 23 months, carbohydrate exceeds the amount of lipid; subsequently, there is then a major change in proximate composition. Presumably, the larvae are dependent on lipid reserves, as are the young spat. At some point during their second year, there is a change to a carbohydrate-based metabolism. Since there are few data on proximate composition of the soft tissue of juvenile sea scallops, the following study was conducted to provide this information and, in doing so, investigate Manning's hypothesis.

3.2. Methods

3.2.1. Scallops

Juvenile sea scallops (*P. magellanicus*) were taken from aquacultured animals grown in Passamaquoddy Bay in pearl nets (Dadswell and Parsons 1991). These scallops, set as spat, were sorted by age for this work. Samples were taken at 9, 12, 15, 18, 21, and 24 months of age. Two different year classes (1988 and 1989) were sampled, at three month intervals, meaning that the 9 and 21 month samples, and the 12 and 24 month samples, were taken at the same time.

3.2.2. Tissue Samples

The scallops were dissected and soft tissue was divided into four categories: adductor muscle, digestive gland, gonad and viscera (mostly mantle). Tissues within samples were pooled to ensure sufficient sample size for proximate analysis. For the 24 month samples, three groups of three were pooled; for the 21, 18 and 12 month samples, three groups of five were pooled; for 9 and 15 month samples, three groups of eight were pooled. Dry weights were determined to constant weight at 90°C, followed by ashing in a muffle furnace at 450°C. The remaining tissues were lyophilized in a freeze dryer and then ground to a powder using a mortar and pestle. These were stored temporarily in a desiccator, then placed in sealed, labeled vials until analyses were completed.

3.2.3. Biochemical Analysis

The ash determination was made on the oven-dried samples. These were placed in preweighed ceramic crucibles, and reweighed after combustion in a muffle furnace at 450°C. Lipid extractions were done on freeze dried tissue using a modified Folch extraction procedure (see Appendix 4). The extracted lipid was then stored in chloroform in sealed

vials under nitrogen gas at -40°C. The samples were later dried under nitrogen to remove the chloroform. For lipid recovery purposes exactly 0.5 ml of chloroform was added (under nitrogen) and mixed with the sample. Aliquots of 100 µl were then placed into a preweighed platinum boat which was placed on a hot plate to evaporate the chloroform. The boat was then reweighed with the lipid fraction included and the total amount of chloroform-soluble lipid recorded. (The balance used for all weights was a Sartorius Type 2492.)

Protein determinations were done using a CHN analyzer. For details see Appendix 3. The resulting data were used to calculate µg N per weight of sample. A conversion factor of 5.8 was used to calculate protein as recommended by Gnaiger and Bitterlich (1984).

The remaining component, carbohydrate, was estimated by difference. The ash, protein and lipids were totaled. An additional 6% of this total was added to account for bound water for mollusc tissues as per the estimates of Gnaiger and Bitterlich (1984). The sum was then subtracted from 100% to give the amount of carbohydrate. The standard deviations were calculated following the methods of Baird (1962).

3.3. Results

The proximate composition expressed as a percentage of the dry weight of soft tissue is shown in Figures 3 through 8 inclusive for months 9, 12, 15, 18, 21 and 24. Tables of data are shown in Appendix 2. Protein was the major constituent in all samples and in most of the tissues. The second highest component was ash. It was usually higher in the viscera component than in other tissue types. (The 'viscera' component represents the remaining tissue including mantle and gills.)

The data for the adductor muscle (Figure 9) indicate that there was little change in the total amount of lipid from 9 months to 24 months. In the spring of the second year, between 15 and 18 months of age, there was a large increase in the amount of carbohydrate. The amount at 18 months was tripled in comparison with the amount at 12 months. Within the 6 month time interval, there is a concomitant decrease in the amount of protein. By fall, the amounts had returned to their previous levels.

Data for the digestive gland (Figure 10) indicate a higher lipid level than in the adductor muscle tissue. The amount of lipid varies slightly at 9 months and 12 months; the lowest levels occur at 18 and 21 months, the spring and summer months. This small decline in lipid (from 12 to 18 months) is coincident with a large decline in protein and an increase in carbohydrate levels. Protein drops by 50% and carbohydrate increases by over 100%. As in the case of the adductor muscle, by 24 months (the fall) the nutrients are restored to their previous levels.

Overall, the lipid in the gonad was greater than that of the adductor tissue and similar to that found in the digestive gland. From 15 to 18 months there is a sharp increase in the amount of protein. The levels of the major nutrients remain about the same from 18 to 24 months.

The nutrient composition of the various organs changes very little in the viscera (Figure 12). Differences between the Manning (1985) data and these data are shown in Figures 13 through 15.

Figure 2 Scallop Shell Height vs. Dry Weight of Tissue (error bars are one standard error)

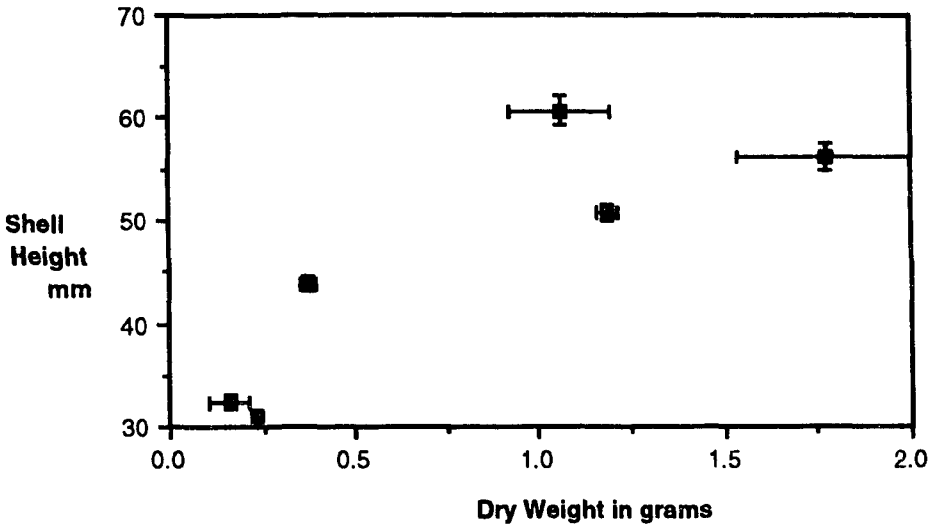


Figure 3 Proximate Composition of Scallops at 9 Months. (error bars are one standard error)

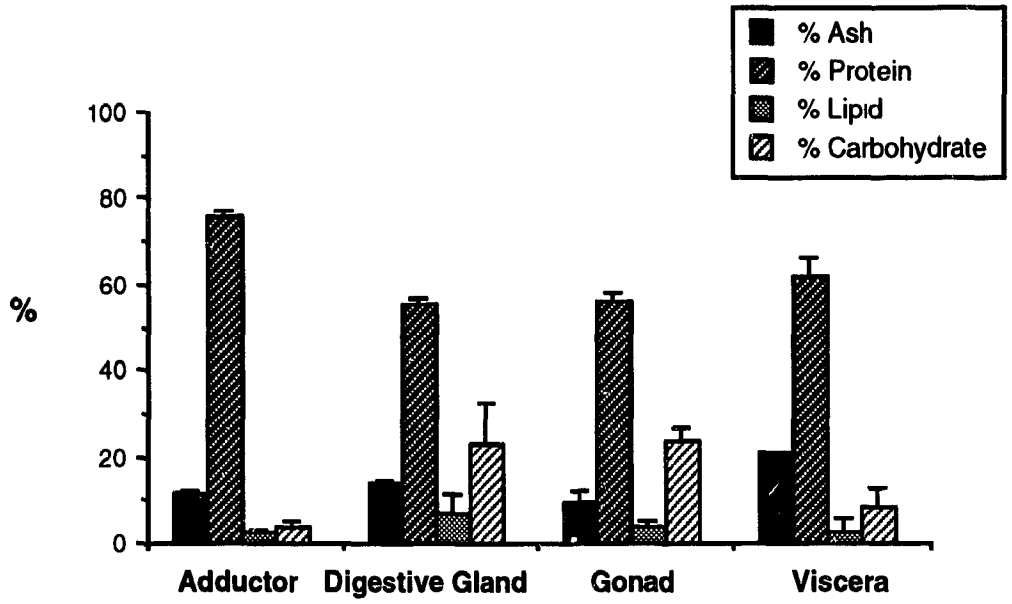


Figure 4 Proximate Composition of Scallops at 12 Months (error bars are one standard error)

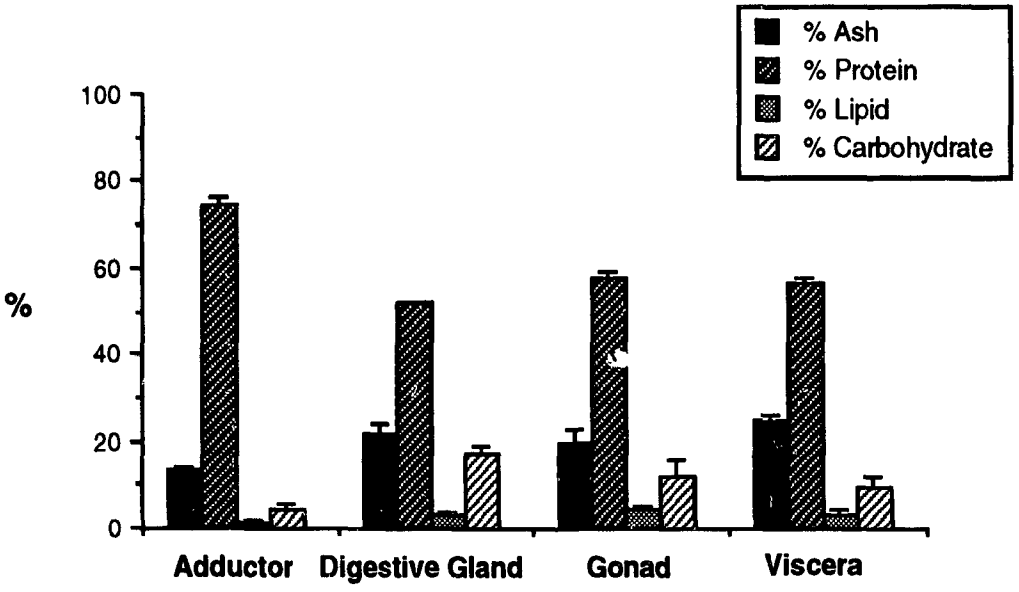


Figure 5 Proximate Composition of Scallops at 15 Months (error bars are one standard error)

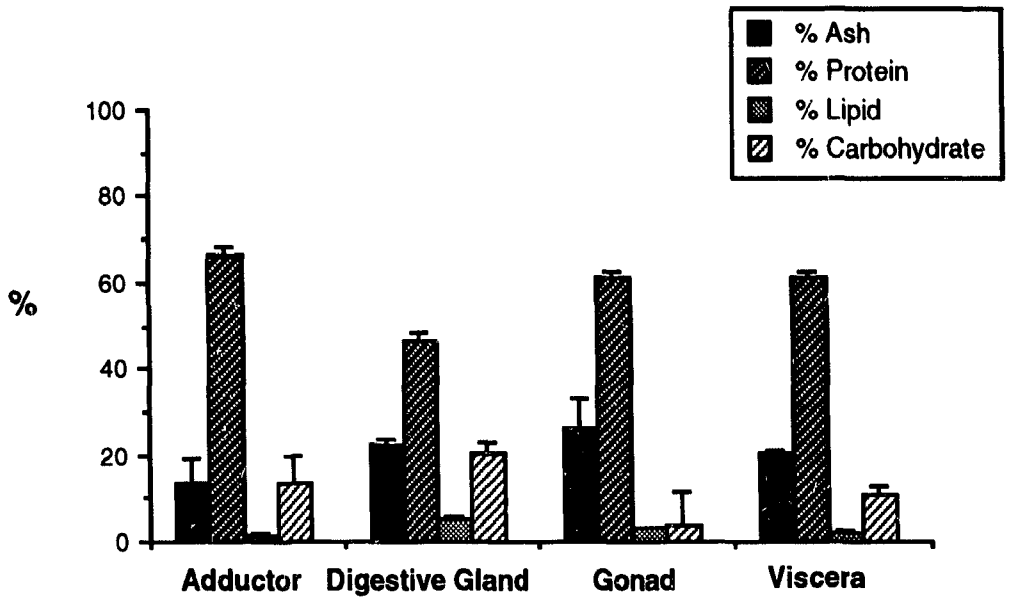


Figure 6 Proximate Composition of Scallops at 18 Months (error bars are one standard error)

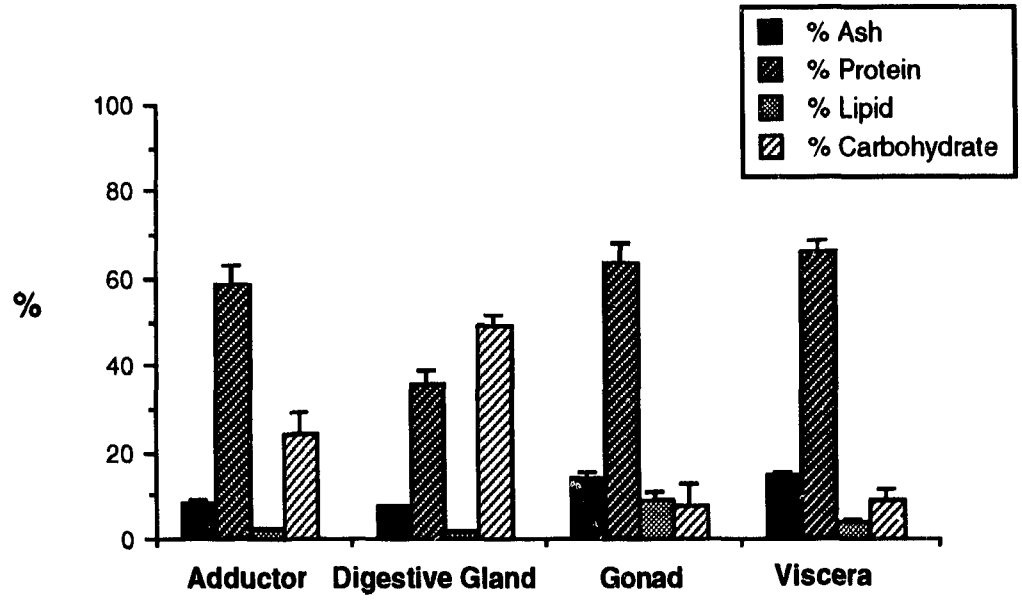


Figure 7 Proximate Composition of Scallops at 21 Months (error bars are one standard error)

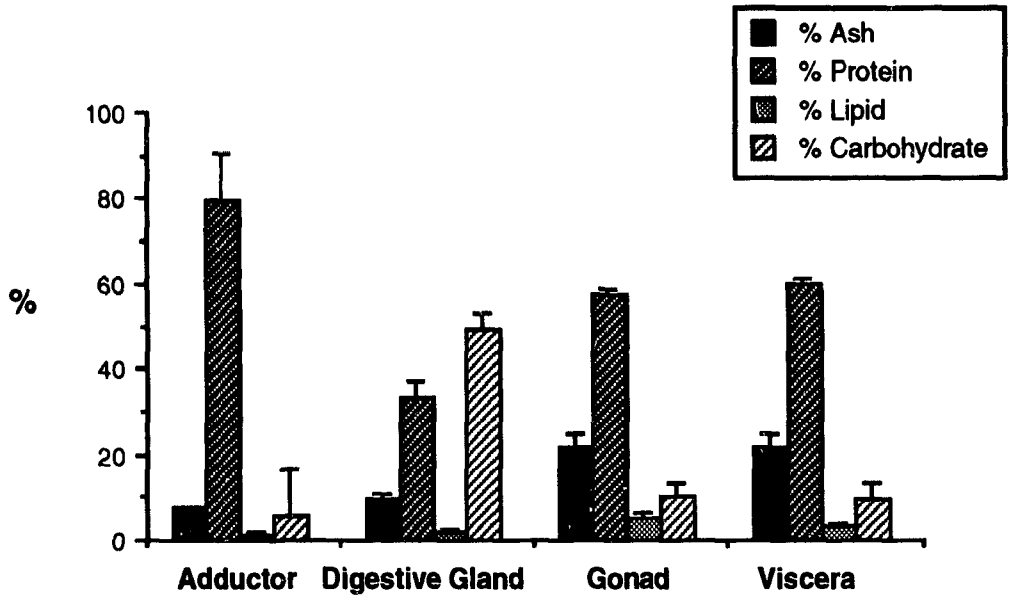


Figure 8 Proximate Composition of Scallops at 24 Months (error bars are one standard error)

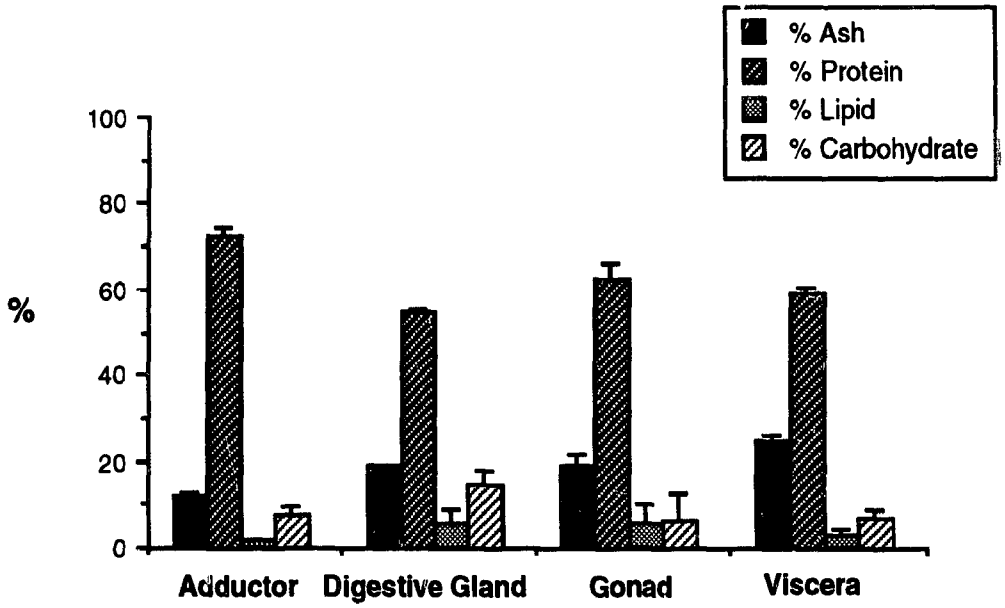


Figure 9 Proximate Composition of Adductor Muscle by Month (error bars are one standard error)

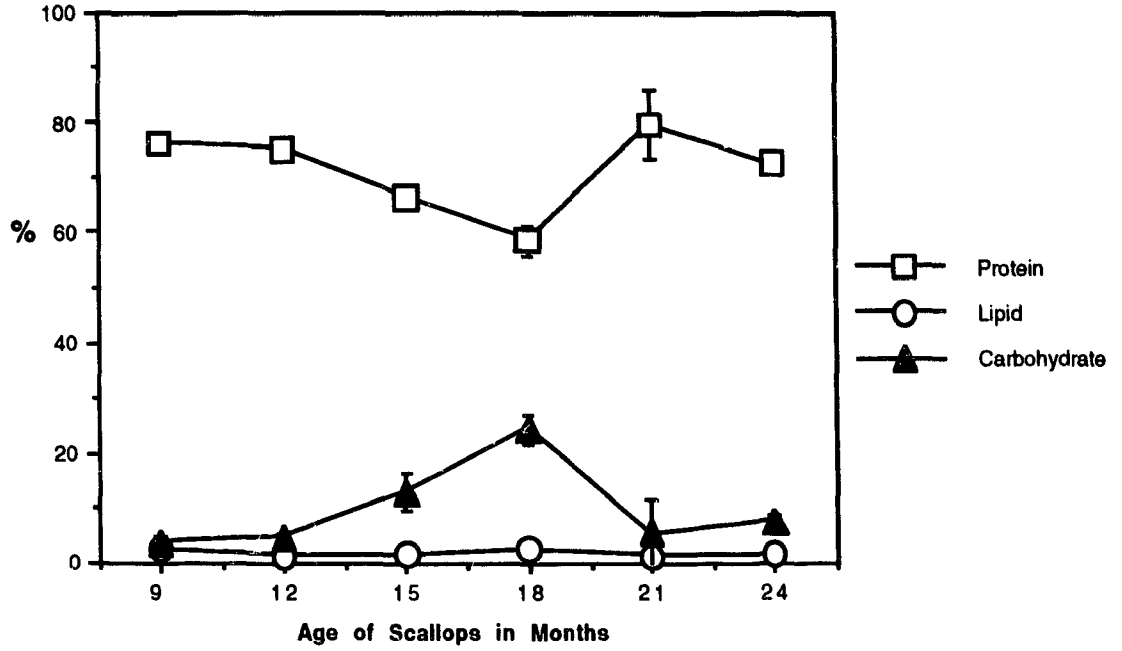


Figure 10 Proximate Composition of Digestive Gland by Month (error bars are one standard error)

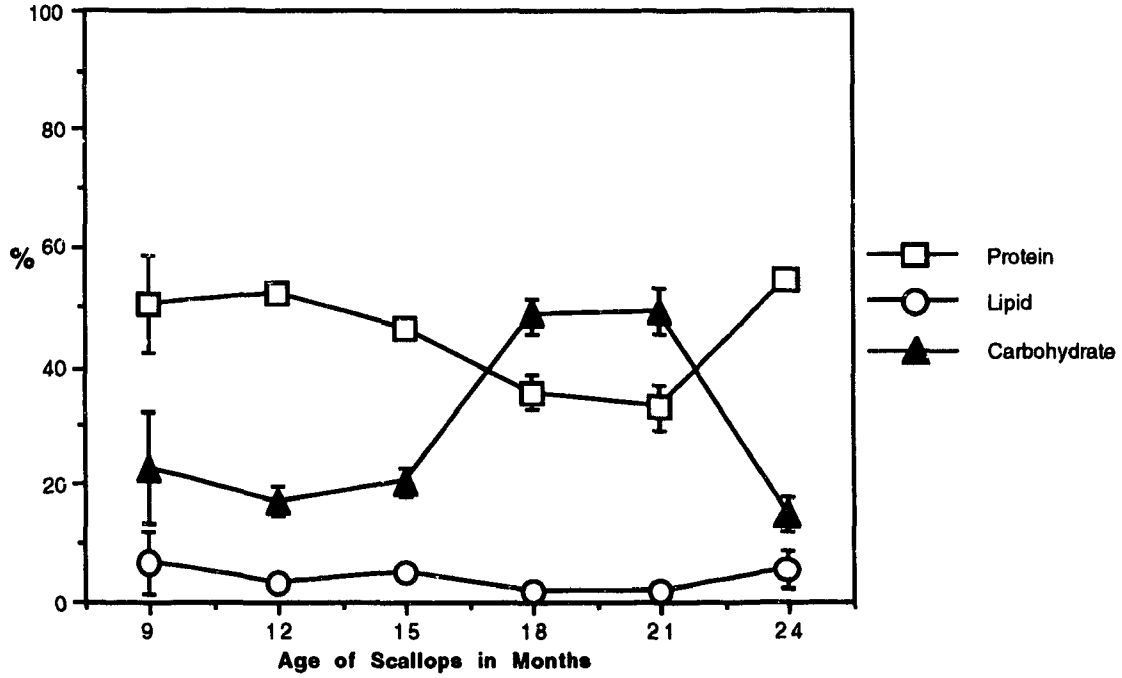


Figure 11 Proximate Composition of Gonad by Month
(error bars are one standard error)

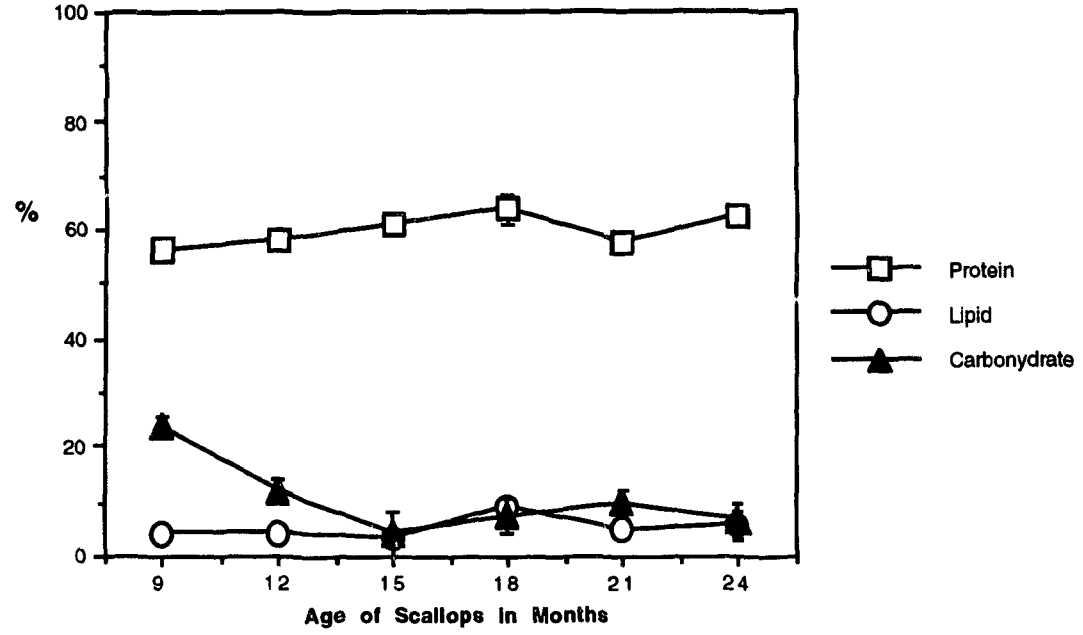


Figure 12 Proximate Composition of Viscera by Month
(error bars are one standard error)

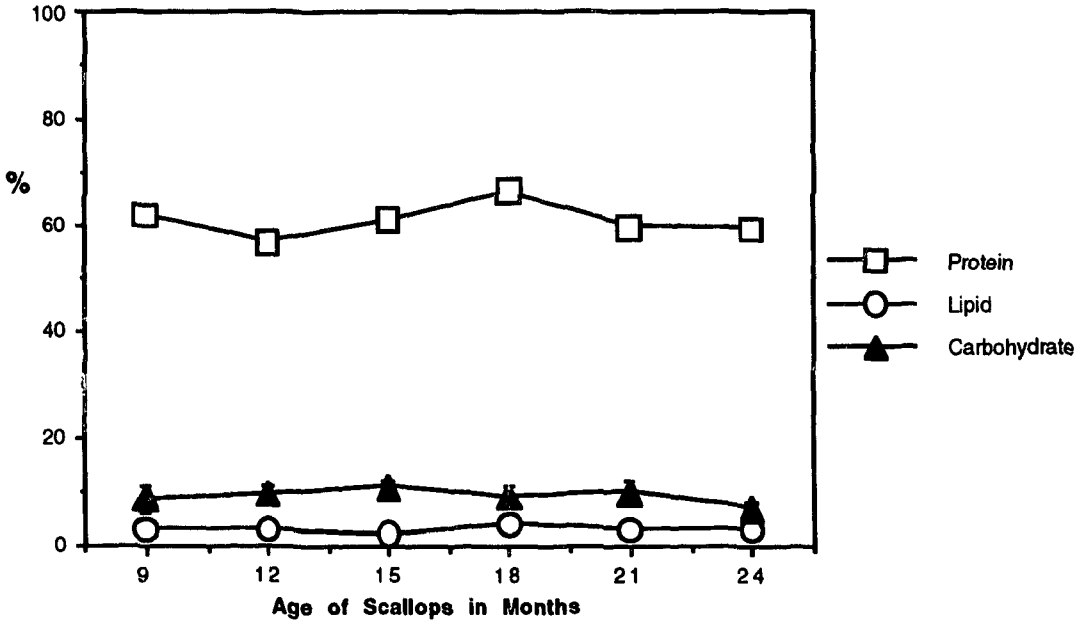


Figure 13 Percentage of Lipid in Different Tissues (Dry Weight Basis) (Compiled from preceding figures).

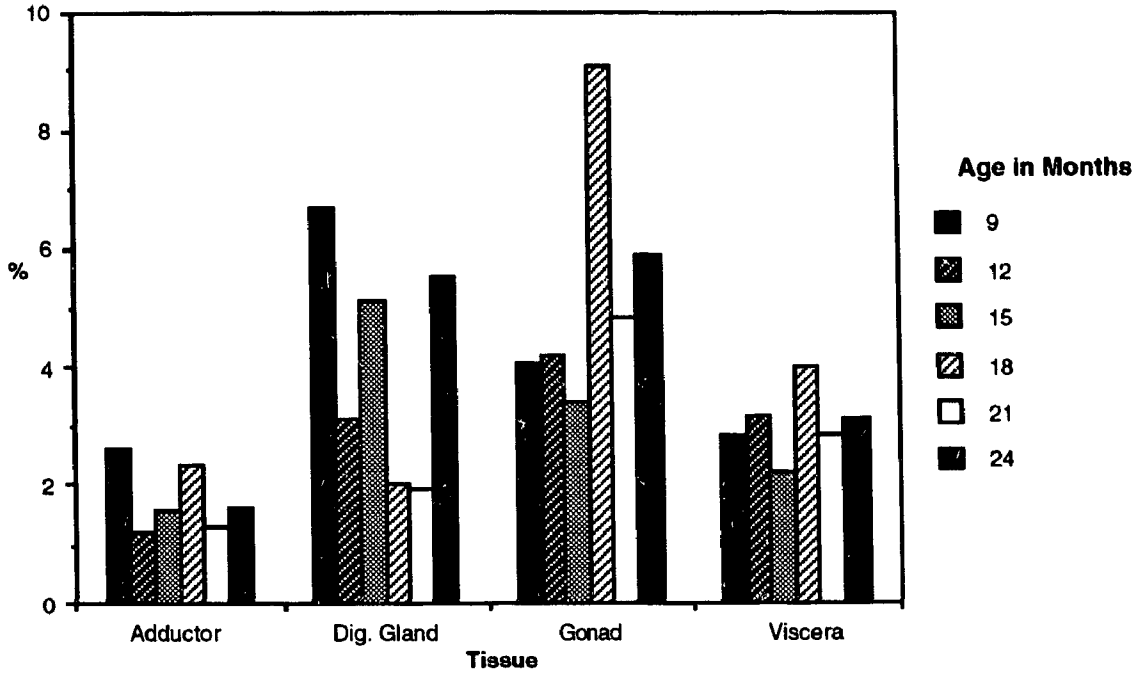


Figure 14 Proximate Composition of Newfoundland *Placopecten magellanicus*
Data are from Manning 1985.

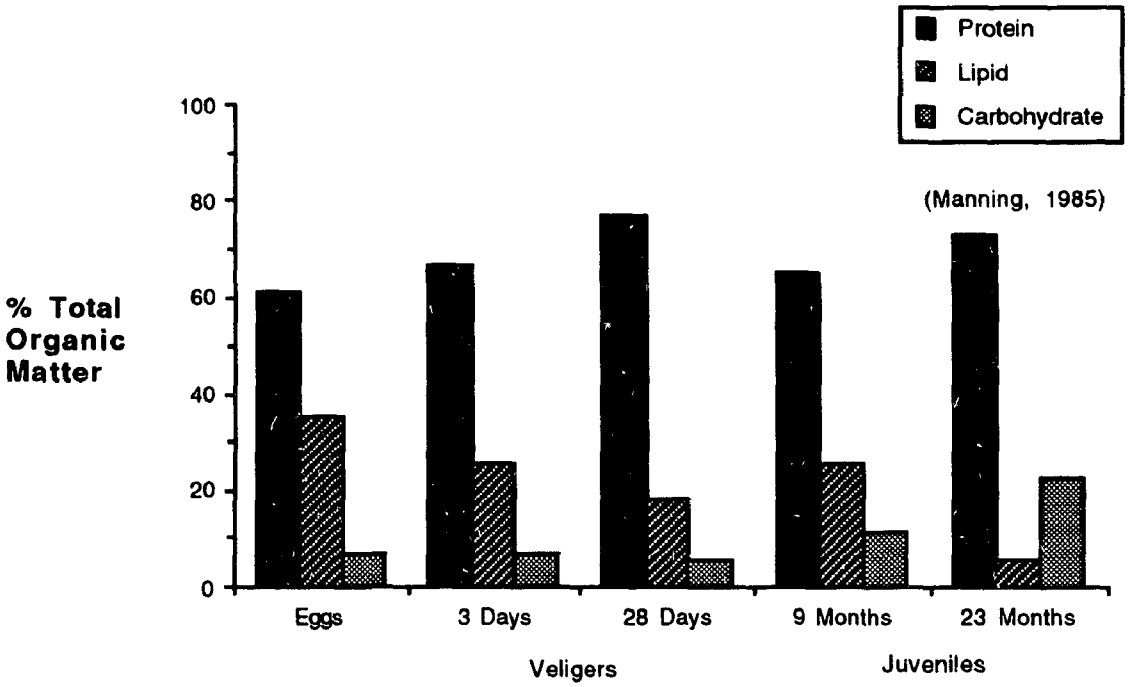
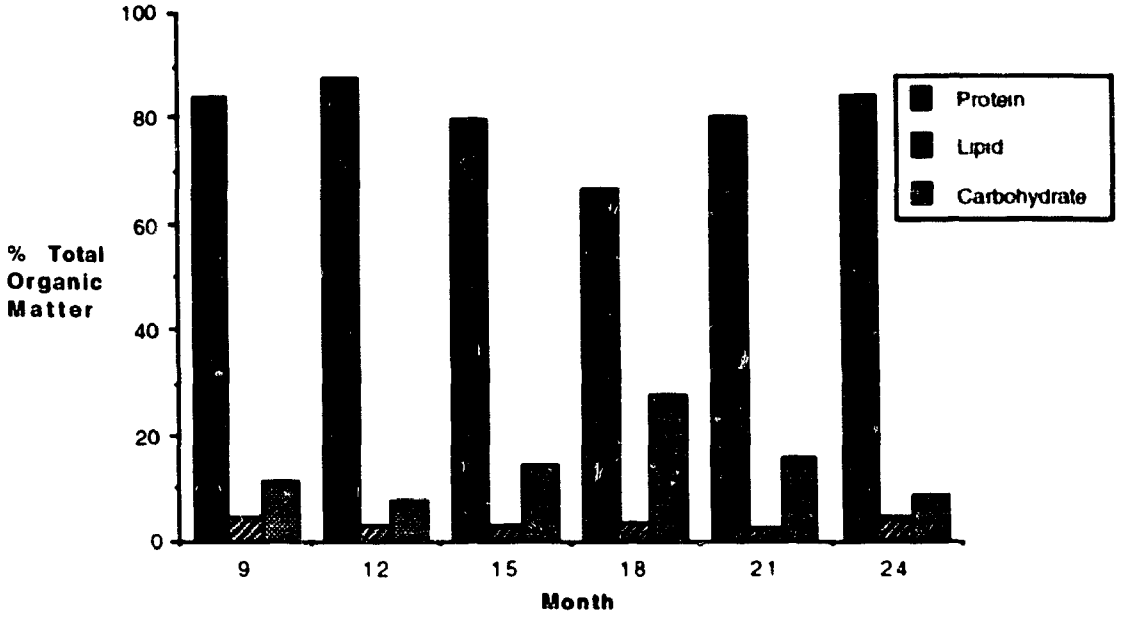


Figure 15 Proximate Composition of Bay of Fundy *Placopecten magellanicus*
These data are shown in Figures 3 through 8, totaled and adjusted as percent organic matter for comparison with Manning (1985)



3.4. Discussion

Napolitano (1991) completed analyses on adult sea scallops and concluded that, based solely on lipid content, there were two tissue types. The first are the lean organs: the adductor muscle, gills, male gonad and mantle. These had little total lipid and exhibited small seasonal variations. The digestive gland and the female gonad were high fat organs. He concluded that in mature scallops, the digestive gland was the only organ involved in storage of lipid reserves, primarily in the form of triglycerides.

In this study on juvenile (non-reproductive) scallops, there appears to be three categories of organs. The first is the lean category, as in adults showing little total lipid and minimal seasonal variability. The adductor muscle is the only organ in that category. This is consistent with the data for adults and with much of the existing literature.

The high fat organs for the juvenile scallops are both the digestive gland and the gonad. In the juveniles, it should be noted that there was no distinction between male and female specimens; therefore lipid content represents a pooled sample of both sexes. This finding for the digestive gland is similar to that found by Napolitano (1991) for the adults. It is interesting that although gonad samples were pooled and had not necessarily reached maturity, this tissue still reflects a high lipid content. Some of the scallops sampled may have been sexually active, being then at the end of their second year (personal communication, Dr. Michael Dadswell). Although Dr. Dadswell expressed this possibility, there was no indication of sexual maturation. If that is the case, then the finding is consistent with that of the adult data.

There is apparently a third organ grouping found in the juvenile scallops. The viscera has intermediate fat levels -- not as low as the adductor nor as high as the fat organs. Since this group contains both mantle and gills, one would expect it to fit into the lean classification as with the adult gill and mantles, but that is not the case. The results do not necessarily mean that this represents a lipid storage site. There are two possible explainations. First, there is little variation throughout the year. Second, the measurement made was for total lipid and not specifically for triglyceride. The viscera may have a higher lipid content than the adductor muscle because both mantle and gills have a high surface to volume ratio and contain many membranes. The data therefore may reflect a higher content of phospholipids, which are typically the structural (membrane) lipids.

The work of Martinez (1991) indicates that in non-reproductive Chilean scallops there was a considerable variation in biochemical constituents. The adductor muscle levels showed little annual variation in protein and lipids but the carbohydrate content was greatest in spring and much reduced in summer. In this study, there were also variations in the lipids of non-reproductive sea scallops. The adductor muscle levels of lipid, as in the Chilean juveniles, did not change much through the year. Protein, in contrast, dropped substantially in winter and spring. Juvenile sea scallops showed little variability in the gonad tissue while the Martinez (1991) data indicate that Chilean juveniles had an increase in gonad protein, gradually accumulating from summer to spring. The carbohydrate showed an increase in autumn and the lipid showed a slight decrease in winter. Since Martinez analyzed mantle tissue (not viscera as in this study) and did not analyze digestive gland tissue, other comparisons cannot be made. It is

apparent, however, that intraspecific variations can occur in the biochemical composition of various tissues. The Martinez (1991) data support the findings of Manning (1985) that these variations can be age dependent.

These data are consistent with the concept of a biochemical metamorphosis occurring within the bivalve's life history. It is important to identify these variations not only for our basic understanding of bivalve physiology but also to determine the basic nutrient requirements and how these may change as the animals grow. For example, for both species (the Chilean and the sea scallop) there was little annual variation in the content of the juvenile gonads. This is in sharp contrast to the variability found in the adults. Lipid reserves are needed for development of eggs and the subsequent support of larvae. The lipid content of a broodstock synthetic diet should therefore be higher than that of a juvenile diet.

4. Microencapsulation Procedure

4.1. Introduction

Microencapsulation is a type of miniature packaging in which liquids or particulate materials are enclosed within a specially designed wall or capsule (Meyers *et al.* 1971). Methods of producing microparticulate diets of major interest are: nylon-protein microcapsules; microgel particles; and gelatin acacia walled microcapsules (Langdon *et al.* 1985). Nylon-protein microparticles were used by Chang *et al.* (1966), in their development of artificial red blood cells. This technique was later applied by Jones and his co-workers (Jones *et. al.* 1974) for growth studies with *Artemia*. Jones later used them for studying nutrition requirements of various crustacean species (Jones *et al.* 1979). Levine *et al.* (1983), investigated the effectiveness of modifications of the diet in nutrition studies with crustacean larvae. Nylon-protein capsules were tested by Kanazawa *et al.* (1984) in their attempt to culture the noble scallop, *Mimachlamys nobilis* Reeve, using synthetic diets. Scallops fed the nylon-protein diet did not perform as well as those animals fed a carageenan microbound diet. Chu *et al.* (1987) were the first to be successful in rearing oyster larvae (*C. virginica*) through metamorphosis on nylon-protein diets. Only 1% of the larvae made it through to viable spat and the larval development did lag considerably behind the algal controls (9 to 14 days). Gabbott *et al.* (1976) reported limited growth of *C. gigas* juveniles fed on a nylon-protein capsule containing 10% hemoglobin (w/v), 10% starch and 0.2% cholesterol. Similar poor results were found using nylon-protein diets for larvae and juveniles of *C. gigas* by Langdon (1977).

High molecular weight, water soluble nutrients such as protein and starch can be trapped in gels of various materials including calcium

alginate, gelatin, carageenan, chitosan, agar or calcium carboxycellulose. Many of these agents have been routinely used in preparation of diets for nutrition of finfish. Levine and Sulkin (1984) used calcium alginate capsules for nutritional studies with crab larvae. With this method, diet ingredients were mixed with a selected gelling agent and the mixture was sprayed into a calcium chloride bath (Levine *et al.* 1983). Other types of microgel particles have been made and tested with aquatic species. Kanazawa (1983) used carageenan gel diets containing soy lecithin and ω3 fatty acids which enhanced the growth and survival of *Penaeus japonicus.* Teshima and Kanazawa (1983) used a carageenan gel diet to grow prawn larvae (*P. japonicus*). One of their findings was the importance of freeze drying in maintaining the nutritional value of the diet. Bautista *et al.* (1989) found that a microgel diet could be used as a partial or total substitute for rearing *P. monodon.* Chu *et al.* (1987) reported that when oyster larvae were fed a gelatin acacia alginate bound diet there was a 10% survival to the eyed larval stage and concluded that the calcium alginate bound diet may be acceptable to bivalve larvae. Lipid microspheres can be used to encapsulate specific fat soluble nutrients (Robinson 1992, Heras *et al.* 1994).

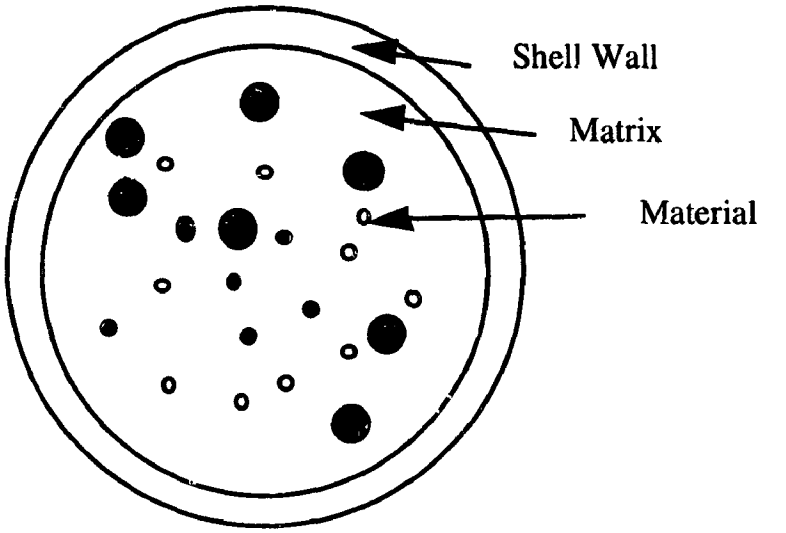
Gelatin-acacia microcapsules are formed by a simple co-acervation reaction between gelatin and acacia at an aqueous-solid or aqueous-lipid interface (Langdon *et al.* 1985). These particles are stable and can be autoclaved. These were used by Langdon and Waldock (1981) to encapsulate specific dietary lipids fed as nutrient supplements with algal diets. Chu *et al.* (1982) found that gelatin-acacia microcapsules were accepted and ingested by the larvae of *C. virginica.*

One procedure which reduces the loss of leachable water soluble nutrients involves adding a lipid wall to the microparticle. This was used successfully by Chu *et al.* (1982) in work with oyster larvae. Langdon and Seigfried (1984) fed lipid-walled capsules to juvenile oysters and found that growth was 73% of the algal controls.

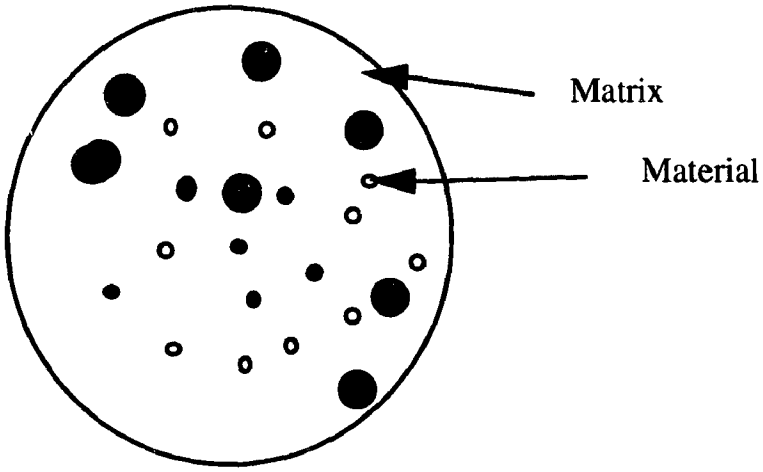
Strictly speaking, unless the microparticle has a shell wall, be it of lipid or another substance, it is correctly called a microsphere. Only walled microparticles are called microcapsules (See Figure 16).

In conclusion, there has been some limited success in the use of microparticulate diets in rearing penaeid larvae in recent years; however, currently, there is no commercially satisfactory artificial diet to replace algae in supporting the growth and development of bivalve larvae.

Figure 16 Microcapsules vs. Microspheres



Microcapsule



Microsphere

4.2. Formulations Tested

As stated earlier, diet formulation was based on the knowledge of proximate composition of scallops and their algal foods as well as on previous nutrition studies on bivalves. In the diets tested on both crustacean and bivalve species, as described in the previous section (3.1), the most abundant nutrient was protein, which was almost always greater than 50% of the diet. Carbohydrate was plentiful and often a component of the microencapsulation procedure (e.g. carageenan and sodium alginate). Various vitamin and mineral premixes were used. Many of those used in bivalve work were based on diets for crustaceans, which in turn were derived from fish nutrition research. Lipid components often included lecithin which could have a beneficial effect as a nutrient (phosphatidylcholine) or for its physical properties as an emulsifier. The importance of polyunsaturated fatty acids was also noted and menhaden oil in particular was popular as it is rich in 20:5ω3 and 22:6ω3 (Ackman and Burgher 1965). The work of Reid (1983) showed that many digestive enzymes are present in bivalves and no limitations were noted.

Accordingly, the overall balance of the formulation selected was based on the work done in John Castell's lab (DFO Halifax) on crustacean nutrition.

Having identified the major nutrients, it was necessary to identify any minor nutrients which may be of benefit in the diet. Chu *et al.* (1987) identified the need to investigate the possible role of attractants or phagostimulants in bivalve diets. There is little reported evidence that chemical signals are important in the feeding and selection processes of bivalves. There seemed to be stronger evidence that selection is based on

physical properties of particles (Gallager 1988). There was one report that dextrose increased the pumping rate of *Mytilus edulis* and 1% was added to the formulation.

4.3. Criteria for Selection

Before selection of a suitable microencapsulation procedure, the following criteria were evaluated:

1. Ability to carry (deli[illegible]) nutrients.
2. Water stability.
3. Suspension stability.
4. Ingestiblity.
5. Digestiblity.

All three procedures -- nylon-protein, microgel, and gelatin acacia walled microcapsules -- had been demonstrated to carry nutrients, be stable in water and stable in suspension. It was difficult to evaluate the next criterion: ingestion. Whether or not a food item is ingested depends on whether or not it meets the criteria for selection. Aside from size (< 100μ for scallops), the selection criteria for bivalve food are poorly understood. Whether a given microencapsulation procedure makes a diet more or less acceptable than another is largely speculative. The final criterion, digestibility, could be approached by looking at the capsule material and deciding whether or not it was theoretically digestible. The work of Reid (1983) indicated that bivalves contain alginase; therefore, a sodium alginate bound diet should be digested. The nylon-protein diets were reported to be more difficult for bivalves to digest (C. Langdon, personal communication). Acacia gel microparticles had been used by this author in an earlier, unpublished, study with *O. edulis* adults and the results were

not encouraging. The decision reached, therefore, was to use the sodium alginate binding method as developed by Levine in 1983.

During Dr. Levine's postdoctoral work in a private company laboratory the methodology had been improved and a patent was pending. The company, Collaborative Labs of Long Island, New York, was willing to co-operate in this research project provided the patent pending procedure was kept confidential. A more detailed description of the diet preparation is therefore not possible until the patent has been granted.

Preliminary work on diet preparation and testing for ingestion and digestion was completed at Collaborative Labs and is discussed in the following section.

4.4. Preparation Procedures and Modifications Required

For these preliminary ingestion studies, fluorescent bead tracers were incorporated into the formulation prior to encapsulation. Sodium alginate-bound microparticles containing the revised formulation (see Part II section 1.3) were tested for acceptability by bivalves at Collaborative Labs, N.Y. to confirm that they were readily ingested and digested.

In collaboration with Dr. M. Bricelj (State University of New York), the fluorescent labeled microparticles were fed to adult mussels (*Mytilus edulis*) over a 2 hour period. Four animals were individually housed in 2 l beakers containing 1500 ml of filtered sea water and about 20 mg of the fluorescent microparticles. Magnetic stir bars kept the microparticles in suspension yet did not appear to disturb the animals. Observations were made on fecal production. and feces were collected and examined under an ultraviolet microscope.

Mussels expel pseudofeces from their inhalant siphon and real fecal material (e.g. digested food) is discharged at the exhalant siphon.

Pseudofeces have a characteristic fluffy, clumped appearance. In contrast, real feces are produced in compact, clearly defined ribbons. Because of these distinctive appearances, it could be readily observed whether a food item is being rejected as pseudofeces or accepted and ingested.

Food was added at time zero. Initially no real feces were produced but large amounts of pseudofeces were observed. It is important to note that the reason for this was apparently the quantity of the ration. Mussels normally produce pseudofeces when given food at a concentration higher than 3 mg dry wt per liter. Over 7 times that amount had been presented. The large ration was to optimize the opportunity of the microparticles being ingested.

About 30 minutes after time zero, some fecal material was observed. It was dark brown, brittle, and contained in a rigid ribbon. The feces were cleaned in filtered sea water and smeared on a slide. Examination of this fecal product under the microscope revealed the remains of many unicellular algae, particularly diatoms. This is the normal diet for these mussels, and the animals had been fed prior to the experiment.

The production of pseudofeces declined, and gradually there was a change in the appearance of the fecal strands. They assumed a pale yellow coloration instead of dark brown. Examination under the microscope revealed that these fecal strands contained a few of the fluorescent beads. Diatoms, however, were still present. (see Plate 1)

About one hour after time zero, there was a pronounced change in the feces. They became not only paler in color but also exhibited a concomitant change in texture. They appeared as smooth gelatinous strands rather than brittle ribbons. This alteration of physical appearance coincided with a substantial increase (800% - 1000%) in the fluorescent

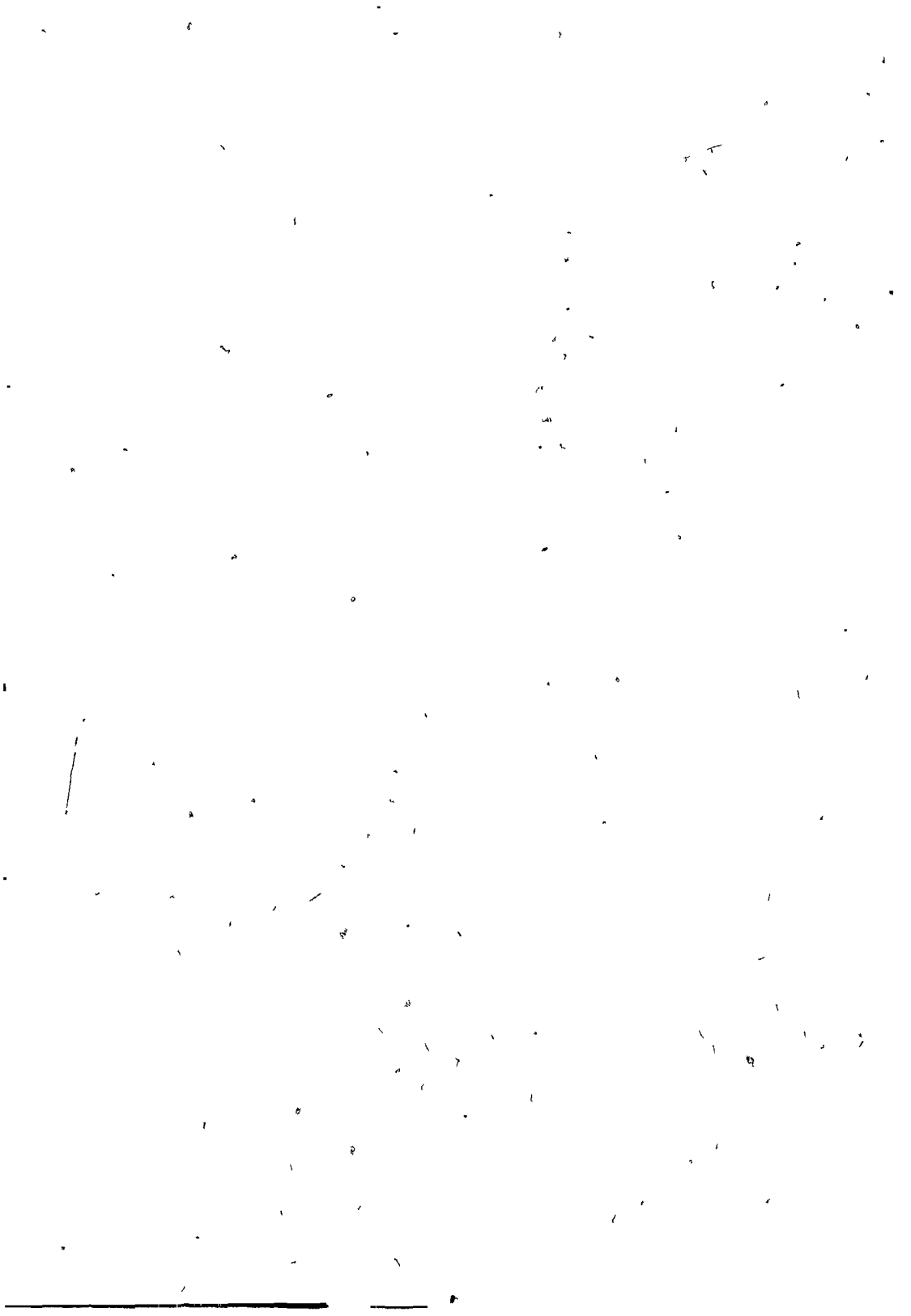


Plate 1 (a) Intact microparticlate diet containing 1 µm latex beads. (b) Fecal material from mussels showing that the latex beads have been released.

beads entrained in the fecal ribbon. These observations confirm that the diet was ingested and caused production of distinctive fecal material.

These data also yield information in addition to the defined objective. Careful examination of the feces (see plate 1b) revealed that the beads were no longer contained inside the microparticles, implying that the microparticles were at least partially digested. Very few intact microparticles were seen in feces. This was an exciting result, and indicated that the microparticle shows potential for delivering nutrients to bivalves.

5. In Vitro Digestion of Sodium Alginate Particles

5.1. Introduction

Although it is important to test for ingestion of food items, if these items are to be of nutritional value, they must be digested. This requires that suitable digestive enzymes be present in the animal. From Reid (1983) it appeared that since many bivalves contain alginase, they would be capable of digesting a sodium alginate microparticle. A more direct measurement of digestibility, however, is preferred.

One approach, recommended by Dr. C. Langdon, Oregon State University, is to place a coating of dye on the microparticle. Once the dye is chemically bound to the surface of the microparticle, it can be released only when the latter is digested. The release of dye can be quantified and comparisons made of various enzyme mixtures. The recommended dyes, Procion dyes (azo dyes) are characterized by an N=N bond to benzene or napthalene rings.

The following *in vitro* experiment was conducted to test the effect of scallop digestive enzymes (from the diverticula and crystalline style) on dyed microparticles, and compare their action on the microparticles with that of commercially prepared enzymes. This work was carried out at the Hatfield Marine Science Center, Newport, Oregon.

5.2. Materials and Methods

5.2.1 Enzyme Mixtures

Tris-thimerosal seawater medium (incubation medium) was prepared by first adding 500 mg of thimerosal to 500 ml of filtered (0.2 μm) seawater. To this was added 3.037 g of Tris buffer (pH of 10) and the pH was then lowered (using concentrated HCl) to pH 7 for amylase, protease,

and seawater controls; and to pH 6 for style, diverticula and the other seawater controls (pH based on known requirements for commercial enzymes and direct measurements for style and diverticula).

The amylase used was Sigma #A6380 (12 mg of enzyme in 5 ml of incubation medium). The protease was Sigma #P4630 (37.5 mg in 5 ml) and to it was added Sigma chymotrypsin #C-4129 (5.9 mg in 5 ml). The amylase was from *Bacillus* species and the protease and chymotrypsin were both from bovine pancreas. These enzymes mixtures were halved and one portion was boiled for 15 minutes to deactivate the enzymes.

The style and diverticula enzyme extracts were prepared from tissues dissected from *P. magellanicus* adults provided from Maine by Dr. S. Shumway. Immediately upon arrival, 4 sea scallops were dissected and styles and diverticula removed. These were placed on ice and the pH was measured. The pH of the styles was pH 6, and of the diverticula close to 6, at pH 5.5. The style material (0.65 g wet weight) was later hydrated with 3 ml of incubation medium, pH 6. The mixture was homogenized (Ultra-Turrax Model SDT-1810) for 1 minute and then centrifuged at 800xg for 15 minutes (Beckman Model TJ-6). The supernatant was decanted and centrifuged again. The resulting supernatant was made up to a 5.0 ml total with 2.0 ml of incubation solution. The preparation for the diverticula extract was similar, starting with 1.0 g wet weight in 3.0 ml of incubation solution. After the second centrifugation, the diverticula extract was opaque and a brown color, so it was passed through a glass filter (pore size about 1 μm), resulting in a clear yellow liquid. In both cases, portions were set aside and boiled for 15 minutes to deactivate the enzymes. These boiled enzymes acted as controls during the incubation.

5.2.2. Procion Blue Stained Particles

The procedure for producing the stained microparticulate substrate is shown in Figure 18. The sodium alginate microparticles were stained with Procion Blue mx-2G (Blue 109, Polyscience). First, 100.0 mg of diet was added to 100.0 mg of Procion Blue 109 in a test tube. To this was added 5.0 ml of distilled water. After a 5 minute wait, 17.0 ml of NaCl (20% solution) was added, and after a further wait of 30 minutes 1.0 ml of $Na_2 CO_3$ (7.2% solution) was added. The tube was then incubated at 55°C for 10 h. (overnight) and then centrifuged for 20 minutes. The supernatant was decanted and the diet washed again with 2% NaCl and centrifuged for 3 minutes. The supernatant was discarded and the dyed capsules retained. Two test tubes of stained microparticles were prepared in this manner. 1.0 ml of capsule suspension (wet wt of capsules 0.05 g) was suspended in 10 ml of incubation medium (pH of 7.0). This was split into two 5.5 ml aliquots. One was added to 35 ml of incubation medium at pH of 6.0, the other to 35.0 ml of incubation medium at pH of 7.0. The result here was 40.0 ml of Procion blue dyed microparticles at pH values of 6.0 and 7.0 respectively. For each assay, 1.0 ml of microparticle suspension was used.

5.2.3. Incubation Procedure

This procedure is shown in Figure 20. First, one ml of stained microparticle suspension was measured into each microfuge tube. To triplicate tubes, 100 µl or 200 µl of the following enzyme preparations was added: style extract, diverticula extract, protease-trypsin mixture, amylase enzyme, boiled style extract, boiled diverticula extract, boiled protease and boiled amylase. To the 1 ml of stained microparticles, 100 µl and 200 µl of seawater at both pH 6.0 and pH 7.0, were also added. For time 0

controls, 100 µl of incubation solution (in triplicate for each pH) was added to 1 ml of microparticles and centrifuged for 5 minutes.

All tubes were incubated for 24 h on a rotary table (Boekel Ind. Inc., Orbitron Model 260200) at 60°C. After incubation the tubes were centrifuged at 1500xg for 2 minutes (Beckman Microfuge Model E) after which 0.5 ml of supernatant was removed from each tube and the absorbance read at 620 nm on a Beckman DI-6 spectrophotometer. Before reading samples, a scan indicated that the maximum absorbance for Procion blue 109 was 620 nm (Figure 17). This setting was therefore used for all samples.

Table 6 Measure of Dye Release with Various Enzymes. Absorbance ± Standard Deviation.

Enzyme Type	Deactivated	Active	Net Effect
PH[1]	0.029±.004	0.632±.054	0.603±.051
PL	0.030±.001	0.686±.043	0.655±.043
AH	0.123±.012	0.148±.034	0.024±.047
AL	0.143±.017	0.135±.023	-0.006±.007
SH	0.036±.005	0.145±.014	0.109±.011
SL	0.013±.022	0.148±.009	0.135±.021
DH	0.044±.002	0.110±.016	0.067±.015
DL	0.048±.004	0.108±.008	0.060±.012
Seawater Controls, pH=6 & pH=7			< 0.010

1 Mean and S.D. of three replicates

P Protease **H** 200µl
A Amylase **L** 100µl
S Style Mixture
D Diverticula Mixture

Figure 17 Absorbance Spectrum for Procion Blue 109

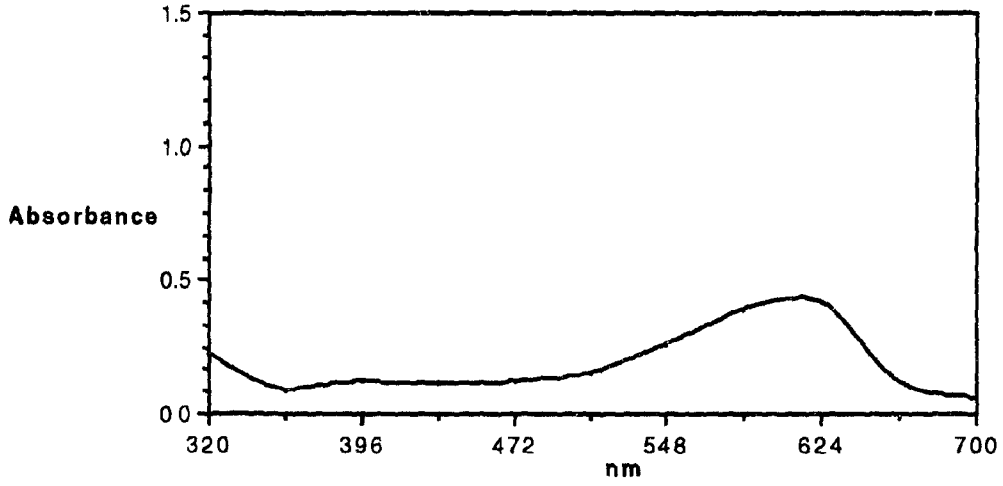
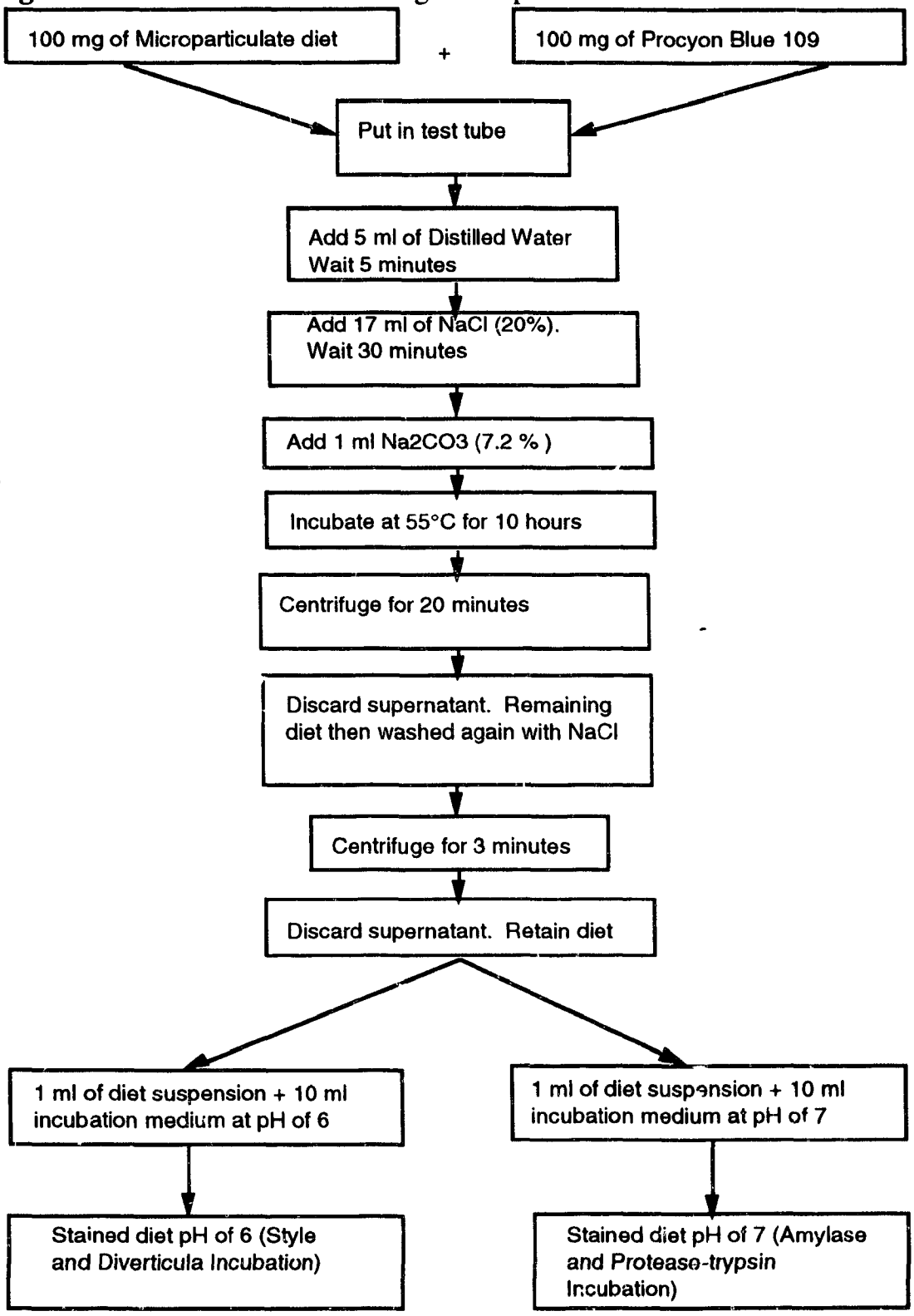


Figure 18 Procedure for Staining Microparticulate Diet



Both pH 6 and pH 7 used for seawater controls

Figure 19 Incubation Procedure

1 ml of stained microparticulate diet was put into each of 60 microfuge tubes. There were 6 tubes for each of the 10 treatments:

3 tubes with 200 µl of each of the following (High amount H) and 3 tubes with 100 µl each of the following (Low amount L):

1. Protease-trypsin mixture
2. Amylase
3. Style mixture
4. Diverticula mixture
5. Boiled protease-trypsin
6. Boiled amylase
7. Boiled style mixture
8. Boiled diverticula
9. Seawater pH=6 (time 0)
10. Seawater pH=7 (time 0)

10 treatments x 6 tubes = 60 tubes.

All tubes put on the orbitron for 24 hours.

After 24 hours, 9 and 10 repeated.

9. Seawater pH=6
10. Seawater pH=7

The difference between the seawater samples would indicate if dye release occurred without any enzymes.

5.3. Results

Table 6 shows the dye released from the microparticle/enzyme mixture and that it varies depending on the enzymes used. The deactivated column are the results from incubation with boiled enzymes. The net effect is the difference between the results for the active and inactive incubations.

The maximum dye release was with the protease enzyme at the lower (100 µl) level, although the result for the high (200 µl) level was similar, 92.1% of the maximum.

The lowest dye release was found with the amylase enzymes (3.6% and 0). It can be seen from Table 6 that the highest deactivated release is with the amylase treatments.

In the case of the style enzymes, there was some dye release; however the net effect was 20.6% and 16.6% of the maximum, for high and low respectively. The diverticula had some dye release as well, however, the net results were about 10% of the maximum.

In all cases where both levels of enzyme were used (H and L) there were only minor differences between the results.

Some of the diet had also been incubated in seawater only to test for leakage of the dye in the absence of any enzymes (Figure 19). Both results were under 0.010 and considered negligible.

5.4 Discussion

These data represent a novel method for measurement *in vitro* digestibility by the scallop style and diverticula. It is essential to note that *in vivo* the microparticulate diet can be broken down by pH changes, mechanical rupture (e.g. grinding by crystalline style) and bacterial action,

as well as by enzymatic action (Meyers *et al* 1971). The *in vitro* assay reflects only a part of the natural digestive processes. I suggest, then, that the digestion of the microparticulate diet would be more extensive *in vivo.*

It is important to note that this new method gives some indication of digestive capability and enzymes from any bivalve can be incubated and tested. This technique can also be used to take enzyme extracts from various parts of the digestive tract (mucus, style, diverticula, esophagus, etc.) and evaluate their impact in breaking down a synthetic diet. Some studies have looked at dye release from inside a microparticulate diet. This is the first study where the dye has been chemically bound to the microparticle.

The Procion dye used in this experiment was a dichlorotriazine dye. The design of this experiment might have been improved, if the mechanism of binding the dye were known. For example, was the dye bound to the carbohydrate or protein moiety of the microparticle? A call placed to the Procion dye manufacturer with a request for further information resulted only in the response that Procion dyes are used in the manufacture of clothing and that their structures are considered proprietary information.

The results for the deactivated enzymes are highly variable but show that some enzymatic activity occurred causing dye release. If the enzymes had been rendered inactive, then a passive leakage of dye would have resulted in similar data for all enzyme mixtures.

The method used to deactivate the enzymes was to place them for 15 minutes in a boiling water bath. This worked well for most enzymes where only a small amount of activity (based on dye release) remained. The protease showed little activity after boiling; however for the amylase results, there is little difference between the active and deactivated. There

are possible explanations for this. First, the protocol for deactivation in boiling water was insufficient. For example, it is possible that the amylase tubes were in the centre of the array of tubes and the heat distribution around them was poor or that the amylase had a high thermal stability. Other reasons focus on the nature of the amylase tested. Often enzymes contain impurities and one of these in the amylase could have promoted dye release. It is also possible that the absorbance read in the deactivated column was not a measure of the Procion blue dye but caused by the absorbance of the amylase itself, or a contaminant, at 620 nm. A sample of amylase alone was not read on the spectrophotometer. Doing that reading on all enzyme mixtures without the substrate and making sure that deactivated enzymes had been rendered inactive would greatly improve the experimental design, but there was no opportunity to repeat this experiment

Two quantities of enzyme were tested (100 µl and 200 µl); however the results were similar in all cases. In retrospect, it appears that the 100 µl amount was sufficient to release all accessible dye and cause some breakdown of the microparticulate diet. Doubling this amount did not change the end result. However, if the amount of dye released had been monitored over the incubation period, the 200 µl quantity might have given a faster result. The 100 µl level may have taken longer to digest the substrate and yielded a result similar to 200 µl only after many hours of the incubation had elapsed. Additional tests on the specific nature of the binding action between the diet constituents and the Procion dye are required before this can be used as a standard procedure.

In vitro digestibility experiments are a useful approach in determining whether or not the necessary enzymes are present. Some

work has been done on the digestive enzymes of bivalves (see the review by Reid 1982) but very little specifically on sea scallops. Wojtowicz (1972) looked at the carbohydrates in the digestive gland and crystalline style of sea scallops and found several in the digestive gland: α-glucosidase, β-glucosidase, laminarinase, β-galacosidase, and chitobiase. He noted that the crystalline style contained very high activity for both α-glucosidase and laminarinase. The optimum pH for these enzymes varied from 4.0 to 6.5. The pH values used for tissue extract incubations in this experiment were based on measurements of the freshly prepared extracts. It was not assumed that these were pH optima; however, it was assumed that the diverticula and style enzymes were not inhibited.

Even in view of the variable results with the deactivated enzymes, the data, based on the tissue enzymes (style and diverticula) clearly demonstrated that sea scallops are capable of at least partially digesting the sodium alginate-bound microparticulate diet. This is a significant result since the microparticulate diet, once ingested, must be digested.

6. Feeding Behavior and Food Presentation for Juvenile Scallops

6.1. Introduction

Microparticle diets with inert markers have permitted us to design experiments which identify suitable conditions for diet presentations. Because sea scallops extract food from water flowing over the gills, factors which determine successful grazing include food particle concentration and current velocity. Most bivalves adjust their feeding rate in response to the quantity and quality of the seston. In general, ingestion rate rises to a plateau level as particle concentration increases (Newell 1981). Excessive amounts of seston can lead to the production of pseudofeces and a decline in the ingestion rate. As long as no pseudofeces are produced, the amount of food ingested is proportional to the amount of water filtered (Winter 1978). However, Shumway *et al.* (1985) have shown that *Placopecten magellanicus* selects and utilizes only some of the algal species it filters.

Robinson (1983) had previously attempted to use inert spheres, suspended in the water with algae, to quantify ingestion in *Mercenaria mercenaria* and found that the algae were selected preferentially. By incorporating the inert indicator Fluoresbrite™ beads inside food particles, it was possible to estimate ingestion rate accurately under a variety of conditions.

Aside from food concentration, high current velocities cause less food to be ingested by scallops due to inhibition of filtration (Kirby-Smith 1972, Wildish *et al.* 1987, Eckman *et al.* 1989, Cahalan *et al.* 1989). Growth of *Placopecten magellanicus* is inhibited at flow velocities exceeding 10-20 cm sec^{-1} (Wildish *et al.* 1987). Scallops may also alter their orientation in response to current velocities. Eckman *et al*, (1989)

tested bay scallop orientation and found that over a wide range of velocities, the preferred orientation was posterior pointed downstream.

Using the labeled microparticulate diet as a tool, experiments were conducted to determine the optimal food concentration and flow rate for delivery of the diet. Ingestion was measured directly by counting the Fluoresbrite™ beads found in the digestive tract. These results provide useful information on the application of food and the determination of protocol for long term nutrition studies.

6.2. Materials and Methods

6.2.1. Microencapsulated diet

Microparticle diet was prepared and encapsulated at Collaborative Labs, Long Island, New York. The diet contained nutrients, sodium alginate and latex beads (Polyscience Inc.) stained with a fluorescent dye (Table 7). The diet was examined under a fluorescence microscope to ensure that during preparation the dye had not migrated from the Fluoresbrite™ latex beads; it was then screened through a 40 μm mesh before use.

Table 7 Formulation of the Diet (prepared by Collaborative Labs) used in Ingestion Experiments.

Ingredient	Dry Weight
Cornstarch	20.5
Dextrin	1.0
Crab Protein[1]	30.0
Vitamin Mix[2]	2.0
Mineral Mix[3]	5.0
Lecithin	7.0
Menhaden Oil Fatty Acids[4]	10.0
Cholesterol	1.0
Carboxymethyl Cellulose	3.5
Total	80.0%[5]

(1) Supplied by Dr. J.D Castell, Dept Fisheries and Oceans, Halifax.

(2) Diluted with cellulose to provide the following (mg/kg diet): thiamine HCl, 64; ascorbic acid, 1200; riboflavin, 144; niacinamide, 512; biotin, 1.6; calcium D-pantothenate, 282; pyridoxine HCl, 48; folic acid, 19.2; menadione sodium bisulfate, 16; cobalamine (vitamine B_{12}) 43.2; L-mesoinositol, 2500; calciferol (Vitamine D_2, 400,000 IU/g), 4.9; vitamine A acetate (850,000 IU/g), 100; p-aminobenzoic acid, 400; butylated hydroxyanisol, 150; butylated hydroxytoluene, 150.

(3) Diluted with cellulose to provide the following: (mg/kg diet); citric acid, 100; cupric citrate, 46; ferric citrate, 279; magnesium oxide 1250; magnesium sulfate 417.5; potassium iodide, 0.5; potassium phosphate, 4050; potassium sulfate, 3400; sodium chloride, 1530; sodium phosphate, 1070; sodium fluoride, 1; cobalt chloride, 10; zinc citrate, 66.5.

(4) Supplied by Dr. R.G. Ackman, Technical University of Nova Scotia, Halifax, and includes antioxidant.

(5) Remaining 20.0% is sodium alginate, spiked with 1 µm latex beads coated with Fluoresbrite™.

6.2.2. Scallops

Scallops collected in February from an experimental field station in Passamaquoddy Bay, New Brunswick by Mr. Michael Dadswell, Acadia University, were held in flowing ambient seawater and fed unicellular algae, *Isochrysis* aff. *galbana* (clone T-ISO) and *Chaetoceros muelleri* for one week. Prior to the trials, animals were not fed for 24 hours to allow for gut clearance, thereby eliminating gut fullness as a factor affecting feeding. Forty-five juvenile scallops with an average shell height of 3.8cm (0.29sd) and wet weight of 5.17 g (1.3 sd) were used in these experiments.

A series of preliminary experiments was executed at 4° C to estimate the first appearance of feces after feeding. In most cases, feces were produced after 1 h, although in one case feces appeared in 45 minutes. Experiments were therefore run for only thirty minutes to ensure that all ingested food remained inside the scallops.

6.2.3. Flow Tank

The flow tank used for these experiments was the same as that used by Okamura (1984), itself a modification of a tank developed by Vogel and LaBarbera (1978). It consists of a rectangular Plexiglas feeding chamber (13 cm x 13 cm x 40 cm) joined on either end by an ABS pipe (total capacity = 22 l). The tank was filled with filtered (5 µm) seawater which averaged 31 ppt salinity. A styrofoam jacket was used to insulate the flow tank and keep the temperature at 3-4°C. A rotary motorized shaft was used to drive a propeller on the far side of the outflow baffle. Current velocity was set by adjusting a rheostat controlling the motor's speed. Flow calibrations were made by timing the movement of the microparticles across a measured distance in the tank, 1 cm off the bottom (about the

height of the scallop mantle). Water velocities were also measured using potassium permanganate dye but this proved less accurate due to turbulence.

6.2.4. Experimental Design

The ingestion experiments followed a factorial design with three current velocities and five concentrations of microparticulate diet. The current velocities (5, 10 and 15 cm sec^{-1}) were selected after evaluating data for adult scallops in Wildish *et al.* (1987). The five diet concentrations of the microparticles (5, 10, 20, 40 and 80 mg l^{-1}) were selected after a series of preliminary observations which indicated that no pseudofeces were produced at diet concentrations of 5-80 mg l^{-1} during a 0.5 hour period. At all levels, the amount of food consumed was not sufficient to reduce initial food concentration. Three replicates were completed at each of the 15 treatment combinations. In each replicate, a single scallop was placed in the centre of the flow tank and current velocity was then increased to the test level. After a 5 minute acclimation period, food was added.

Observations on scallop orientations and feeding behavior were recorded. At termination of each trial, scallops were immediately placed in filtered seawater (5 μm) for two minutes to clear food from the mantle cavity. They were then gently shaken to dislodge microparticles from the gills and mantle, removed from seawater, and immediately frozen at -70°C.

6.2.5. Estimation of Ingestion

Scallops were removed from frozen storage, dissected, the digestive tract excised, and homogenized in 5 ml of distilled water. For each scallop, 10 samples of the homogenate were taken and placed on a standard haemocytometer. The fluorescent beads were counted under a fluorescent

microscope (Large Fluoresence Zeiss). The sampling and counting procedures were determined empirically from the method outlined in Cassel (1967).

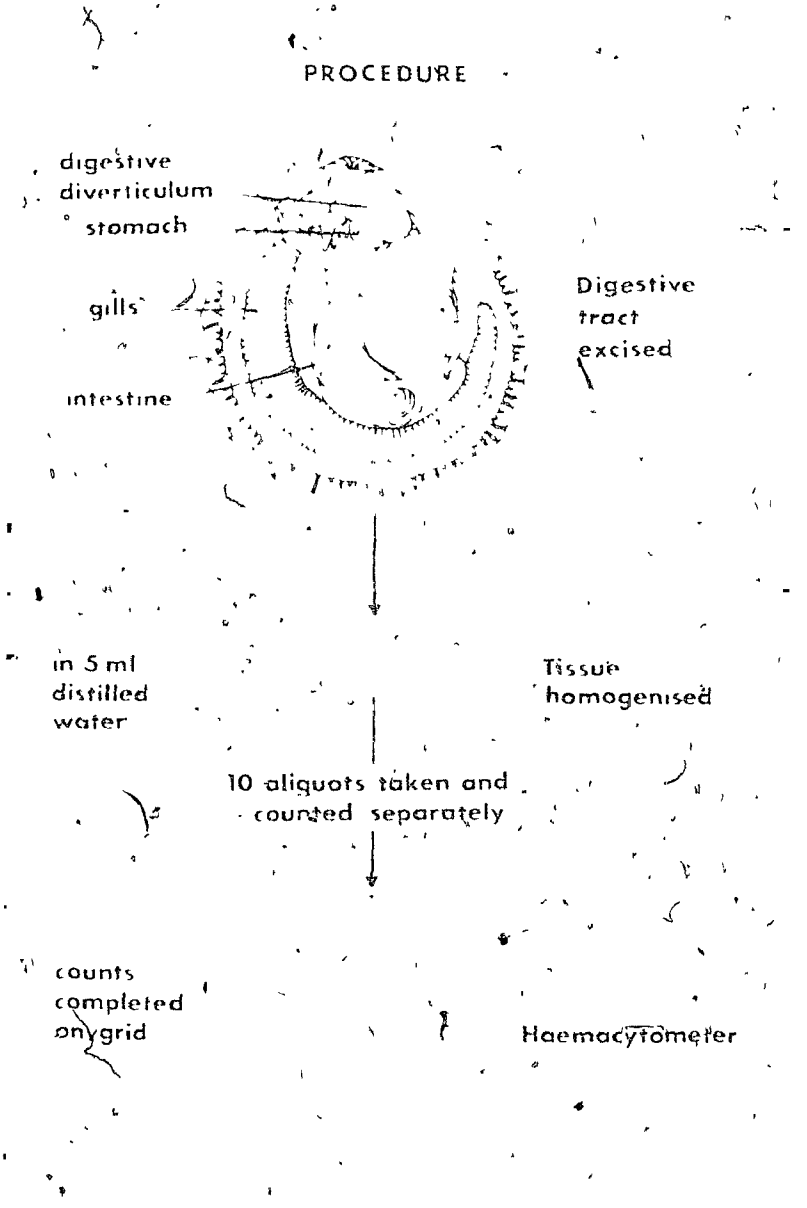
For some scallops, a second set of samples was taken from the homogenate and counted to determine precision. These results differed by less than 10% from the initial observations.

Subsamples for counts were taken only from well-mixed homogenate samples. Counts on the homogenate showed that even after 5 minutes, some settling of the microparticles had occurred.

Although care was taken to completely excise the intestinal tract, there was a possibility that beads might have been present in other soft tissue (e.g. gills, mantle). In three specimens, the washing of the remaining tissue and examination of the resultant suspension revealed no beads.

The ingestion data were analyzed with a two way ANOVA using SYSTAT (Wilkman 1987) after verifying normality and homogeneity of variance for the data set within each concentration.

Figure 20 Protocol for Estimating Ingestion

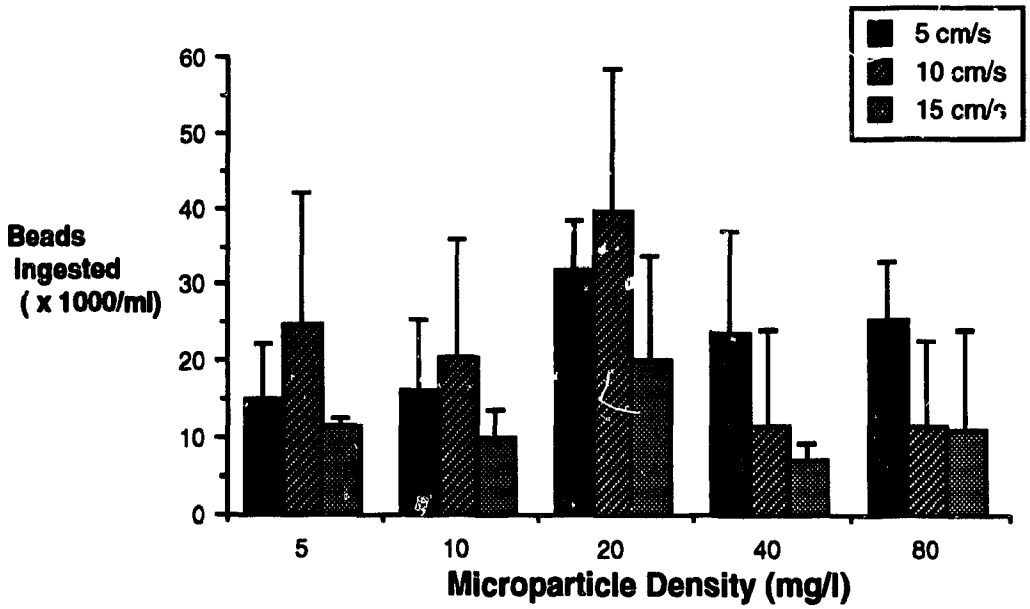


6.3. Results

A two way ANOVA (untransformed data) revealed significant differences in ingestion rate for the food concentration treatment ($p=0.035$). The flow treatment was marginally significant ($p=0.052$), but this result is less critical for the practical reasons below. There was no statistically significant interactive effect between treatments ($p=0.445$) (see Appendix 8.

At 5 cm sec^{-1} (Figure 21), much of the microparticulate diet settled out of suspension. Thus the amount of food available to the scallops was less than at the higher velocities. At 15 cm sec^{-1}, most of the scallop were transported to the downstream end of the tank, pressed against the outflow baffles, and often remained closed. The somewhat lower ingestion levels were a result of the physical stress to which the animals were subjected. In contrast, scallops at lower velocities moved about and often reoriented during the test period. At 10 cm sec^{-1}, scallops were open and appeared to be feeding. Moreover this velocity was sufficient to keep all the microparticulate diet in suspension.

Figure 21 Ingestion vs. Flow Rate



Linear contrasts between means for the various concentrations indicated that the ingestion rate at 20 mg l^{-1} was significantly higher than at any of the other concentrations ($p=0.003$). Pairwise contrasts between ingestion at the other concentrations (i.e. excluding 20 mg l^{-1}) indicated no significant differences ($p>0.38$). There was no correlation between the orientation of scallops and food concentrations at 5 cm sec^{-1} and 10 cm sec^{-1}.

6.4. Discussion

Our results indicated that at flow rates of 15 cm sec^{-1} feeding was inhibited by physical dislodgement. Had scallops been given sufficient time to establish byssus threads to the tank floor feeding at the highest velocity, 15 cm sec^{-1}, might have been different. Evidence of reduced feeding by scallops at current velocities of 15 cm sec^{-1} was found by Wildish and Kristmanson (1988). They showed that at higher flow rates, the growth of *Placopecten magellanicus* is inhibited, possibly due to a pressure differential between inhalent and exhalent siphons which interferes with filtration.

The hydrodynamic conditions present in the flow tank are unlike those of the natural benthic boundary layer. The absence of sediment means that scallops are not recessed in their characteristic way (Caddy 1968) or exposed to boundary layer roughness. These results, therefore, should not be considered to represent wholly the natural feeding ecology of scallops, but rather are useful in designing laboratory experiments for nutrition research. Within a culture system, current velocity at the scallop will vary depending on the structure of the boundary layer. Our measurements at 1 cm height were generally within the freestream flow

such that the application of these velocities to culture containers should be considered in terms of average flow rates.

The absence of observable orientation patterns suggests that a half hour test period may be insufficient for the scallops to establish a permanent orientation. On the other hand, juvenile scallops may be opportunistic and continually reorient to optimize feeding conditions. Eckman *et al.* (1989) have concluded that changes in orientation of bay scallops may provide a significant means of maintaining high growth rate under field conditions.

Ingestion of the microparticulate diet was greatest at a concentration of 20 mg l^{-1}. Due to sinking of food at the 5 cm sec^{-1} flow rate and transport of scallops at 15 cm sec^{-1}, 10 cm sec^{-1} was selected as a suitable starting point for long term growth experiments. Modifications may be required if other factors such as assimilation efficiency and scallop biomass are shown to affect assimilation. For example, bigger scallops may be more stable at higher flow velocities. Studies of ingestion rates of attached or recessed scallops are desirable, since high velocities and turbulence are effective at keeping particles in suspension and delivering them to the animal.

These results demonstrate that the microparticulate diet containing the Fluoresbrite™ bead marker was an effective tool in examining the effects of current speed and food concentration on scallop feeding rates. This method quantifies food intake directly and was clearly more accurate than methods which rely on monitoring the disappearance of food from suspension.

The microparticulate diet was readily accepted under a variety of presentation regimes and is a useful tool for future nutrition studies with

bivalve molluscs. Advances in molluscan nutrition can be made by using this technique for other applications including selectivity based on particle size, chemical attractants, and toxins. Further work should address the effects of diet quality on assimilation, growth, and survival.

Part III Effectiveness of Sodium Alginate Microparticulate Diet in Feeding Experiments with Juvenile Scallops, Bivalve Larvae And Early Spat

1. Feeding Studies with Bivalve Larvae and Spat

1.1. Introduction

The major cost in production of bivalves in hatcheries is the high overhead associated with algal production. Through the use of microencapsulated diets, bivalve larvae may be fed a diet of known composition and subsequent changes in diet formulation can be made to meet the specific nutrient requirements of the larvae. This could mean using some live algae in combination with a synthetic diet or a diet based entirely on a synthetic microparticle. The latter would be readily made, easily stored, and of consistent nutritional quality.

This chapter reports results on the acceptability and digestibility of a sodium alginate microparticulate diet by three species of bivalve larvae. Experiment I was completed in Halifax, N.S. in September of 1988 in conjunction with A. Silva, using the sea scallop, *Placopecten magellanicus.* The other two experiments were conducted in Dr. C. Langdon's laboratory at the Hatfield Marine Sciences Centre, Oregon, in October of 1990 using two Pacific coast species: the oyster, *Crassostrea gigas rivularis,* and the Manila clam, *Tapes philippinarum.*

1.1.1. Experiment 1. Scallop Larvae (*Placopecten magellanicus*)

A sodium alginate microparticle carrying the diet formulation described in Table 7 was used to study microparticle acceptability by veliger larvae of the sea scallop reared under laboratory conditions. The presence of a fluorescent dye (Table 7), enabled the ingestion of particles to be seen in the larvae under a fluorescence microscope (Large Fluorescence Zeiss). Two preliminary studies were completed. First the larvae were observed while capturing and ingesting the particles. Photographic and videotape records were kept. Second, a short term feeding trial was run using three treatments: the microparticulate diet, an algal diet and an unfed control.

1.1.2. Experiment 2 Oysters (*Crassostrea gigas rivularis*)

The Pacific oyster is an important commercial species in the western United States and is cultured on a world wide scale. This experiment was designed to test the growth response of oyster larvae to the sodium alginate particle.

In order to keep microparticulate diets in suspension, the water has to be kept gently moving. In some experiments diets have been presented to larvae in glass jars fitted with aerators. The aerators are effective in keeping particulate diets in suspension but the bubbling can cause the larvae to move around considerably. Preliminary tests were conducted using magnetic stir bars and these proved unsatisfactory. This experiment was designed to test the use of an orbital shaker to keep diets in suspension. The shaker was run at low speed throughout the trial and one replicate, the algal treatment, was kept on the counter and aerated according to the normal procedure.

1.1.3. Experiment 3 Clams (*Tapes philippinarum*)

The Manila clam (*Tapes philippinarum*) is cultivated world wide and is a very hardy species for laboratory studies. This experiment was begun using late larvae, many of which were starting to settle. By the end of the experiment metamorphosis was complete. It was not critical, therefore, to test for effectiveness of food suspension as in the previous experiment. The main goal of this experiment was to test the growth response of the clams to the sodium alginate-bound microparticulate diet.

A study was done to test whether or not the sodium alginate microparticulate diet was ingested by the clams. Procion dye stained diet was used and, after feeding, the clams were photographed. The dyed food inside the clams is shown in Plate 3.

1.2. Materials and Methods

1.2.1. Experiment 1 Scallop Larvae (*Placopecten magellanicus*)

Larval rearing: Mature adult sea scallops (*Placopecten magellanicus*) were induced to spawn in the laboratory as described by Couturier (1986). During the following 96 hours fertilized eggs were placed in 20 l plastic buckets at a concentration of 30 per ml to allow egg development into straight hinge larva or early veliger (D stage). All seawater used was filtered through two polypropylene filters of 1 micron pore size each (Filterite Corp., Timonum, Maryland, USA), temperature was kept at 14°C throughout the experiments.

Larvae were washed with filtered sea water and retained in a 44 micron Nytex mesh, then suspended in a 7 liter bucket and sampled for estimations of size and survival. Three samples of 10 ml each were taken,

three subsamples of one ml each were used to estimate total number of larvae. Initial size was estimated from a total 25 larvae.

Experimental design: Straight hinge larvae were distributed in 700 ml glass jars at a concentration of 5 larvae ml^{-1}. Filtered seawater was changed every 2-3 d and food was added according to treatment. Samples for estimation of growth and survival were taken at the beginning of the experiment, and at the end of the first week and the end of the second week. Larvae were concentrated in a 250 ml beaker and a 5 ml sample was taken from each replicate after a few drops of formaline (5%) was added.

Direct observation: larvae were reared for 1 wk on an algal diet (*Isochrysis* aff. *galbana* (clone T-ISO)) and then left unfed for 24 hours. These unfed larvae were fed the microparticulate diet and then viewed under a fluorescent microscope. Feeding was observed directly and photographs were taken. Repeated viewing of the videotape made it possible to make more detailed observations.

To evaluate the acceptability of a microparticulate diet for sea scallop larvae during the first week of development (after fertilization), three treatments were tested. Each treatment included 5 replicates. Treatment I: algal diet, 50,000 cells of *Isochrysis* aff. *galbana* (clone T-ISO). Treatment II: microparticulate diet (see Table 1), 1000 particles (screened to under 20 µm) were suspended in filtered sea water, mixed and added to each replicate. Treatment III was an unfed control. The experiment lasted one week.

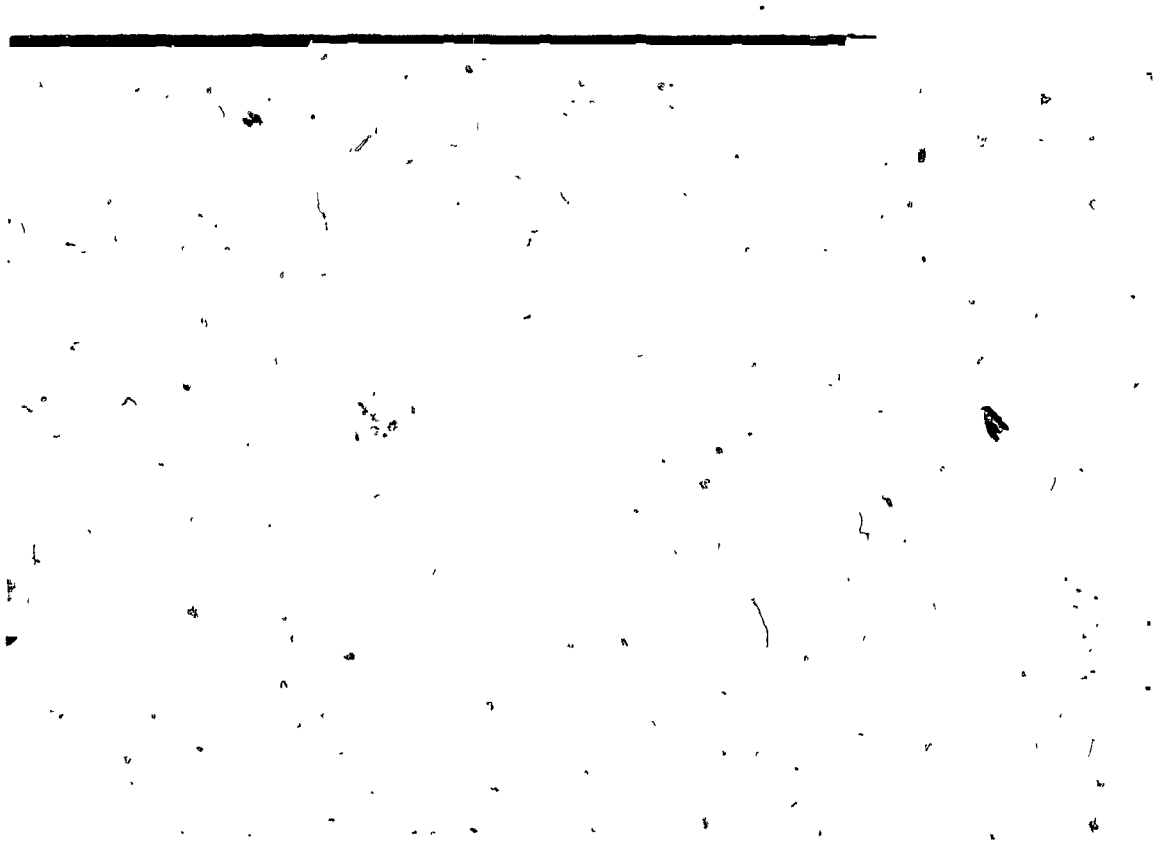


Plate 2 Larva (about 100µ) of the sea scallop *Placopecten magelanicus* showing five 1 µm latex beads.

Plate 3 Newly settled spat of *Tapes Philippinarum* (approximately 300 μ in length) showing ingestion of microparticulate diet stained with procion red (a) and procion blue (b).

1.2.2. Experiment 2 Oysters (*Crassostrea gigas rivularis*)

Larvae of the Pacific oyster (*Crassostrea gigas rivularis*) to be used in the feeding trial, were provided by Dr. A. Robinson. The larvae were observed under a microscope and photographs were taken throughout the experiment. All larvae had vella and up to two days before termination of the experiment no pediveligers were seen. Because these were late larvae, some may have metamorphosed into young spat be the end of the experiment. They were counted, and then allocated to various treatment flasks (500 ml). Stocking density in the round bottomed flasks was 5 larvae ml^{-1}. Each flask contained 350 ml of filtered seawater (0.2 μm) and 1750 oyster larvae. Two replicates were used for each of the following treatments:

A.	Mixed Algal Diet Control, on Counter Top	MAC
B.	Mixed Algal Diet Control, on Shaker	MA
C.	Sodium Alginate Microparticle Only, on Shaker	AP
D.	50% Microparticle, and 50% algae, on Shaker	MAAP
E.	Unfed, on Shaker	U

Treatments B to E, inclusive, were kept on an orbital shaker at low speed (at setting 3.5) for the duration of the eight day experiment. The remaining treatment, A, was kept on a nearby countertop and equipped with an airline. The algal diet was comprised of two species *Pseudoisochrysis paradoxa* (clone VA-12) and a *Chaetoceros* species. Full ration was 80,000 cells ml^{-1} total. All treatments were monitored daily with subsamples taken for counts on the Coulter counter. When the food ration had been grazed down by over 20%, new food was added to bring

the food density up to starting values. At 48 h intervals, each treatment chamber was drained, animals retained on a screen, and were then placed in fresh filtered water (0.2 µm) and given a new food ration. At termination of the experiment, survivors were counted and for each treatment shell length measurements were taken and recorded. An ANOVA was done on these data and the results of comparisons are detailed in Appendix 7A.

1.2.3. Experiment 3 Clams (*Tapes philippinarum*)

Manila clams (*Tapes philippinarum*) were placed into 2250 ml of filtered (0.2) seawater in 3 l beakers. Each beaker received 520 spat and one of the following dietary treatments:

MA	Full Ration	80,000 cells ml^{-1}
AP	Sodium Alginate Particle	Equivalent dry weight of 80,000 algal cells
50%AP	Half Ration	
MAAP	Both MA and AP	
UNFED	Control	

Each treatment was conducted in 2 replicates. All treatments were monitored daily with subsamples taken for counts on the Coulter counter. When the food ration had been grazed down by 20%, new food was added to bring the food density up to starting values. At 48 h intervals, each treatment chamber was drained, animals saved on a screen and re-established with filtered water (0.2 µm) and a new food ration. Each beaker was equipped with an air line to keep food in suspension. Salinity was 35 ppt and temperature was 25°C over the 8 day experimental period. At termination of the experiment, survivors were counted and for each

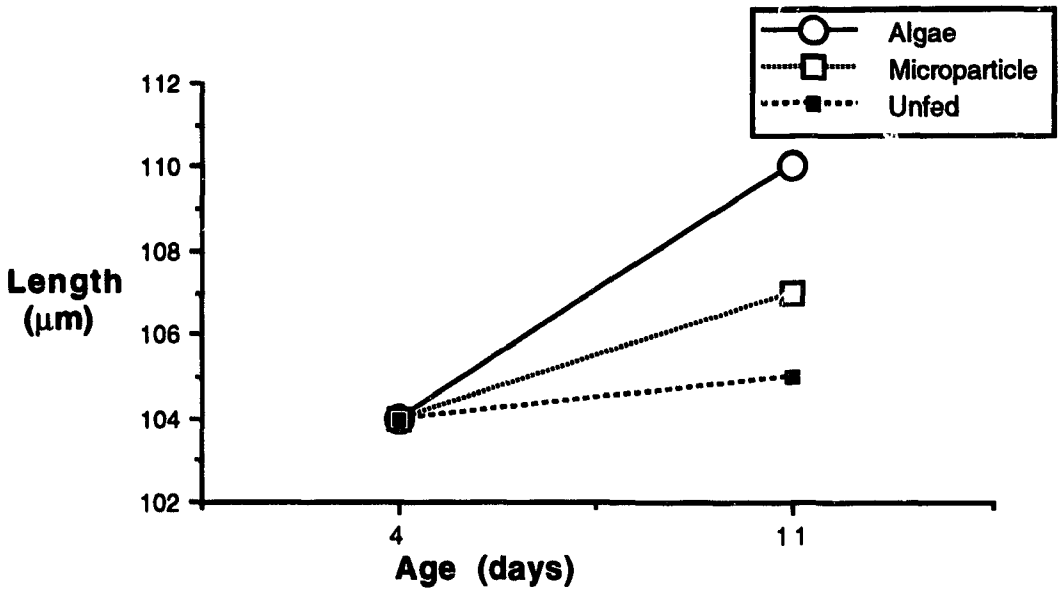
treatment shell length measurements were taken and recorded. An ANOVA was done on these data and the results of comparisons are detailed in Appendix 7B.

1.3. Results & Discussion

1.3.1. Experiment 1 Scallop Larvae (*Placopecten magellanicus*)

Ingestion of the microparticulate diet by the larvae was observed directly under the microscope and subsequently shown in both the photographs and videotapes. Feeding behavior appeared to be unaltered in the presence of the microparticulate diet. The cilia on the velum beat actively and the diet particles were ingested (Plate 2 shows a 1 week old larva, approximately 100 μ). Data from the second part of the study, the growth experiment, are shown in Figure 22. The growth increments on the microparticulate diet appeared to be at an intermediate level between the algal fed treatment and the unfed treatment. The difference between the microparticulate diet and the unfed treatment was not statistically significant.

Figure 22 Veliger Growth



This study clearly demonstrates that veliger larvae of *Placopecten magellanicus* will ingest the microparticles. In the first part of the study, the larvae were observed while feeding and the ingestion of fluorescent labeled microparticles was noted. The use of particles stained with fluorescent substances has been used effectively in other studies to trace and analyze feeding behavior and also to monitor sedimentation and larval settlement (Lindegarth *et al.* 1991). Such observations are limited since only short term measurements can be made as the food is being digested. The benefits are, however, that the details of feeding behavior -- flow stream patterns, velum activity, acceptability and/or sorting of food items, ingestion rate, digestion rate -- can be closely monitored and quantified. In studies like this one repeatedly reviewing the videotape record enables many measurements to be recorded. The fluorescent-labeled sodium alginate microparticle is an excellent tool since it is readily accepted and does not, in the short term, appear to have any deleterious effects on the larvae.

The same microencapsulation process has proved successful in diets for zooplankton (Cary *et al.* 1992). In a study designed to investigate zooplankton grazing, the sodium alginate microparticles were ingested and digested by the copepod *Calanus pacificus* . The authors initially checked for ingestion by using florescent latex beads and then later encapsulated a chlorophyll a-oil solution for their feeding experiments. Their results showed that oil-soluble compounds like chlorophyll a can be delivered using this methodology. It should be noted, however, that other methods for encapsulation of lipids and lipid soluble compounds are available (Ackman and Kean-Howie 1994; Heras *et. al.* 1994).

In the second part of this study, growth was poor. Since the diet contained fluorescent stained latex beads, one can speculate that adequate digestion of the dietary nutrients may have been impaired, resulting in a poorer growth. Diets prepared with no inclusion of the stained latex beads will be used in future studies; this could lead to a better performance of the larvae.

1.3.2. Experiment 2 Oysters (*Crassostrea gigas rivularis*) Results and Discussion

The growth results indicate that some of the larvae may have completed metamorphosis during the course of this experiment. *C. gigas* larvae continue to feed while undergoing metamorphosis and can reach a size of 500μ within a day or two after setting (personal communication, M. Helm). The oysters ingested the sodium alginate microparticulate and demonstrated some growth. This treatment (AP) and the other diet containing the sodium alginate microparticulate diet (MAAP) demonstrated better growth than the unfed controls. These results indicate that the sodium alginate-bound diet is not only acceptable, but since growth was produced, also digested and, to some extent, assimilated (Figure 23).

The oysters fed the mixed algae on the rotary shaker (MA) did not grow as well as the unfed animals on the shaker. Furthermore growth of larvae in this treatment was poorer than that of larvae fed the same diet left on the counter (MAC) with aeration. This treatment (MA) produced poorer growth than the unfed controls. The reason for this is not clear.

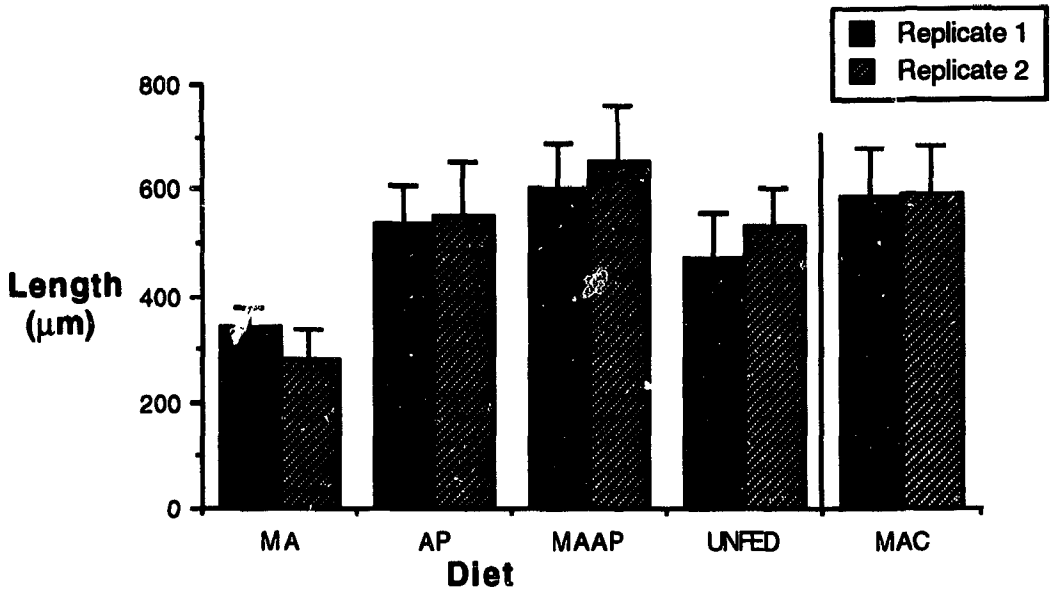
Oyster survival data are summarized and analyzed in Appendix 7C. The results showed no differences amongst AP, MAAP, or the unfed treatments. Survival on MA alone was significantly lower than for all other treatments; the best results were demonstrated on MAC. The

treatment sitting on the counter, receiving only algal food, produced a significantly greater survival than the one on the shaker.

It is notable that the mixed algal diet on the shaker gave both poorer growth and significantly lower survival than the other treatments on the shaker. This result is in sharp contrast to the same diet on the counter. The mixing process may interfere with capture of algal food or may cause mortalities in the oysters. This result may reflect differences in oyster feeding behavior under the two different food presentations.

Although this is a preliminary study, growth and survival of the oyster larvae on the sodium alginate-bound microparticulate diet indicates that this diet is ingested, digested, and to some degree assimilated. The shaker did provide a good means for keeping diet in suspension; however, results with the algal diet on the shaker are anomalous and do merit additional investigation.

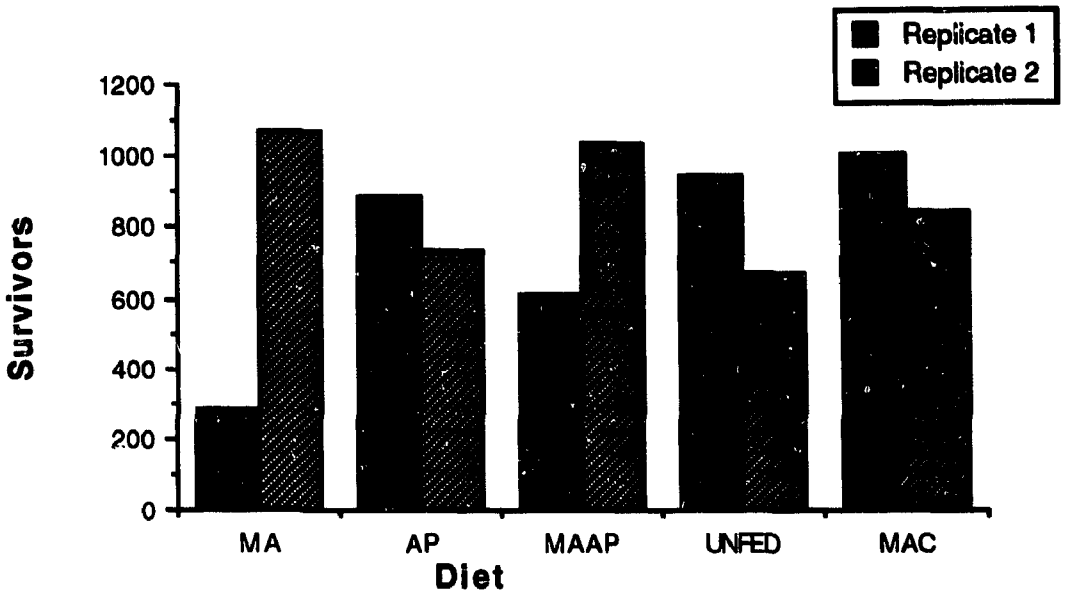
Figure 23 Length of Oysters at Time of Termination
(error bars are 1 standard deviation)



Legend		Statistics[1]
MA	Mixed Algae	a
AP	Sodium Alginate Particle	b
MAAP	Mixed Algae and Sodium Alginate Particle	b
Unfed	On Shaker	c
MAC	Mixed Algae on Counter	b

[1]Treatments with the same letter are not statistically different. Statistical procedures are contained in Appendix 7A

Figure 24 Oyster Survivors at Termination



Legend		Statistics
MA	Mixed Algae	a
AP	Sodium Alginate Particle	b
MAAP	Mixed Algae and Sodium Alginate Particle	b
Unfed	Shaker	b
MAC	Mixed Algae on Counter	c

1.3.3. Experiment 3 Clams (*Tapes philippinarum*) Results and Discussion

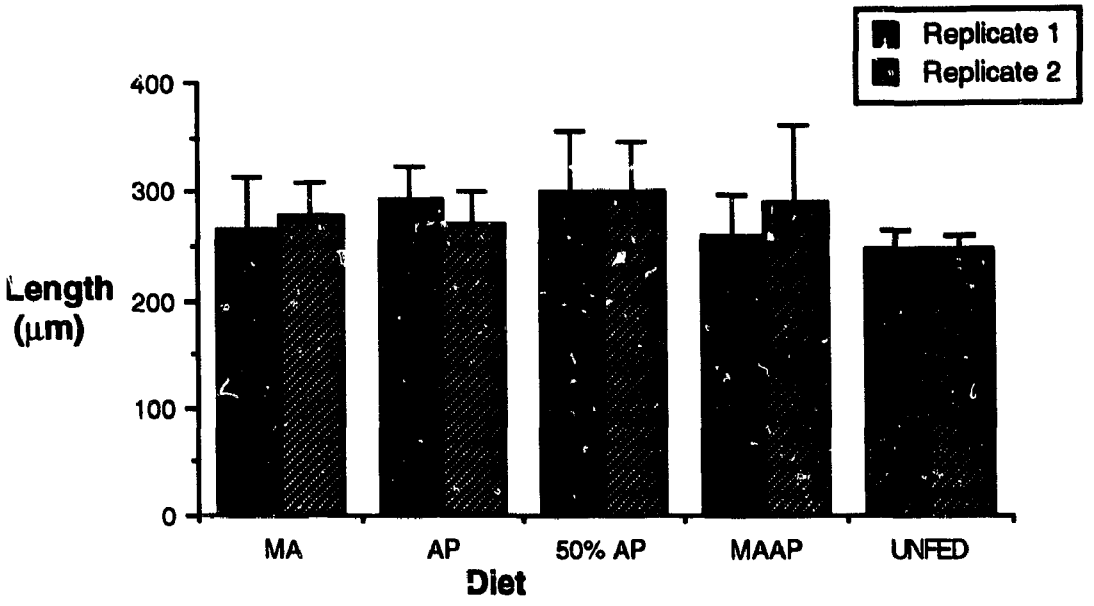
Figure 25 depicts the length of clams at termination. The unfed treatment shows the lowest growth response; the best response with the sodium alginate microparticulate diet at 50%. All dietary treatments produced growth that exceeded that of unfed controls.

Survival data (Appendix 7C) showed that the unfed group, the microalgae group and the sodium alginate particle fed group gave the best survival, with all these treatments at approximatel. 75%. The 50% sodium alginate particle diet gave the best growth results in both replicate groups. In contrast, however, one of these treatments resulted in over 50% mortality. It is possible that a non-dietary factor contributed to this high mortality.

These are encouraging results showing that the sodium alginate particle is readily ingested and digested by the Manila clam spat. Furthermore, halving the ration (to 50%) of sodium alginate microparticulate diet did not adversely affect the result.

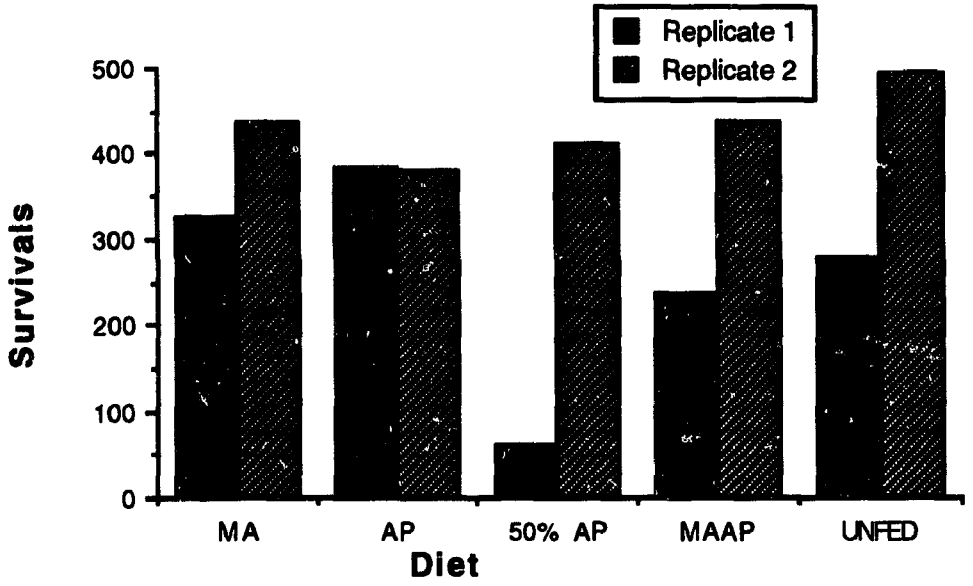
The exciting result is that in this experiment, a microparticulate diet (50%AP) produced a better result than the algae control. This is contrary to most other experiments where the best performance has been on algae diets.

Figure 25 Length of Clams at Time of Termination
(error bars are 1 standard deviation)



Legend		Statistics
MA	Microalgae	a
AP	Sodium alginate particle	a
50%AP	50% of above ration	b
MAAP	Both microalgae and sodium alginate particle	a
Unfed	Control	c

Figure 26 Clam Survivors at Termination



Legend		Statistics
MA	Microalgae	a
AP	Sodium alginate particle	a
50%AP	50% of above ration	b
MAAP	Both microalgae and sodium alginate particle	b
Unfed	Control	a

1.4. Summary Discussion for Bivalve Larvae and Early Spat

The above data verify the successful ingestion and digestion of the sodium alginate microparticle by three bivalve species: the sea scallop, the Pacific oyster and the Manila clam. The fact that growth was supported in both oysters and clams means the diet was digested and nutrients to some extent assimilated.

Other studies have demonstrated that microencapsulated diets can be used as larval feeds. Chu *et al.* (1987) achieved metamorphosis of the American oyster (*Crassostrea virginica*) larvae fed microcapsules. The synthetic diet, however, supported only 36% and 46% respectively, of the growth achieved with algal controls. Southgate (1988) reported the successful use of microencapsulated diets for larval clams (*Tridacna gigas*). Numaguchi and Nell (1991) found that gelatin-acacia microcapsules were useful as a dietary supplement to the Sydney rock oyster (*Saccostrea commercialis*). A protein-walled microcapsule was assessed by Southgate who found (Southgate 1990, Southgate *et al.* 1992) that this diet supported growth in excess of 80% of algal-fed controls (based on shell length).

The growth response by the sea scallop larvae was not significant and growth was better demonstrated in the clams and oysters. There are many possible explanations for this. First, the sea scallop larvae are smaller and may have required a longer time for differences between treatments to become pronounced. Second, the sea scallop work was done at Dalhousie University but the other larvae work was done at the Hatfield Marine Sciences Centre. Any number of differences between the two sites,

especially water quality parameters, could explain the different results. Thirdly, there may be species specific responses to these experiments.

Subsequent to this experiment, efforts were made to repeat the work with sea scallop larvae using a more detailed experimental design. Two such efforts were made, and in both studies high mortalities were experienced. One of these experiments is discussed in detail in Appendix 6. It should be noted, however, that at day four the results with the microparticulate diet produced larvae which were not significantly different from the algae controls, but were significantly different from the unfed treatment. Although the causes for the mortalities in this experiment remain unclear, it appears that growth in *Placopecten magellanicus* larvae can also be supported by the sodium alginate microparticle.

Clearly in these studies the sodium alginate microparticulate diet meets the criteria for selection, ingestion, digestion, and assimilation (as demonstrated by growth) for the clam and oyster larvae. In the past, in efforts to investigate molluscan nutrition, a critical limitation has been the inability to deliver diets of known nutrient composition. In these experiments, with three species of bivalve larvae, the sodium alginate microparticulate diet has led to growth responses. This is one of the most promising recent advances in the development of molluscan nutrition research. The formulation used was not expected to satisfy the nutritional requirements of any one species: it was meant merely as a generic starting point to test the microencapsulation procedure. The formulation for the diet can now be manipulated and different compositions tested for each molluscan species.

The techniques for labeling the diet are also valuable tools for monitoring and measuring various aspects of feeding. For example, the

Procion dye can been seen inside the transparent larvae, and under specific experimental conditions food ingestion can be confirmed immediately. Furthermore, the Fluoresbrite™ beads in the diet could be used to quantify food intake.

In experiments with the early life stages survival is often poor and highly variable. Variable results are common with the larvae and repeated experiments are required to compile a large body of data on which statistical analyses can be based. These growth results are promising, and in future more experiments are required to develop a wider data base.

2. Feeding Trials with Juvenile Sea Scallops and Bay Scallops using Microparticulate Diets

2.1. Introduction

As described in Part I, although much has been accomplished in the general area of aquaculture nutrition, our results have focused on fish and, to a lesser degree, crustaceans, with very little achieved in the field of bivalve nutrition. Many bivalves are reared as part of aquaculture enterprises and there is great interest in understanding their growth and physiology, in particular their nutritional physiology. Research in this area has lagged behind that on fish and crustaceans simply because it is more difficult to do. There are two major difficulties. First, in order to conduct nutrition experiments on bivalves, satisfactory artificial diets are required in order to deliver diets of known nutrient composition. These formulations can then be manipulated to test for specific nutrient requirements. Second, appropriate delivery methods or feed application techniques designed especially for filter feeders must be developed. Both of these requirements mark a major departure from the traditional approach of presenting a pelleted diet to fish or crustaceans.

The criteria for an artificial diet are: the ability to carry nutrients, stability in water and acceptability in terms of both ingestion and digestion. Most importantly, for bivalve filter feeders, the diet must be available in small particle sizes, a few microns (1-20μ) in diameter, to be readily ingested. In recent years those working on diet development have tried several methods for producing such diets. The diet ingredients must first be mixed and then somehow held together as small food particles. There are two basic approaches. First, the nutrient mixture could be bound together, or microbound, using substances like sodium alginate or

carageenan. The resultant microspheres can then be screened to the appropriate size. Another procedure encapsulates the diet mixture inside a shell wall and such diets are called microcapsules Lipid has been used as a shell wall substance and fed, for example, to oysters (Langdon and Siegfried, 1984; Chu *et al* 1984). Microparticulate diets are discussed in detail in section 1 of Part I. Included is the work of Levine *et al.* (1983) who achieved considerable success with a microparticulate diet bound with calcium alginate. A modified, sodium alginate bound, diet has been used in this study.

Delivery of microparticulate diets to filter feeders can be accomplished by producing large volumes of diet and using a flow-through system to culture the animals. This approach is very costly since diets are expensive items to produce. A cheaper alternative is to use a closed or recirculating system. Additional criteria for these recirculating experimental chambers are: a large surface area for dispersion of test animals; inexpensive construction materials; an inexpensive mechanism which will not only recirculate the water but also control flow velocity; a robust design with sturdy components which can withstand frequent handling and cleaning. Units meeting these criteria have been designed for these feeding experiments.

In Atlantic Canada two scallop species, the sea scallop (*P. magellanicus*) and the bay scallop (*A. irradians*) have demonstrated potential as important commercial species for an expanding aquaculture industry. One key requirement for the cultivation of both species is spat supply, specifically, hatchery produced spat. Hatchery production of bivalves requires an understanding of their nutritional requirements, feeding physiology and ecology.

Research on nutrient requirements of bivalves has, to date, been focused on oysters and mussels and has concluded that bivalves in general have a glycogen-based metabolism. Very little has been done on scallops. One exception, the work of Manning (1985), indicates that sea scallops, *Placopecten magellanicus*, start as larvae with a lipid-based metabolism (preferentially use lipid for energy) and by the time they are 2 years old, they possess a carbohydrate-based metabolism. The energy source for juvenile scallops is not clear. Manning (1985) suggests that they keep a lipid-based metabolism long after settlement and well into their second year. Overall, there seems to be a biochemical metamorphosis which occurs during the scallop life history. This implies that nutrient requirements may also be altered. In contrast, another species, the bay scallop (*Argopecten irradians*), utilizes protein as an important energy substrate (Epp *et al.* 1988). One major difference between these two species is their life history strategies -- semelparous vs. iteroparous. Iteroparous organisms have repeated reproductive cycles while semelparous ones have only one brood at the end of their a lifetime.

A feeding trial was conducted in order to compare growth performance of these two species on diets of known composition. Since lipid may play a more important role as an energy source for the sea scallops, the three diet formulations tested contained three different lipid levels. The diets were not isocaloric; therefore, the ratio of protein to total calories was different for each diet.

In addition to the live mixed algae diet, another diet containing half (by dry weight) mixed algae and half a spray dried algae product was also tested. The latter was heterotrophically produced *Tetraselmis suecica* which had been grown on sugars in the dark and subsequently spray dried.

The technology for commercially producing spray dried algae has existed for a few years (see Laing and Gil Verdugo, 1991) and it is hoped that this product will be a suitable, reliable, replacement for live algae.

2.2. Materials and Methods

Microparticulate diets were formulated as shown in Table 8 and the raw ingredients were sent to Dr. Goldstein's laboratory in New York for preparation (as in the previous section.). All diets were freeze dried before shipment back to Halifax. The artificial, spray dried algal diet (*Tetraselmis* species) was supplied by Dr. M. Helm of SFT Ventures, Blandford, N.S.

Live algae used in these experiments were grown by D. Krailo following methods described by Enright *et. al.* (1986a). "Algal diet" means an equal mixture (by weight) of three species: *Isochrysis* aff. *galbana* (clone T-ISO)*Thallasiosira weisflogii,* and *Tetraselmis*. The level of ration was based on the results presented in Part II, section 6 and 40 mg of dry diet per day was administered to each tray of scallops. The ration was first mixed in 100 ml. of seawater using a hand blender (Braun Multipractic) and then immediately dispersed inside the designated tray. In treatments receiving more than one diet source, both were mixed (stirred) inside a beaker before delivery. All diets, equipment, and trays were color coded to minimize the risk of error.

A total of seven dietary treatments were tested:

1.	Microparticluate Diet 1	M1
2.	Microparticulate Diet 2	M2
3.	Microparticulate Diet 3	M3
4.	Algal Diet	AC

5	50% Microparticulate Diet 1 50% Algal Diet	MA
6.	50% Spray Dried *Tetraselmis* + 50% Algal Diet	SA
7.	Unfed Control	U

Table 8 Diet Formulations

Diet Formulations			
Ingredients	Percentage		
	Diet 1	Diet 2	Diet 3
Corn Starch	24	24	24
Dextrin	1	1	1
Sodium Alginate	20	20	20
Crab Protein[1]	30	30	30
Vitamin Mix	2	2	2
Mineral Mix	5	5	5
Menhaden Oil Fatty Acids[2]	10	5	2.5
Lecithin	6	6	6
Cholesterol	2	1	0.5
Energy Content (Kilojoules)	2070	1730	1560

[1] Supplied by Dr. J.D. Castell, DFO Halifax

[2] Supplied by. Dr. R.G. Ackman, T.U.N.S.; Include 1% Vitamin E

2.2.1. Experimental Animals

The sea scallops were supplied by Dr. Michael Dadswell (Acadia University) and were cohorts from the 1988 fall spat. The bay scallops were from the 1989 spring spat produced by M. Helm at SFT Ventures, N.S. These were sorted and distributed equally by weight among the experimental treatments. Mean wet weight (total) of bay scallops was about 60 mg each with 30 scallops stocked in each tray. Mean wet weight of sea scallops was about 200 mg and mean shell height was about 1.2 cm with 22 scallops per tray.

Over sixty recirculating units were designed and built as (see Plate 4) specifically for the feeding trial. A prototype was developed in conjunction with fellow researchers. The requirements for these units were identified and met as follows. A large surface area was supplied on pieces of eavestroughing, manufactured for household gutters. These had been used successfully by colleagues in flow through systems for bivalves. The eavestroughing was cut into 1m lengths which were inexpensive and easy to handle.

Because experimental synthetic diets are costly and require that water in experimental chambers be recirculated the eavestroughs had to be closed in. This was accomplished by attaching end pieces to the trough. Then two holes were cut in the bottom, one for the w ter intake, the other for the outflow. To provide for even circulation of the recirculated water, large tygon tubing with an interior diameter (ID) of 19 mm, was used.

Next a means for recirculating the water had to be devised. Based on earlier results (Part II, section 6) the inflow mechanism had to disperse water into the trough at speeds approaching 10 cm s^{-1}. These earlier data

had indicated that speeds close to 5 cm s^{-1} resulted in some settlement of the synthetic diet. Several delivery tubes for the inflow were tested but the resulting velocities were too low. The PVC T-split shown in plate 4 was equipped with two channels: one across the top, 6.5 cm in length, and the other perpendicular to it, 3 cm in length. The resulting T-shaped outlet was 3 mm in diameter. This T had an ID of 15 mm.

A method to keep the water flowing at the required velocities was required. An airlift was selected since it was inexpensive, reliable, and readily effective. An air injection tube (ID 2 mm) was glued into a piece of cork placed into the PVC joint, under the inflow. This injection tube was in turn connected to the laboratory air supply.

Because the experimental units had to be robust to tolerate daily cleaning, only some of the connections were permanently sealed. Some of the tygon was pressure fitted over the PVC connectors to permit the tubing to be disconnected for draining and cleaning. All other connectors were glued in place with epoxy.

The airlift kept the 2 l of filtered seawater flowing over the scallops and recirculating via the tygon tubing which sloped down to the airlift to avoid trapping particles. The velocity inside the tray was found to be 8 to 9 cm. per second. Each unit was drained, cleaned, and refilled daily. A measured 40mg of diet, which had been screened to under 40 μm in size, was then added. Room temperature was maintained at 14-15°C by an air conditioner, and this proved an effective way to control water temperature. The sea scallop trays were kept at 14° and the bay scallops at 15°C.

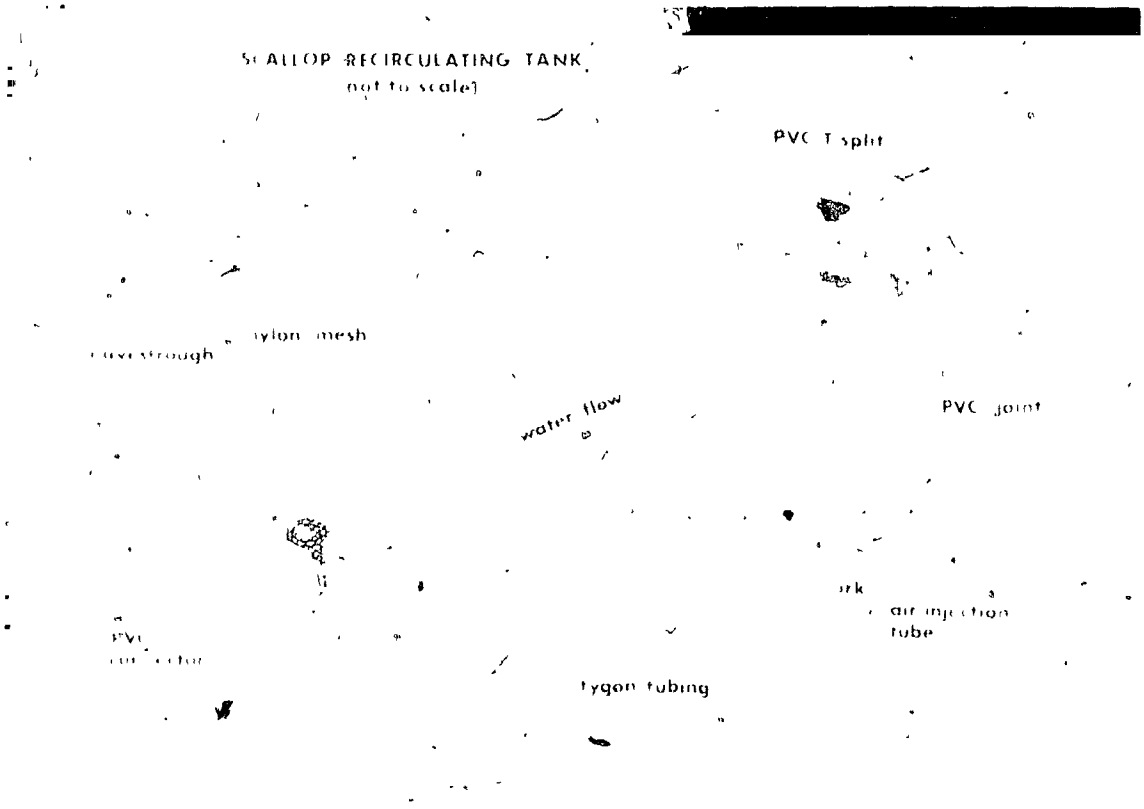


Plate 4. Recirculating tray designed for nutrition experiments with juvenile bivalves.

Four replicate units of each species of scallops (total of 28 units) were used for each dietary treatment. All 56 units were serviced daily for the 9 week experimental period. These experiments were conducted at Dalhousie University in one of the Aquatron climate control laboratories. All water there is pumped from the Northwest Arm and filtered through pressurized sandbed filters to about 50μm. Inside the wetlab all water was passed through a 1μm. cartridge filter before it was used.

2.2.2. Analytical Methods

Chemical methods are discussed in detail in Appendices 2A and 2B. ANOVA was completed on the data for both survival and growth. Contrasts were evaluated using Rodger's F (Rodger 1969) at $P \leq 0.05$.

2.3. Results

The sodium alginate bound diets were readily prepared, handled, and delivered to experimental animals in these feeding trials. The freeze-drying process gave them a long shelf life and, in the absence of moisture, made them relatively easy to screen to size. The one exception was diet 1 which did on occasion clog the screen and require more time to prepare. The rations were well dispersed in the seawater, did not clump and appeared to remain in suspension.

The recirculation trays designed for these experiments were easy to service and maintain. Occasionally, some connections needed repairs; however this was easily accomplished. There are two important observations on the interaction between the diets and this mode of diet presentation: the diet did not settle around the experimental animals and furthermore the diet did not accumulate, clog or interfere with flow in the tray area, tubing or couplings of the unit.

One result which was unexpected was that the two species of scallop responded differently to the disturbances which occurred during the daily cleaning routine. Although done gingerly, the tipping and draining of the trays, caused some of the scallops to be displaced. These animals subsequently clumped together at the outlet end of the tray. The sea scallops remained in clumps for several hours and often did not move back upstream. In contrast, the bay scallops redistributed themselves, in a very even, regular distribution. The experimental design did not include criteria for evaluating such behavior but repeated daily observations always gave the same result.

Differences between the two species were also observed in the growth data. The shell heights of bay scallops (Figure 27) receiving the algae, as all, (AL) or part, (AS, MA), of their diet were significantly greater than that of those fed the microparticulate diets (M1, M2, and M3). The animals fed the microparticulate diet with the lowest lipid content (M3, Figure 28) did have shell height greater than that of the unfed control.

Dry weight increases were substantially higher in animals fed the diets containing algae (AL, AS and MA, Figure 29). All three microparticulate diets showed a dry weight increase above that of the unfed group. The three diets including algae gave significantly better weight gain than the microencapsulated diets. Statistical comparisons could not be made between the latter and the unfed treatment because there were no survivors in three of the four trays.

The survival data (Figure 29, Appendix 7E) show very few mortalities amongst the algal fed animals (AL, AS and MA) and very few in the treatment receiving microparticulate diet #3. The unfed controls experienced a high mortality with only 33% surviving. Although only

small size differences were shown between scallops receiving M1 and M2 (Figure 27), the survival was considerably better than the unfed controls, 60% and 50% respectively. The contrasts revealed the poorest survival in both the unfed and the M2 treatment. Survival for M1 (Appendix 7F) was significantly better than for M2 and there was no difference in the survival data between M3 and the three diets containing algae (AL, AS, and MA). The scallops which showed the poorest growth and survival, the unfed group, had the highest lipid level (24%). Lipid levels were also higher in the scallops fed microparticulate diets M2 and M3.

Figure 27 *Argopecten* Shell Height
(error bars are 1 standard deviation)

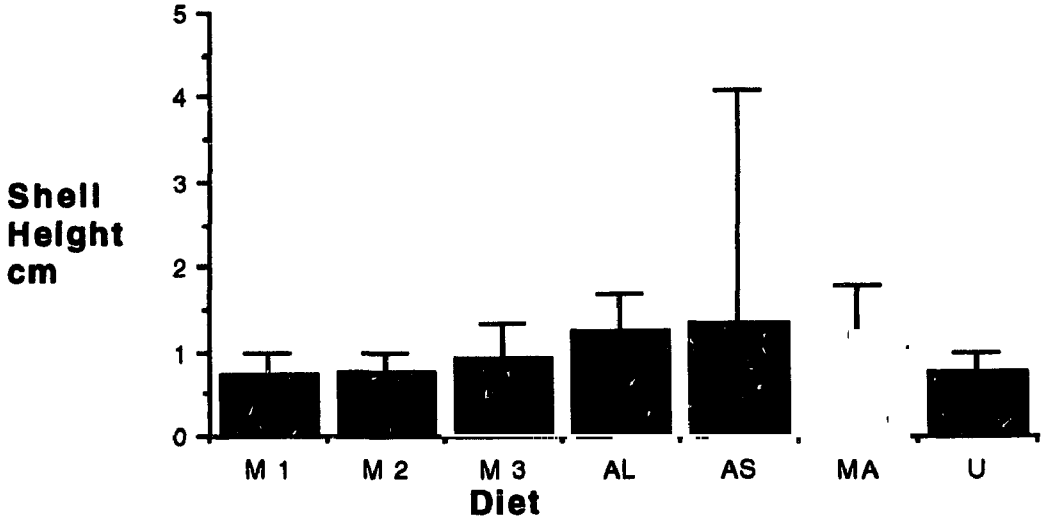


Figure 28 Proximate Composition of *Argopecten* as % of Dry Tissue Weight

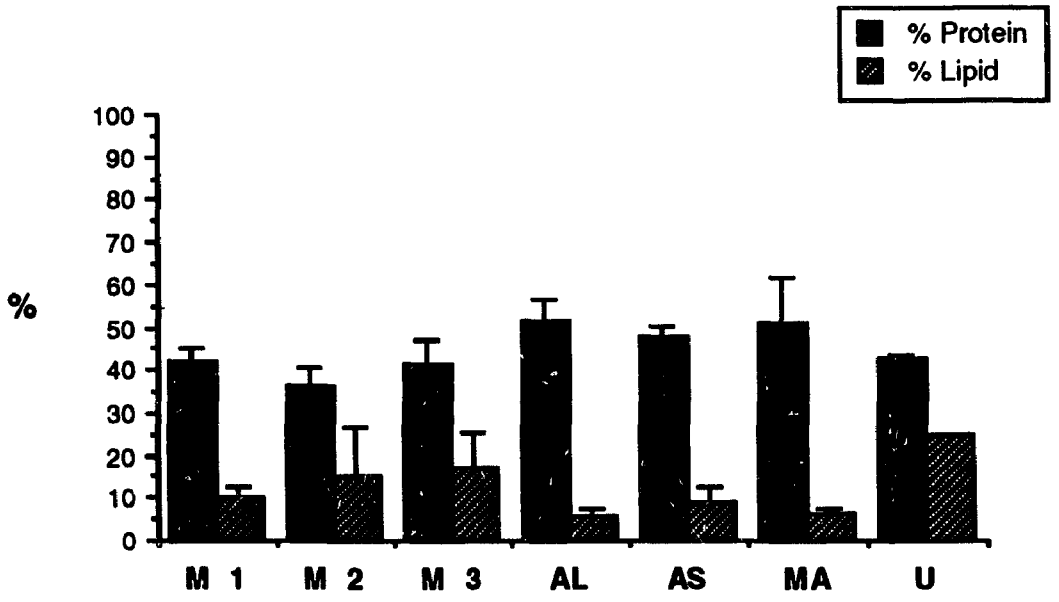


Figure 29 Survivors for *Argopecten*

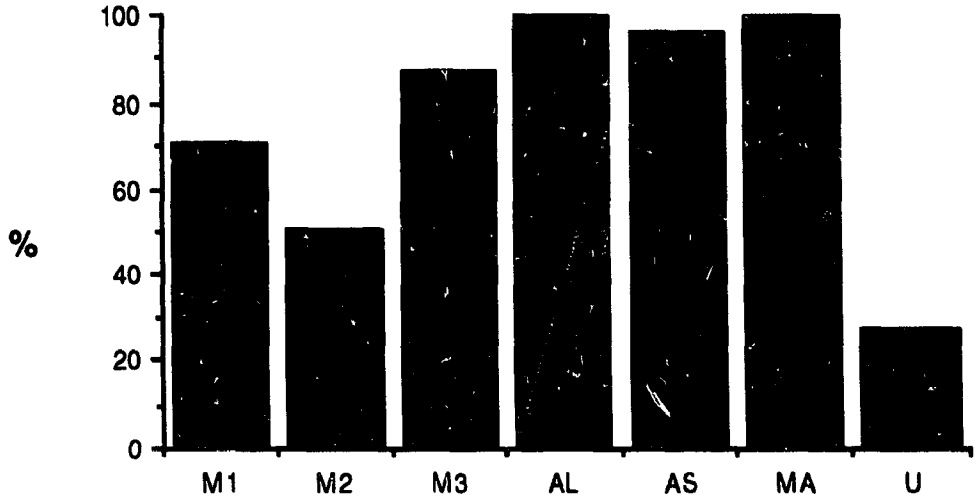


Figure 30 *Argopecten* Dry Tissue Weight

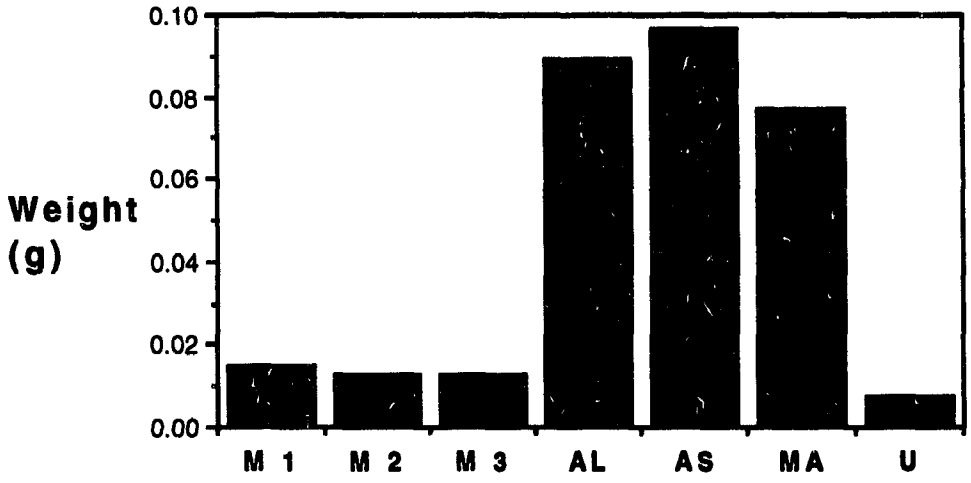


Figure 31 *Placopecten* Shell Height
Error bars are one standard deviation

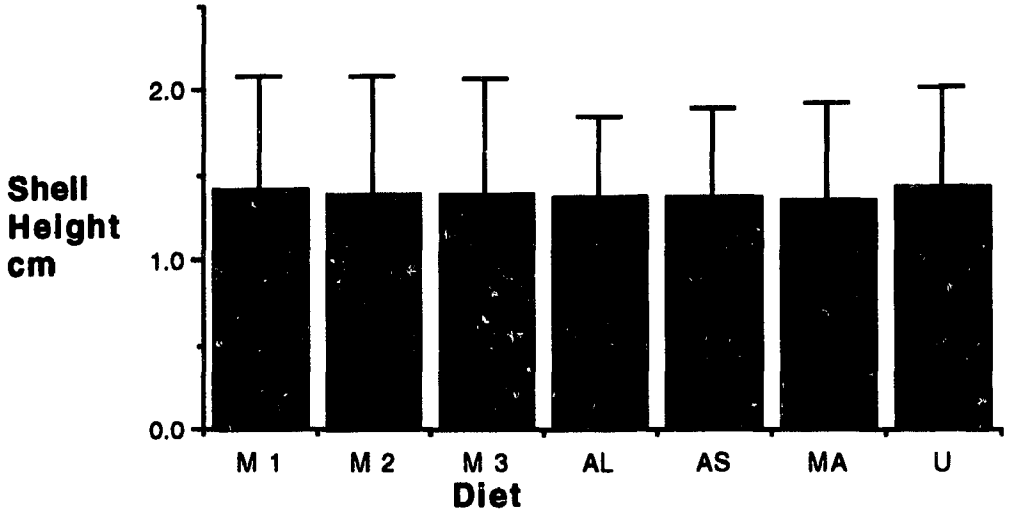


Figure 32 Proximate Composition of *Placopecten*
Error bars are one standard deviation

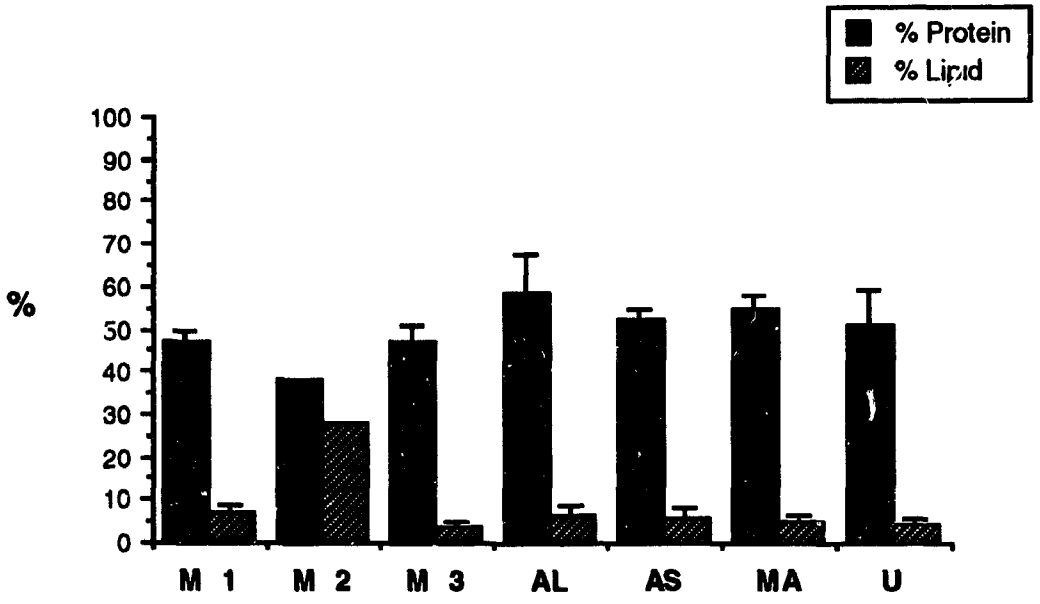


Figure 33 Dry Tissue Weight of *Placopecten*

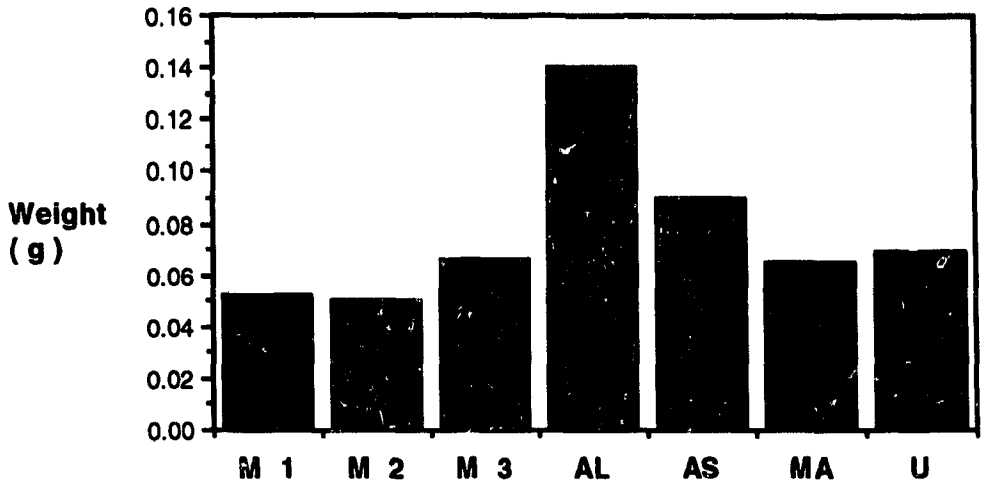
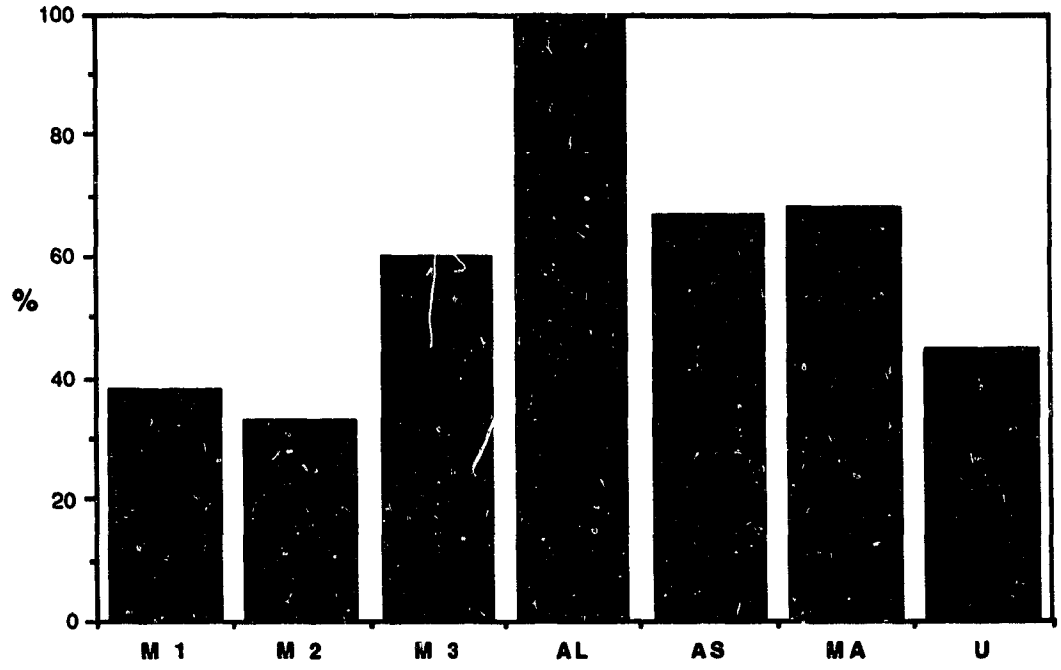


Figure 34 Survival of *Placopecten*



2.3.1. *Placopecten magellanicus*

The shell heights for *Placopecten* juveniles were similar (around 1.4cm) for all dietary treatments (Figure 31) and do not reveal any differences between dietary groups.

The dry weight data and the survival data (Figures 33 and 34) indicate that, again, the best diet was the live algal control diet. The diet of algae alone (AL) produced twice the mean scallop dry weight of most other treatments. The diet mixture of live and spray dried algae (AS) gave a poorer result than AL but it was still significantly better than all the other groups (M1, M2 M3, MA, unfed) as evaluated with Rodger's F (see Appendix 7D). Analyses of the survival data indicated no difference amongst M1, M2, and the unfed group (Figure 34). Similarly there was no significant difference between M3 and all the diets containing algae (M3, AL, AS, MA). Again in these data, it is apparent that diet M3 gave better results for survival than the other microparticulate diets.

2.4. Discussion

The sodium alginate diet was successfully presented to both bivalve species using the recirculators which had been designed specifically for these nutrition experiments. Both the diet and the delivery method proved to be easily used and effective tools. The sodium alginate diets were easy to prepare and maintain and the only problem, that of screening diet 1, is explained by the high lipid content of this diet. Similar problems of screening high lipid diets were experienced early in the development of the diets. This physical restriction may impose an upper limit on the amount of lipid which can be included in diet formulation. This will not be a problem if high lipid levels are not essential. The lipid levels found in

natural bivalve food items, (i.e.. phytoplankton species) is highly variable, ranging between 8% and 25% (Whyte, 1987), and it is therefore unlikely that the dietary requirement is near the 18% found in diet 1. More important than the quantity of lipid is the quality of lipid as represented in their fatty acid composition, especially the polyunsaturated fatty acids (Ackman and Kean-Howie, 1994). Efforts have been made to encapsulate omega-3 fatty acids and use them as nutritional supplements for bivalves (Robinson, 1993; Heras *et al. 1994*). These fatty acids can be encapsulated using the sodium alginate-bound diet and then used in feeding studies with bivalves.

Although there were no measurements made on the digestibility of the sodium alginate-bound diets, it is known that bivalves contain the alginase enzyme which is necessary to break down these microparticles. Furthermore, the results from Part 2, section 6, well demonstrated that the sodium alginate diet was ingested in all cases by all test animals used for those experiments. It is important to note that further experiments should focus on hypotheses relating to digestibility. This is especially important when evaluating improvements in digestibility based on specific nutrients and their levels of inclusion.

All diet formulations prepared for this study were easily encapsulated. One can conclude therefore that the encapsulation methodology would be suitable for other nutrient formulations. The only restriction is the premature cross-linking which can occur with calcium, as discussed in Part 2, section 2.3. It is unlikely however that bivalves are calcium limited, given the nature of their surroundings.

Results with the recirculating trays indicate they were useful tools in presenting the microparticulate diets to the scallops. One important result

was that the food did not settle-out. Deposition of microparticulate food could cause two problems. First, it would reduce the amount of ration in the water and thereby reduce the uptake of food. Second, pockets of microparticulate diet could provide excellent media for growth of bacteria. The presence of the latter can have a negative impact on the experimental animals. A solution is to use antibiotics to keep microbial populations in check. This is undesirable because it not only adds another variable which has to be quantified and monitored but also antibiotics can eliminate the gut bacteria which may be needed by the scallops to digest their food.

In the bay scallop growth data, some small differences in shell height between the dietary treatments were observed. It is the mean dry weight data, however, which indicate that bay scallop juveniles grew much better on the diets containing live algae -- algae alone, algae plus spray dried *Tetraselmis*, or the algae plus the microparticulate diet #1. This is an important observation since shell height is routinely used as a measure of growth in bivalves. Measuring shell height is convenient and simple to perform whether used on field specimens or on laboratory animals. More importantly, it is non-destructive and the bivalves can be returned to the environment for future study. This criteria alone, however, is not an accurate assessment of growth. The dry weight data in this study vary by over five-fold while shell height remains close to 1 cm ± 50%.

The inaccuracy of shell height as a growth indicator has been noted by Southgate *et al.* (1992) in their work with rock oyster (*Saccostrea commercialis*) larvae. These authors recommend the use of ash free dry weight (AFDW) determinations as a suitable criterion in nutrition studies.

As bivalves grow there is a partitioning of energy between shell and soft tissue. The relative proportions of energy are not known. It is,

however, assumed that the cost of shell deposition for juveniles and adults is a small one (personal communication, Dr. S. Shumway; Dr. B. Barber). Most energy is diverted toward soft tissue growth. If that is the case, the soft tissue and its associated indices (AFDW, protein, etc.) are better choices for growth measurements.

The data on bay scallop survival (Figure 29) show no significant differences between the three diets containing algae and microparticulate diet 3 (between 120 and 130 survivors). Also, the result for microparticulate diet 3 is better than that for M2 or the unfed group. This is in sharp contrast with the results for dry weight. It appears these diets are yielding sufficient nutrients to sustain these scallops, yet not enough to support new tissue growth. Since it is diets 1 and 3 which support survival better than diet 2, this improvement does not appear to be related to lipid composition nor to caloric content. Diet 2 contains more lipid and fewer calories than diet 3 but less than diet 1.

Sea scallop juveniles also grew better on algal based diets. This was especially true in the case of the live algae (AL). Other algal diets and microparticulate diet 3 did, however, promote better survival than the other microparticulate diets. This result of improved survival is comparable to the results for bay scallops. In this case, it is also unclear if the lower lipid and or the lower caloric content of M3 are contributing factors. In the treatment where half the live algae is replaced by M1, the results were similar to the AS and M3 treatments, not the AL treatment. In this case the live algae were by far the best diet.

The contribution of the microparticulate diet could be direct or indirect. These diets can be, and are, ingested directly by the scallops. However, since a recirculating system is in use, the possibility that the

scallops may be indirectly benefiting from the diets through the uptake of dissolved organic matter cannot be ruled out. Recent studies have shown that some bivalve species can remove organic matter from seawater and these dissolved nutrients can be a substantial diet supplement (Welborn and Manahan 1990). The uptake of nutritionally significant dissolved nutrients (including L-methionine, L-lysine HCl and choline chloride) has been demonstrated in rock oysters (*Saccostrea commercialis*) by Nell and Wisely (1983). Oyster larvae (*Crassostrea gigas*) have been shown to take up various sugars (monosaccacharides and complex sugars) in experiments using an innovative HPLC methodology developed by Welborn and Manahan (1990). There is still, however, much to be done for a better understanding of the role of DOM in molluscan nutrition.

For both scallop species, diets containing algae gave a better growth result than the microparticulate diets. For the latter, it is not clear whether the poorer result for dry weight is due to less food being ingested as compared with algal diets or less food being digested and assimilated. The same diet, M3, was sufficient to improve survival in both species. Since results for both species are similar, there are no differences which can be related to life history strategies or preferred substrates. Additional experiments on diet uptake and digestibility are required to explain these results.

The spray dried algal product, used as a partial (50% dry weight) replacement for live algae, gave a good result for bay scallops. There were no discernible differences between it (AS) and the live diet (AL) with respect to growth or survival. Helm and Hancock (1990) indicated that spray dried *Tetraselmis* may be a good diet for bay scallops. Their data indicated that it worked best in combination with *Isochrysis* aff. *galbana*

(clone T-ISO). In contrast, the sea scallops in our experiment did not grow as well nor was survival as high on the AS diet compared with the AL treatment. In fact, based on these criteria the spray-dried algae results were similar to M3 and MA (the mixture of M1 and live algae). In conclusion, under these experimental conditions, there are differences between the ability of the two species to utilize this product. The results for bay scallops are encouraging, indicating this species may be similar to the other bivalve species including *Ostrea edulis, Crassostrea gigas, Mercenaria mercenaria, Tapes philippinarum,* and *Tapes decussata* (Laing and Gil Verdugo, 1990; Helm and Hancock, 1990;Laing and Millican, 1992) in its ability to utilize the spray dried *Tetraselmis.*

Part IV Summary and Recommendations

In this thesis, I have focussed on the development and improvement of the techniques used to study molluscan nutrition. Our understanding of molluscan nutrition has progressed slowly because of the many difficulties inherent in this work. The test diets must not only carry nutrients in an aquatic environment, but also be readily accepted and ingested by the bivalve filter feeders. This necessitates the development of new techniques for diet application and evaluation.

A sodium alginate-bound microparticle was formulated, tested and shown to be an excellent tool for nutrition research. This microparticulate diet was ingested and digested by the larvae of sea scallops, pacific oysters and Manila clams. In addition, this diet has shown good growth results with juvenile bay scallops and somewhat lesser growth with juvenile sea scallops. This is a surprising result since the initial formulation was done without benefit of details on specific nutrient requirements. The results with the larvae and newly settled spat are promising, especially the superior response by the clams.

In the course of this research program significant advances have been made to methodologies for conducting research with synthetic diets. First, it has now been demonstrated that ingestion can be observed and monitored in larvae through the incorporation of markers--the Fluoresbrite™ beads and Procion dyes--in these synthetic diets. During the course of these experiments, the fluorescent beads allowed us to observe larval feeding on the synthetic diets. Observations recorded on videotape confirmed ingestion of the synthetic diets prior to the subsequent

feeding trials. These labeled diets could be used for more extensive investigations of factors controlling larval feeding.

The Fluoresbrite™ bead markers were successfully used to quantify diet uptake by juvenile bivalves. The information on flow rate and food concentration established during the ingestion experiments was used to set parameters for a long term feeding trial. This method for determining ingestion is more accurate than others which rely on indirect measurements, or which deliver beads in conjunction with food, not in it. In future studies, this direct method for quantifying ingestion can be used to evaluate the impact of various abiotic factors (temperature, turbulence, salinity, etc.) as well as to generate more information on food density and flow velocity. Using the controlled conditions of constructed flumes and the labeled diet, many aspects of feeding ecophysiology can be investigated. In addition it is now possible to examine how variations in diet composition affect ingestion. For example the effect of phagostimulants such as algal exudates on ingestion can be quantified using this method.

In these experiments, an *in vitro* method of determining digestibility has been described. For the first time Procion labeled food has been incubated with enzyme extracts to quantify digestibility. Future potential applications of this Procion dye labeling are numerous. For example, enzyme digestibility *in vitro* experiments could be done with virtually any aquatic species to determine the species' capability of digesting a microparticulate diet prior to *in vivo* testing. More specifically, this method could also be used to determine the digestibility of various diet formulations. Of course, such *in vitro* analyses do not replace efforts to develop procedures for *in vivo* digestibility analysis, but should accelerate them.

The recirculating system designed to deliver the sodium alginate microparticle.to the juveniles was effective and efficient. The diet did not settle out, the units were easily serviced, and maintained. These recirculating units are also suitable for extended experiments on juvenile feeding ecology. Presentation protocols devised for the juveniles were successful, but more work is needed to improve on food presentation protocols for the larvae.

Future studies to determine the physical characteristics of the sodium alginate microparticle are recommended. For example, leaching experiments are needed to test retention of water soluble nutrients. Shelf life should be determined by testing the resistance of the particle to oxidation. Because hand-screening of the diet to the appropriate particle size is extremely time and labor intensive, a more efficient screening mechanism should be investigated.

This research has established a base formulation for a synthetic diet which is accepted by bivalve species and supports their growth. This formulation can now be modified to determine nutritional requirements for optimal growth. These data on nutrient requirements can then be used in the selection of less expensive diet ingredients for production of practical diets. Improved production techniques will then be required to test the feasibility of scale up to commercial production. Whether or not this specific microparticle becomes the "clam chow" of choice, it is a valuable tool for nutrition research, and fundamental knowledge about nutritional requirements can contribute to future studies of bivalves in nature and culture.

Future Research Directions

In this study, the microencapsulated diet was demonstrated to be an effective way to deliver dietary nutrients to both bivalve larvae and juveniles. It has great potential for future investigations into bivalve nutrient requirements, feeding responses and metabolism. For example:

1. Laboratory experiments on bivalve feeding with respect to current velocity, food concentration, size of bivalve, etc., can be completed using the Fluoresbrite™ labeled diet. This diet can also be used in studies on larval feeding ecology.
2. Phagostimulants (including algal exudates) can be added to the diet formulation. This would not only improve the acceptability of the diets but also give some insight into the selection processes involved in bivalve feeding.
3. Additional studies are needed with bivalve larvae in order to generate a large data base since results are often highly variable.
4. An *in vitro* method for assessing digestibility was described; however it is important to develop a readily applicable methodology for evaluating the *in vivo* digestibility of the microparticulate diets.
5. It is critical to evaluate the physical and chemical properties of the microparticulate diet. For example, do the water soluble nutrients readily leach? Can lipids be included in greater amounts?
6. Few data are available on the proximate composition of young bivalve spat. Additional data for different species are needed in order to prepare better diet formulations for testing. The

juveniles grow quickly and nutritional deficiencies can be more readily identified.

7. Alterations in the diet formulation can be made in order to assess the impact of specific nutrients and their levels of inclusion. This would result in improved diet formulations. As specific nutrient requirements are determined it will be possible to prepare more nutritionally complete semipurified diets and also to identify suitable practical low-cost feeds. The The latter will have immediate value in bivalve hatcheries.

8 Finally, is the alginate diet sufficient for nutrient delivery or is there a benefit in applying it in conjunction with one (or more) of the other microencapsulation methods? Perhaps the lipid can be incorporated inside small (2-3μ) lipid microspheres and these, in turn, mixed with other nutrients inside the alginate matrix.

APPENDICES

Appendix 1 Feeding in Bivalves

The following originated as an assigned essay submitted to fulfill the PhD preliminary exam requirements of the Biology Department. In its present form it has been accepted for publication by ICES and is included as an Appendix since it is not yet published.

Kean-Howie, J.C., D.J. Scarratt, R.K. O'Dor (in press). The Evolution of Feeding Strategies Throughout the Life Histories of Bivalves with Emphasis on Ontogeny and Phylogeny. ICES Publication. Rapport et Process - Verbaux.

Introduction

In an 1883 article for Encyclopedia Britannica, Lancaster citing Verrill (1896), described a hypothetical schematic mollusc or 'archemollusc' furnished with a radula. For over a century Lancaster's depiction of a rasping, grazing, protomollusc has been accepted as the primitive molluscan form and, as represented in various textbooks, has altered very little. According to Purchon (1968) bivalves evolved in the early pre-Cambrian, feeding directly on organic material. The earliest bivalves, he postulates, were protobranchs which collected food directly from the substrate with ciliary organs. They gave rise not only to later protobranchs but also the septibranchs and polysyringia (lamellibranchs). Purchon gives detailed accounts of functional morphology and describes the resultant adaptive radiation. He stresses the importance of the capacity to filter food.

Marine bivalves have characteristic mixed life histories. First, fertilized eggs hatch into a swimming trochophore, a form present in many protosomes. The next stage, the veliger larva, is symmetrical and eventually secretes two valves. Food particles are collected by the trochophore with a girdle of cilia called the prototroch, and by the velum of the veliger. At metamorphosis the velum is shed. Few detailed studies have been done on feeding modes during these early stages of development; however, Lucas *et al.* (1986) have shown that in *Mytilus edulis* the sequence of phases are: endotrophy for the first two days followed by mixotrophy from the second to eighth day at which point the animal becomes extrophic.

The following evaluates some of the conventional theories and traditional information used to describe bivalve evolution. The objective is not to give a comprehensive summary and critique of each area but to briefly review and reevaluate them in light of recent findings, and identify future research areas.

Recapitulation Theory and the Origin of the Trochophore

In reviewing phylogeny one approach is to apply Haeckel's Biogenetic Law "ontogeny recapitulates phylogeny." This implies that by examination of successive embryological stages, one can infer details of adult ancestors. What is uncertain is whether or how this linearity is preserved when animals have mixed life histories. Do the developmental stages of larvae represent earlier adult forms or have the larvae evolved independently of changes in adult morphology?

In the case of bivalves, the distinct planktonic and benthic phases of the life history are separated by metamorphosis. Because larvae have adapted to a pelagic life and adults are specialized for a benthic environment, the result is a morphometric, anatomical, and behavioral divergence. Each stage has adapted independently to its environment.

Traits are not necessarily restricted to one stage because mechanisms exist whereby features that evolve in one phase can be transferred to another. With acceleration of morphological development (termed adultation by Jägersten 1972), adult features can shift to the larvae. Through paedomorphosis adults can retain formerly juvenile traits; however, when a radical metamorphosis separates the stages, comparisons are difficult. The incomplete morphogenesis of a larval gill, for example, renders impossible any comparison with the adult feeding mechanism.

Barrington (1967) states that the dual functions of larvae are to ensure development of the individual and dispersal of the species. Because the larvae undergo independent adaptations in fulfilling these roles, they cannot be precise guides to the organization of adult ancestors. Indeed Barrington stresses the great importance of the larval capacity to evolve independently of adults. Larval development then should not be interpreted as strict recapitulation, in the Haekelian sense, of phylogenetic history.

Although a contemporary larva cannot be regarded as an exact recapitulation of its ancestral history, larvae can and do demonstrate features of ancestral adults. Jägersten (1972) gives the example of the veliger shell: if this means the ancestral adult in fact had a shell, then it may be valid to say that the larva recapitulates this adult feature from the ancient life cycle. Recapitulation theory may give some insight into specific mechanisms or common organizational plans.

If the veliger larva can recapitulate phylogeny, what of the trochophore? The similarity of trochophore larvae found in different phyla has been stressed by many workers (Jägersten 1972; Giese and Pearse 1974-79; Chia and Rice 1978). Why do so many diverse phyla (including annelids, molluscs and echinoderms) have such larvae?

Until 1987, two explanations had been offered. The first, convergent evolution (Hadzi 1963), presupposes that independent evolutionary lines have developed organisms similar in appearance and function at the same life stage, the trocophore. Yet, when examined in detail, it is clear that convergence is never total. Dawkins (1986) warns against assuming that two lines of evolution should converge at exactly the same endpoint from different starting points. He states that it is not only

unlikely from the examination of points of detail but also statistically unlikely that the same evolutionary pathway should be traveled twice. Although trochophore larvae are similar in form and placement within the life histories of different phyla, they are not necessarily the result of convergent evolution.

The other explanation, common ancestry, would mean that the trochophore stage is a homologous feature indicating common descent. A review of the animal kingdom phylogenetic tree indicates that protostomes (including the molluscs) and deuterostomes (including the echinoderms) diverged from a common flatworm ancestor (Barnes 1980). What is unclear is why a few phyla with this common flatworm ancestry would retain a trochophore when others do not. More recently, Field *et al.* (1988) have used ribosomal RNA sequencing to examine metazoan phylogenies. Their work suggests complex relationships between annelids and molluscs and a separate lineage for the echinoderms. Yet modern echinoderm classes have either a trochophore or the distinctly different echinopluteus larva.

In 1987, a major departure from conventional thinking was offered by Williamson who suggested that some invertebrates have hybrid life histories combining veliger larvae and adults from different ancestries. Williamson maintains that the trochophore has been incorporated into several phyla, including the molluscs, recently in geological time. This is in contrast to the conventional view of phylogenetic genealogy, that the archetypal trochophore was either the adult or the larval form of a species which gave rise to a most diverse set of phyla, before or during the Cambrian. Why else would an ancestral larval type remain unchanged while the adults diverged? Larvae are also subject to adaptive pressures.

If the trochophore has not remained as an unchanged ancestral form, then the alternative explanation is that it has been incorporated within several phyla adding a new larval phase and delaying, but not supplanting, all subsequent stages.

Williamson speculates that heterosperm fertilization is a possible mechanism for transfer among the phyla of sufficient genetic material to introduce a trochophore stage. There is some evidence to support this. For example, similar nucleotide sequences have been found in cells of distantly related species (Lewin 1982). Alternately, transfer by vectors such as a virus or bacterium is also possible.

Additional support for Williamson's theory can be drawn from the work of Taylor and Margulis (see Margulis 1970). Taylor said that eukaryotic cells were formed by symbioses of various prokaryotic cells. This was called the Serial Endosymbiotic Theory. If production of new species is technically feasible from such symbioticism is it not a feasible mechanism for creating a mixed life history?

If such transfers have occurred, then where a new larval phase has been added to the life history of a bivalve, the inserted larva is always a 'first' larva: (Williamson 1987). Acceptance of this theory necessitates a rigorous re-evaluation of traditional views on phylogeny and ontogeny. While early larval forms may represent adult stages of ancestors, these ancestors may be a distantly related or unrelated species.

The morphological similarity of trochophore larvae from different phyla can also be considered from a different perspective. Why do these larvae have adults with such a wide variety of structure and organization? If larval traits, not those of adults, were the primary taxonomic tools, this

would be a large phylum with radically differing adult forms - and many anomalies.

Paleobiology and the Resultant Phylogenies

Traditionally, the study of characteristics shared within and between species has relied on phenetic analysis. Nineteenth century biologists relied heavily on anatomy and used comparisons of form and structure of adults as their phylogenetic tools. If relationships of organisms are determined by similarities of shared, homologous features then determining the source and divergence of these features reveals the relevant phylogenies. This approach was challenged by Newell and Boyd (1978), who recommend adapting a more flexible evolutionary systematics, and the development of models consistent with many hitherto unconsidered sources of evidence. Their perspective follows that of Mayr (1963) who encouraged this type of flexibility.

In the past, the phenetic analysis of bivalve evolution has been reviewed and discussed using two types of information. First, paleobiology describes the fossil record of bivalve molluscs in an attempt to assign a phylogenetic chronology. Second, ecophysiology describes adaptations that have evolved in response to selection pressures in the environment. Each of these approaches is characterized by inherent perspectives and interpretations. These will be discussed and some alternative approaches identified.

The first problem encountered when evaluating paleobiological data is that of clandestine evolution. Very little is known about the evolution of larval forms and the non-muscular soft parts of adult bivalves. Without this knowledge, it is difficult to trace the relationship between the anatomical features of larvae and adults. A new approach in describing

larval development of ancestral species has resulted from the study of modern larval shells. Jablonski and Lutz (1983) state that bivalves are well suited to this type of study since the early stages are calcified and remain that way as the organisms grow. Also the presence or absence of yolk determines egg size, which in turn affects larval shell morphology. This is especially true of the prodissoconch I, or D-shaped veliger, the first shelled stage. Large diameter eggs produce large diameter prodissoconch I larvae which are usually lecithotrophic. Often the index used to determine the likelihood of the larvae being lecithotrophic is the size ratio of prodissoconch I to prodissoconch II. In bivalves possessing planktotrophic larvae, the prodissoconch II is large compared with the prodissoconch I (Ockelmann 1965). Jablonski and Lutz (1979 and 1983) suggest that modes of development can be identified by comparing larval shells of one species to those of a congeneric or confamilial species whose development characteristics are known. At these taxonomic levels the similarities of shell morphology likely indicate similarity in developmental history.

In interpreting paleobiological data, there is also a difficulty with the time scale chosen. Geologist's and biologist's perspectives are quite different since the former typically use large increments of time (millions of years instead of days or months) when viewing the past. The timing and intervals of speciation and extinction events are subject to interpretation. Is evolution the result of a slow step by step change (phyletic gradualism) or does it occur in distinct jumps (Punctuated Equilibrium Theory)? It is now clear that when mass extinction events have occurred, (caused by a catastrophe, e.g. large meteorites striking the earth), the adaptations previously accumulated by a species may have been useless. Jablonski (1986a, 1986b) has demonstrated that the rules of molluscan evolutionary

survival changed during a mass extinction event 65 million years ago: a species past ability to adapt to environmental changes did not ensure survival during a collapse. New lineages are unable to retain any memory of former ones (Morrell 1990); evolutionary adaptations of species that do not survive will neither be retained, nor reflected in the ontogenies of newly evolving forms.

An additional problem in timing evolutionary events is the great limitation imposed by the nature of the observer, in this case an anthropoid relatively new to the planet. As pointed out by Dawkins (1986), human brains are "built to deal with events on radically different timescales from those that characterize evolutionary change." Since we are tuned to work within mere decades, it takes great imagination to evaluate processes which occur over millions of years.

Time and scale should also be considered together in interpreting speciation events. Some species have evolved through repeated isolation, reunification and hybridization (Newell and Boyd 1978). Other species evolved completely independently and without genetic remixing. Mayr (1963) stresses the importance of peripheral isolates in producing diversity. Tracing fossil fauna in time through one narrow area may not be the best way to determine its origin.

Much of the information used by paleobiologists is derived from a study of hard parts. This reliance can be deceiving since other factors, (e.g. reproductive mode, life history strategy, etc.) can play a major role in evolutionary adaptations. Using only hard parts can reduce the study of speciation and extinction events to a study in shell morphologies. To what extent must shells differ in order to define them as distinct species?

Morphometric evidence is also unreliable because of polymorphism; variations in shell shape and size within individual species. These are often the result of environmental factors. Sexual dimorphism in the gastropod *Margarites vortificarus* is so pronounced that male and female were identified as different species (see Rhoads and Lutz 1980). Hedgecock (1986) cautions against using poorly defined morphometric data to determine speciation and extinction rates because of the widespread existence of cryptic sibling species. The range of polymorphism within a species should be identified. To what extent do changes in shell morphology occur in response to changes in selection pressures (e.g. crowding)? Only after the parameters for sub species are quantified, can interspecific variations be defined. The newly developed techniques for RNA and DNA sequencing may promote a better understanding of the differences between closely related species and provide greater insight on past phylogenies (Field *et al.* 1988).

Feeding Modes- Larvae

The feeding mechanisms and evolution of invertebrate larvae are rigorously reviewed by Strathmann 1978. He categorizes larval feeding mechanisms into two groups; the first has a single ciliary band system as found in the oligomera. Particles are retained on the upstream side of the cilia, probably due to a local reversal of beat. The second, the spiralia (including the molluscs) possess a double band of cilia: there is a protoconch comprising a band of long, compound cilia parallel to a band of shorter cilia in the metraconch. Between these two is a food groove with smaller cilia. Some support does exist (Jägersten 1972) for the double band being a primitive trochophore feeding mechanism. For detailed descriptions of these structures and their functions, see Nielsen (1987).

Bivalve larvae may develop completely within an egg mass or while brooded by an adult. This direct development requires a good reserve and eliminates the need for a free living feeding phase. Lecithotrophic larvae can be of two types. In primary lecithotrophy, the early stages depend on yolk from the egg. In secondary lecithotrophy, non feeding larval stages develop on energy reserves accumulated by preceding planktotrophic larval stages.

Planktotrophy has its advantages: a given amount of energy can be apportioned among numerous eggs giving greater opportunity for dispersal, more time to select settlement site, and a large size at settling. The disadvantages are a greater susceptibility to predation and abiotic perturbations. Lecithotrophy, by contrast avoids direct competition for food between larvae and adults and does not leave the larvae subject to variable food supplies. Egg number tends to be smaller, however, and a larger amount of nutrient is invested per egg. (compiled from Barrington 1967; Vance 1973 a,b; Jackson 1974; Spight 1976; Strathmann 1978,1980; Jablonski and Lutz 1980, 1983; Kempt and Hatfield 1985; Scheltema 1986).

Vance (1973) assumed that the evolution of mode of development is determined largely by the availability of energy. This concept was criticized by Underwood (1974) who maintained that other factors may be important. For example many marine invertebrate larvae show delays in metamorphosis until presented with a suitable substrate for settlement, therefore, selection pressure has presumably fostered the evolution of dispersive larvae. Vance (1974) replied that the phenotype that satisfies a given demand with the smallest amount of energy is favored by natural selection because it has the most energy left to devote to other energy-demanding tasks.

A modification of Vance's model is discussed by Strathmann (1985), who altered it to include parental investment and various combinations of planktonic and benthic development. The original prediction for extremes (either planktonic or benthic) remained. Strathmann (1985) points out that this is not rigid because some species (e.g. *Conus* spp.) exist which display a continuous series from entirely benthic to mostly planktonic. He concluded that a feeding planktonic larval stage must be regarded as "a migration" into the plankton during early development, for feeding and safety. In his view, extensive dispersal is "an accidental by-product that increases gene flow and geographic range and hence affects speciation, extinction and degree of adaptation to local conditions."

The traditional view has been that early molluscan larvae were planktonic and planktotrophic. However, Cambrian molluscs were small in size and small molluscs are usually brooders with lecithotrophic larvae. Recently, this question has been considered by Chaffee and Lindberg (1986) who concluded that early molluscs had large eggs and that lecithotrophic development was therefore the ancestral form. The benthic lecithotrophic condition therefore presumably led to planktonic lecithotrophs from which were derived planktotrophic larvae. This novel concept of how an original epifaunal habitat was exploited is also supported by Tevesz and McCall (1976), and to some extent by Salvini-Plawen (1969; 1980) who, although supporting lecithotrophy as the ancestral condition, adhere to the concept of a planktonic ancestral form.

Planktotrophic forms assist in dispersal but it has been documented by Runnegar (1982) that early molluscs were limited geographically. This argues against the presence of planktonic larvae (either veliger or trochophore).

In suggesting planktotrophy as the primitive condition, it is also accepted that loss of a feeding larva is an "absorbing state". Dollo's law, states that an organ, once lost, does not re-evolve in the same lineage. In order for larval evolution to proceed from planktotrophy to lecithotrophy and back to planktotrophy, the larvae would need to re-evolve a new feeding apparatus and digestive tract. No evidence exists that this has occurred.

Furthermore, if loss of the requirement to feed is "absorptive", why do many lecithotrophic larvae retain feeding structures? It is known for example that many encapsulated or lecithotrophic gastropods retain feeding structures (Strathmann 1985). Certainly the cilia may serve to circulate embryonic fluid but the retention of a food groove offers no apparent advantage. Another interpretation is that these forms may be living links demonstrating the transition between lecithotrophy and planktotrophy in molluscs.

Feeding Modes - Adults

Feeding by adult bivalves encompasses a broad spectrum of morphological differences in feeding structures. As indicated by Beninger and Le Pennec (1990) the two approaches commonly used to study bivalve feeding are:

1. Direct observation of structures and behavior of particles on them.
2. Indirect techniques such as observations on particle clearance, gut contents and flow cytometry.

In general bivalves can be grouped into two categories: active and passive suspension feeders. The former actively move water over their gills while the latter depend on ambient water movements. Aspects of filter feeding in molluscs are summarized by Jorgensen (1983) and

Jorgensen *et al.* (1984) with emphasis on fluid mechanics. Very little is known about food selection processes in bivalves. For example, how does selection occur both before and after ingestion? Some factors which affect food selection have been examined (Shumway *et al.* 1985; Kean-Howie *et al.* 1990)

It is apparent that a complete understanding of particle capture requires a detailed description of the participating organs anatomical structures. The structure of bivalve pallial organs has been examined by Atkins (1938), Owen (1966), Morton (1983), and more recently by Beninger and Le Pennec (1990). The latter suggest that if sea scallops can exercise selection in a mixed suspension, the site or sites of this selection have yet to be identified. Beninger *et al.* (1988, 1990; Beninger and Le Pennec 1990) have searched for the necessary anatomical detectors and effectors on the gills and peribuccal organs of scallops. To date, no chemo-sensory cells have been revealed (Beninger *et al.* 1988, 1990; Beninger and Pennec 1990).

The bivalves are represented in a review paper on molluscan diets by Graham (1955). Various feeding habits including microphagy (detritus and algae); macrophagy (by both carnivores and herbivores) and even parasitism are discussed. There are, however, few data on what bivalves feed on. Although unicellular algae are a major food item, other sources have been investigated including: macrophyte detritus (Stuart, Field and Newell 1982); sediment (Grant and Cranford 1989); bacteria (Berg and Alatalo 1984); dissolved organics (Manahan 1983) and artificial diets (Laing 1987). Very little is known about specific nutrient requirements or how larvae, juveniles and adults partition their energy.

The various digestive enzymes found in molluscs have been reviewed by Reid (1983). Other factors must not be neglected. For example, a lectin recently found in sea scallops (*Placopecten magellanicus*) may play an important role in feeding (Gill 1987).

In bivalves, there may be many common features shared by the veliger and adult forms, such as the criteria for particle selection and the associated selection mechanisms as well as metabolic pathways, especially for substrate utilization.

The traditional view of molluscan evolution starts with the grazing, rasping 'Archemollusc'. Lancaster's original statement in 1883 was "Such a schematic mollusc is not to be regarded as a archetype, in the sense which has been attributed to that word, not as an idea present in a creating mind, nor as an epitome of developmental laws" (from Verrill 1886). Even though the originator did not consider it an archetype, it is remarkable that it has remained so for over a century. An alternative is discussed by Tevesz and McCall (1976) who maintain that the first bivalves were not burrowers and that the bivalve shell evolved for protection and improved suspension feeding in epifaunal habitats.

It is notable that one of the most primitive bivalves, *Pseudomyona queenslandica* (discussed in Runnegar 1982), is D-shaped and spiculose and bears a striking resemblance to the bivalve veliger. The idea that a veliger-like form was the molluscan archetype is not new, having been promoted by Verrill in 1896. Thus, one can speculate that molluscan archetypes were planktonic veligers which presumably adopted a benthic habit during the early Cambrian after changes in seawater composition promoted the formation of heavy shells. A major change would not be needed to induce shell formation. Recently Gallagher *et al.* (1988) have found that

strontium is required in artificial sea water for embryonic shell formation by bivalves. One may conclude then, that the formation of calcified shells in early molluscs may have been due to a minor change in seawater composition. Runnegar (1982) noted that gross macroevolution resulting in speciation may not be caused by rapid changes but by minor transitions intra-specifically within a lineage.

Summary

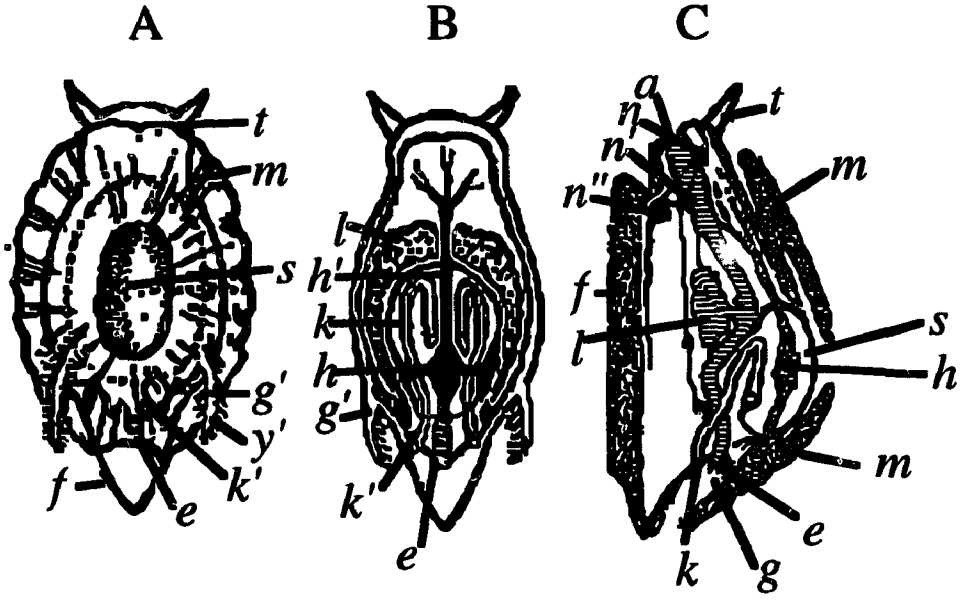
Several traditional views on the origin of feeding mechanisms in bivalve molluscs have been challenged and the following conclusions reached.

1. The archetypical mollusc may have been a D-veliger and not the long accepted rasping, grazing archemollusc. This D-veliger, originally of planktonic origin, settled as a result of shell formation but remained a filter feeder.
2. The original mode of nutrition of protomolluscan larvae was lecithotrophy, not planktotrophy.
3. The bivalves remained holobenthic until they acquired trochophore larvae by insertion into existing life histories. Trochophore larvae were planktotrophic and displaced the veliger as the 'first' larva. This acquisition promoted dispersal and led to the mixed life history of bivalves.
4. Ontogeny of bivalves recapitulates phylogeny only to the second larval stage. The trochophore may represent distantly related species with a different phylogeny.

In view of these new insights, a flexible approach to bivalve phylogeny is encouraged, one that objectively views a variety of information sources including anatomy, life histories, nutrient

requirements, macromelecular structures, biochemistry and stratigraphy. This will result in a more holistic view of evolutionary systematics and life history strategies.

Figure 35 Schematic Mollusk after Lancaster, from Verrill 1886



A, dorsal; **B**, horizontal section; **C**, longitudinal section. ***f***, foot; ***m***, ***m***, mantle; ***s***, shell; ***t***, tentacle; ***g***, gill-cavity; ***g'***, gill; ***a***, mouth; ***e***, anus; ***h***, heart, ***h'***, aorta; ***k***, nephridium; ***k'***, nephridial duct; ***l***, liver; ***i***, gonad; ***y'***, genital pore; ***n***, ***n'***, ***n''***, ganglions.

Appendix 2 Proximate Composition of Juvenile Scallops Collected in the Field

Proximate Composition of the Adductor Over Time (expressed as a percentage)

Age (months)	Protein		Lipid		Carbohydrate	
9	76.08	± 0.07	2.65	± 0.41	3.57	± 0.86
12	74.82	± 0.77	1.22	± 0.26	4.56	± 0.84
15	66.12	± 1.02	1.57	± 0.20	13.09	± 3.66
18	58.68	± 2.66	2.39	± 0.13	24.45	± 2.67
21	79.65	± 6.15	1.32	± 0.17	5.62	± 6.15
24	72.69	± 1.14	1.63	± 0.13	7.53	± 1.17

Proximate Composition of the Digestive Gland Over Time (expressed as a percentage)

Age (months)	Protein		Lipid		Carbohydrate	
9	50.56	± 4.69	6.67	± 2.85	22.73	± 5.52
12	52.20	± 0.17	3.16	± 5.56	16.97	± 1.14
15	46.59	± 1.14	5.09	± 0.54	20.29	± 1.47
18	35.67	±1.70	2.07	± 0.07	48.80	± 1.70
21	33.06	± 2.09	1.95	± 0.40	49.33	± 2.21
24	54.62	± 0.35	5.54	± 1.82	14.74	± 1.85

Proximate Composition of the Gonad Over Time (expressed as a percentage)

Age (months)	Protein		Lipid		Carbohydrate	
9	56.26	± 1.02	4.09	± 0.81	23.83	± 1.83
12	58.00	± 0.87	4.18	± 0.47	11.96	± 2.15
15	60.90	± 1.05	3.38		3.92	± 4.19
18	63.70	± 2.62	9.05	± 1.15	7.50	± 3.05
21	57.61	± 0.48	4.87	± 0.69	9.89	± 2.14
24	62.54	± 2.07	5.89	± 2.46	6.49	± 3.44

Proximate Composition of Viscera Over Time (expressed as a percentage)

Age (months)	Protein		Lipid		Carbohydrate	
9	61.96	± 0.19	2.85	± 0.05	8.42	± 2.38
12	56.55	± 0.12	3.20	± 0.56	9.58	± 1.29
15	60.90	± 0.20	2.21	± 0.35	10.73	± 1.23
18	66.41	± 0.64	4.00	± 0.32	9.05	± 1.59
21	59.74	± 0.57	2.89	± 0.53	9.73	± 2.21
24	59.06	± 1.13	3.18	± 0.75	6.89	± 1.25

Shell Height and Dry Weight Over Time (expressed as a percentage)

Age (months)	Shell Height		Dry Weight		Wet Weight	
9	31.15	± 0.44	0.2292	± 0.0089	1.4366	±0.0415
12	43.82	± 0.65	0.3712	± 0.0204	2.7102	±0.0689
15	32.45	± 0.64	0.1585	± 0.0527	0.8144	±0.0885
18	50.85	± 0.74	1.1845	± 0.0283	5.8432	±0.1729
21	56.15	± 1.32	1.7788	± 0.2414	8.6179	±0.6568
24	60.77	± 1.38	1.0569	± 0.1390	6.7344	±0.6568

Appendix 3 Preliminary Feeding Trial

1. Determination of Ration

Juvenile scallops (*Placopecten magellanicus*) provided by Dr. Micheal Dadswell (Acadia University) from Bay of Fundy sites were held in a Dalhousie University Aquatron wet lab. After sorting into size classes, a stratified random sample of 30 individuals was taken for each of the experimental treatments (two replications of 30 per treatment). The average weight at time zero (T_0) was approximately 0.22 g and the average height was about 1.2 cm. At T_0, a Dremel tool was used to notch all animals.

Each test group of 30 scallops was held in a mesh container submerged inside a 7 l bucket. Air lines into each bucket kept water circulating through the mesh container. All buckets were held in a constant temperature bath kept at 11.5°C ± 1.0°C. (Water cooled by a submersible cooling unit was kept circulating by a pump).

All buckets were cleaned daily using Sunlight detergent. While rearing buckets were cleaned , the scallops were transferred to a holding tank. During transfer, holding baskets were brushed vigorously to remove any remaining algae. After cleaning, each bucket was filled with seawater (Dalhousie University Aquatron -- Northwest Arm) which had been filtered through a 12 µm cartridge filter. Once weekly, all buckets and lines were cleaned with Javex and thoroughly rinsed with hot tap water, then seawater.

Algal counts were determined daily to establish the volume of algal cultures required for each treatment. The predetermined algal densities were: *Isochrysis* aff. *galbana* (clone T-ISO) 0.5 x 10^5 ml^{-1}, 2.5 x 10^5 ml^{-1},

and 5.0 x 10^5 ml^{-1}.; *Chaetoceros neogracilis* (Chagra): 0.5 x 10^5 ml^{-1}, 2.5 x 10^5 ml^{-1}, and 5.0 x 10^5 ml^{-1}. Two treatments were left unfed. Two supplements of microcapsules, 5 and 10 ml respectively, were added to test groups receiving 2.5 x 10^5 ml^{-1} *Chagra*. These acacia gel microcapsules contained menhaden oil, were prepared weekly, and stored under nitrogen at 4°C between feedings. Measured amounts of food were presented once daily, 7 days a week. Algae were harvested at day 4 or 5.

During the daily cleaning/feeding regime, animals were examined for signs of crowding, mortalities, etc.

During the study animals were weighed and measured twice -- once at 4 weeks and once at 8 weeks. On both occasions, care was taken to cut the byssal threads of each animal with a sharp scalpel before removing it from its basket.

Daily record were kept of amount fed, water temperature, and mortality. Observations were also made of the amount of food remaining.

2. Research Objectives

The objective of the first experiment was to answer the following questions:

1. Is the growth of juvenile scallops affected by the species of algae used as a food source?

   Two monocultures, *Isochrysis* aff. *galbana* (clone T-ISO) and *Chagra*, were tested. A third, *Prorocentrum*, was brought in from the Boothbay Lab but failed to grow well. Good growth was promoted by both algal species during the first four weeks of the feeding trial. In most treatments, however, there was very little growth between four and eight weeks. Although

mortality was not extensive, survival was consistently better in groups fed *Isochrysis* aff. *galbana* (clone T-ISO).

2. Is the scallop growth improved by increasing ration size (algal density)?

   Three feeding levels (determined empirically) were tested for each alga. *Chaetoceros neogracilis* tended to give better growth at the highest algal density. In contrast, increasing the ration of *Isochrysis* aff. *galbana* (clone T-ISO) did not produce marked improvements.

For questions 1 and 2, good growth was promoted by both algae during the first four weeks of the experiment. The animals grew very little after week four, in effect independent of the type or size of ration. The good initial growth could be attributed, at least partially, to reserves acquired in the field; however, the unfed controls did not grow. Since the animals were weighed after week four, the post-weighing stress of regenerating the byssal threads could have diminished growth.

It was apparent that the scallops were ingesting food; they may have had difficulty digesting and absorbing it.

Field data show that those kept on the collectors grew over twice as much during the time span as those kept in the lab (C. Warren-Perry, pers. comm.). Although environmental conditions in the field differ from those in the laboratory and do not form a basis for direct comparison, it is evident that animals have the capacity to achieve better growth than is indicated by these studies.

Modifications to the culture system are recommended. The methods employed work well for oysters but must be altered to work well for

scallops. Although mortality was not a problem, refining the culture system will eliminate the negative effects on growth.

3. Will a lipid supplement improve scallop growth?

   Acacia gel microcapsules containing menhaden oil were made weekly and fed as a supplement to scallops on a *Chagra* diet. No growth enhancing effect was achieved with the microcapsule supplement, however one of the replicates receiving the larger supplement showed a tendency toward better growth than the groups on scallops fed only *Chagra*. The results are difficult to interpret as there was 100% mortality on day 27 of one replicate group. This mortality was attributed to human error (nature unknown).

4. Can individual scallop growth rates be determined using a Dremel-tool notch?

   Scallops shells were notched at time zero and examined under the microscope after termination of the feeding trial. Individual shell growth increments of juveniles can be determined using the notching method. This method shows promise for evaluating growth rates over a long term feeding trial.

Figure 36 Density of T-Iso vs Scallop Height

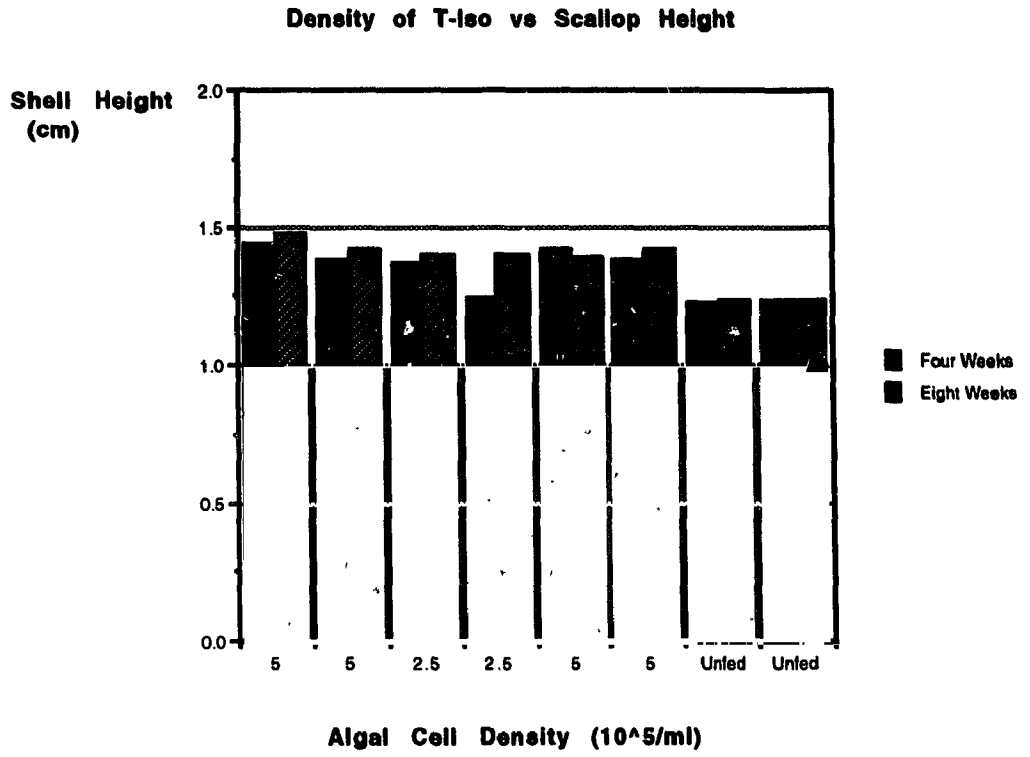
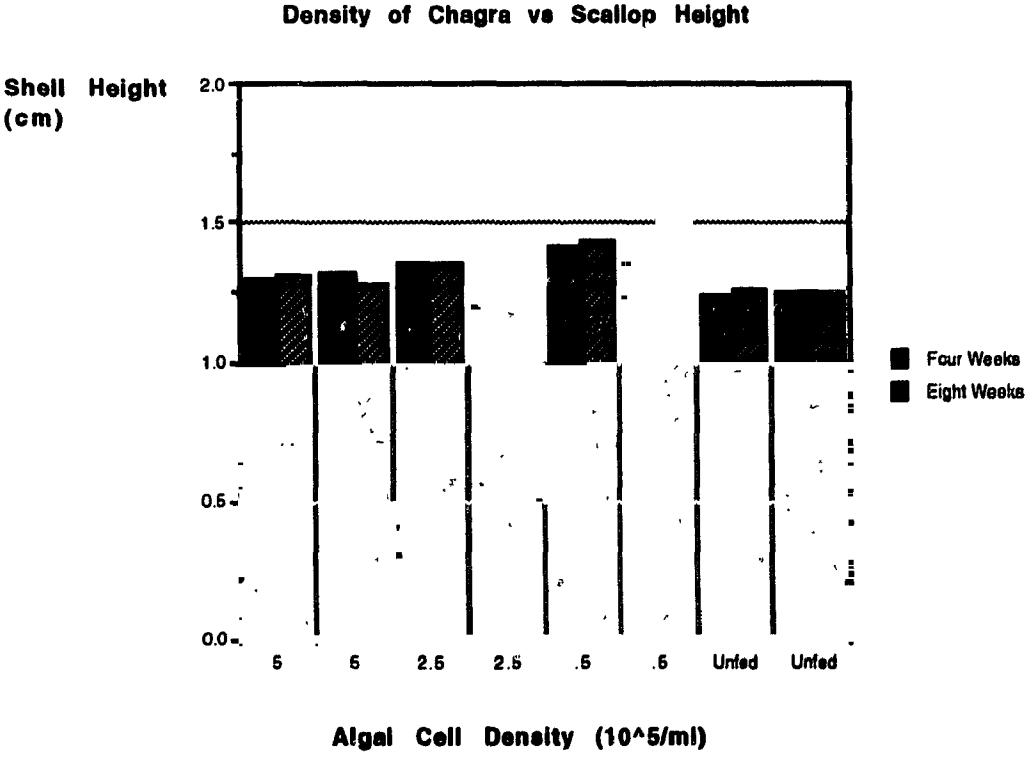


Figure 37 Density of Chagra vs Scallop Height.



Appendix 4 Modified Folch (Folch *et al* 1957) Procedure for Total Lipid Extraction[2]

Equipment	Supplies	Reagents
Homogenizer	Whatman No.1 filter paper	20 ml Chloroform [C]
Homogenizer tube	N_2 Gas	20 ml MeOH [M]
Buchner funnel	glass pipettes	0.1 N KCl [W]
Suction filtration apparatus	glass wool	Na_2SO_4
Vortexer	screw cap conical bottom centrifuge tubes	upper phase solution 47:48:1 [M:C:W]
Centrifuge (Optional)		Lower phase solution 86:14[C:M]

1. Procedure

1. Weigh sample into a tube.
2. Homogenize in 20 vol. (4ml/0.20 g tissue) of 2:1 [C:M].
3. Add 1.1 ml of 0.1 KCl to make 2 phase separation. Vortex 1 minute.
4. Suction filter.
5. Rinse vial with 1.2 ml of upper phase solution and filter.
6. Vortex 1 minute.
7. Centrifuge for 3 minutes.
8. Discard upper layer (water) and transfer lower layer to another tube.

   A little of the water may be retained in B.
9. To clean the water from B: wash with ideal upper phase solution: 1/5 vol. Vortex and separate.
10. Transfer the resultant upper layer from step 9 (water with a little lipid)
11. Re-extract tube C with 1/5 lower phase solution. Remove the upper layer (water) and discard.

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[2]Personal communication, Dr. K.E. Harrison

12. Combine the lower phases of tubes B and C. Dry with $NaSO_4$. Vortex.

Figure 38 Modified Folch Procedure, Initial separation

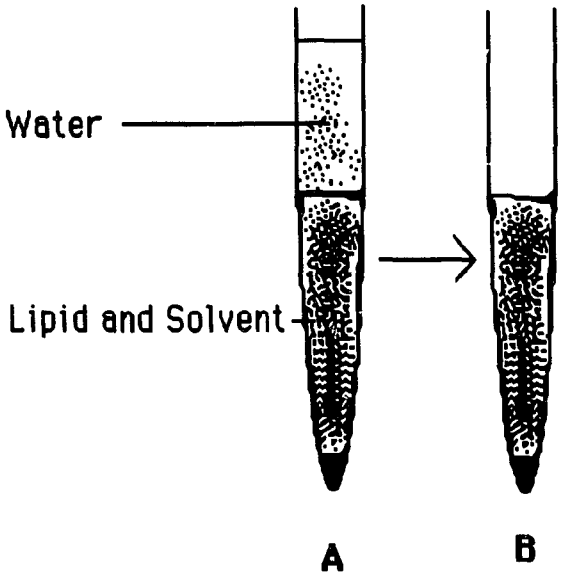
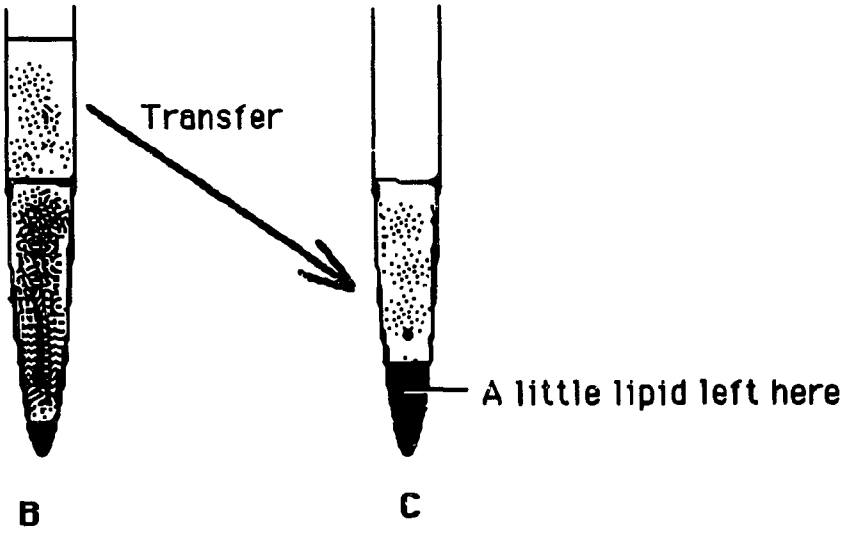


Figure 39 Modified Folch Procedure, Separation to clean water from B.



Appendix 5 Technique on Experimental Analyzer 1106

1. Equipment

Elemental Analyzer 1106	Carlo Erba
Mettler Microgram Balance	No1-912 distributed by Fisher Scientific
Tin Capsules (weigh boats)	Carlo Erba Code no. 240-06400

Analytical standards: Acetanilide BDH 3071610

2. Procedure

The weighed tissue samples inside the tin capsules were placed into an automatic carousel. From here the samples were dropped at specific time intervals into a vertical quartz tube. This is kept at 1030°C and supplied with a stream of helium. When the sample entered, the helium was temporarily enriched with a stream of oxygen and combustion occurs. This is primed by the oxidation of the tin container.

The gas mixture then passed over chromic oxide and quantitative combustion occurs. The gasses then passed over metallic copper at 650°C. This serves two purposes: to remove excess oxygen and to reduce any nitrogen oxides to nitrogen.

The resultant mixture then passed through a Porapak QS column (100°C) and individual components were separated and eluted. These were measured by a thermal conductivity detector whose signal fed to a potentiometric recorder in parallel with the integrator and printout.

Sample Calculation

1 Sample ID	2 Sample Weight	3 Standard Count	4 Sample Area	5 µgN	6 %N	7 % Protein
#1	353	6877	322330	46.87	13.3	77.14

1 ID as per sample label

2 A weighed on balance

3 From printout

4 From printout

5 µgN = (Sample Area) / (Standard Count)
i.e. (Column 4) / (Column 3)

6 %N = µgN/wt(µg) i.e. (Column 5) / (Column 2)

7 % Protein = 5.8 x %N i.e. 5.8 x Column 6

Appendix 6 Sea Scallop Larvae Feeding Experiment, August 1991

1. Introduction

A feeding trial al Dalhousie University (see Part III, Experiment 1.1) was successful in showing that sea scallop larvae could ingest the sodium alginate microparticulate diet. In an attempt to reproduce demonstrate growth on these diets, a second larval feeding trial using similar diets and protocol was done at the Molluscan Nutrition wet lab facility at DFO. Two modifications were made to the experimental design.

1. The containers were larger, 3 l beakers. This was done to improve survival.
2. For each treatment, 4 replicates were run. This was done to increased the database and facilitate statistical analyses.

2. Materials and Methods

Larvae

Five day old larvae of the sea scallop (*Placopecten magellanicus*) were acquired from the National Sea Products hatchery on August 12. They were taken to the Halifax lab and counts were done to determine the number of larvae per ml Based on these counts, the larvae were equally distributed to the various experimental containers. Each 2 l container was stocked with about 5 larvae per ml.

Dietary Treatments

Five dietary treatments were tested. The algal diet, *Isochrysis* aff. *galbana* (clone T-ISO), was fed at $30x10^3$ cells/ml. The microparticulate diet (IX) was based on a dry weight equivalent to $30x10^3$ cells of *Isochrysis* aff. *galbana* (clone T-ISO). The 2x microparticulate diet was twice the dry weight. A mixture of half algae and half microparticulate diet was also

tested (total dry weight = 30,000 cells). The fifth treatment was an unfed group. There were four replicates of each diet.

Experimental Protocol

Larvae were kept in 3 l beakers which were partially submerged in ambient sea water. This kept the larvae in water at about 15°C. The ambient sea water was used since the 15°C seawater was entering the wet lab at 17°C. Each beaker contained an air line terminating in a 2 cm piece of bubble tubing which gently aerated the container.

On alternate days, each beaker was removed, the contents screened to retain the larvae and a small sample of larvae removed for counts and measurements. At this time the beaker was cleaned and filled with sterile (autoclaved) seawater. Larvae were returned and the appropriate food ration was added. These "changes" were done quickly and gently in an effort to reduce stress to the larvae.

On the days between changes 25 ml of seawater was taken from each of the 20 beakers. These samples were screened to retain larvae and the larvae returned to the proper chamber. The remaining solution, containing food but no larvae, was counted on a Coulter counter to measure food density. If the latter was declining, an appropriate amount of food was added to "top off" the container and restore the part of the ration that had been grazed down.

The samples which were removed during the change days were examined for growth and survival.

3. Results and Discussion

The growth data are shown in the attached figure. On day four when samples were measured the best growth was in the algal fed group, followed by the 1x ration of the sodium alginate microparticle. The

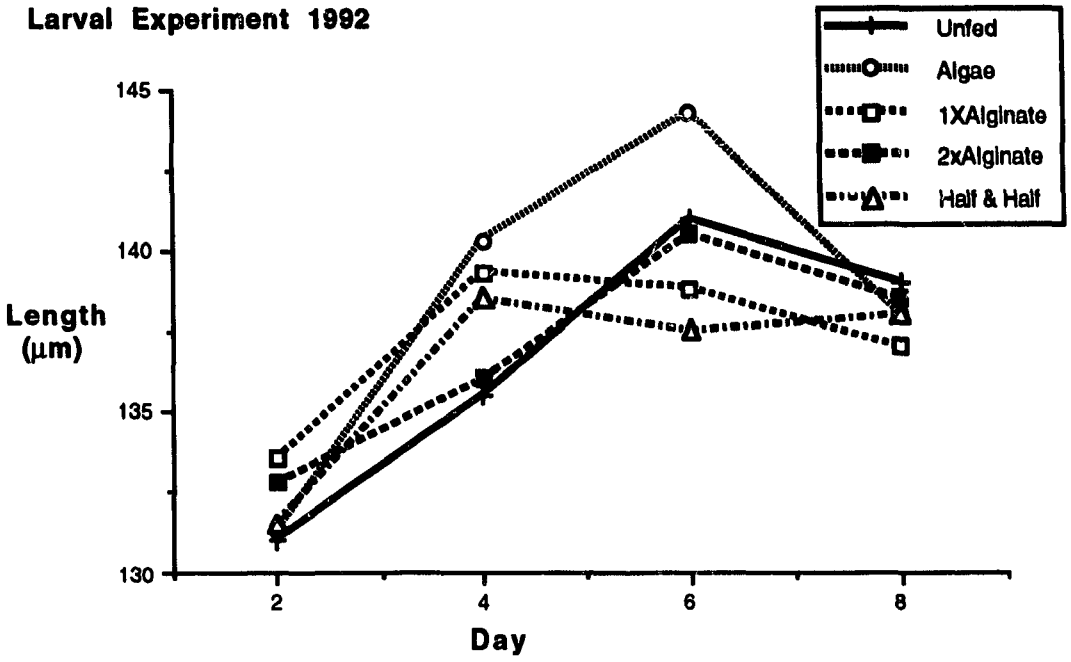
poorest performance was in the unfed and 2x sodium alginate treatments. By day 8 the larvae were showing a decline in growth. Survival data indicated high mortalities in all treatments and the experiment was terminated.

The reasons for the poor growth and survival are not clear. Since an experiment with essentially the same design and protocol was successful in the Dalhousie Aquatron, between site differences were examined. Some disparities between the experiments include.

1. Seawater at Dalhousie was filtered; seawater at DFO was autoclaved. This process could have removed most of the oxygen; however, one would have expected mass mortalities early in the experiment if anoxia was a problem.
2. Containers at Dalhousie were smaller (≈1/3 vol) than those used at DFO. From our experience with larvae, the larger the container the better.
3. There were obviously different "batches" of larvae, and those used in the DFO experiment could have been in some way physiologically inferior.
4. The water supply at both sites is from different sources. There may be beneficial elements in the Aquatron water absent at DFO, or something detrimental in the DFO water. (For example, it could be the presence or absence of a specific mineral element.)
5. Bacteria can pose a problem in larvae trials using synthetic diets. The numbers of bacteria were not quantified at either site; however, poor growth and survival at DFO occurred in all treatments, irrespective of diet.

Whatever the cause, it is apparent that there are inherent difficulties in conducting controlled feeding trials with bivalve larvae. Our knowledge of the critical factors determining growth and survival must be expanded.

Figure 40 Larval experiment 1992.



Appendix 7A Analysis of Variance, Oyster Larvae Size

Source	Sum of Squares	DF	Mean-Square	F-Ratio
Diet	120886.831	4	30221.708	28.842
Error	5239.109	5	1047.822	

where F[0.05]; 4,5 = 3.1

	Group 1 MA	Group 2 AP	Group 3 MAAP	Group 4 Unfed	Group 5 MAC
m_j=	311.11	543.83	625.6	500.48	589.43

Contrasts evaluated by $F = \dfrac{N\left(\sum c_j m_j\right)^2}{\nu_1 s^2 \sum c_j^2}$

where N is number of replicates

ν_1 is numerator degrees of freedom

s^2 is mean square

1. $\mu_{MAAP} - \mu_{AP} = 0 \quad F = \dfrac{2(625.6 - 543.83)^2}{4 \times 1047.822 \times 2} = 1.595$

   Therefore accept null hypothesis.

2. $\mu_{MAAP} + \mu_{AP} - 2\mu_{MAC} = 0 \quad F = \dfrac{2(625.6 + 543.83 - 2 \times 589.43)^2}{4 \times 1047.822 \times 6} = 0.007$

   Therefore accept null hypothesis.

3. $\mu_{MAAP} + \mu_{AP} + \mu_{MAC} - 3\mu_{ma} = 0$

   $$F = \frac{2(625.6 + 543.83 + 589.43 - 3 \times 311.11)^2}{4 \times 1047.822 \times 12} = 27.100$$

   Therefore we reject the null hypothesis. The MA group is smaller.

4. $\mu_{MAAP} + \mu_{AP} + \mu_{MAC} + \mu_{ma} - 4\mu_{Unfed} = 0$

   $$F = \frac{2(625.6 + 543.83 + 589.43 + 311.11 - 4 \times 500.48)^2}{4 \times 1047.822 \times 20} = 0.110$$

Only the comparison of the MA group with the other fed groups reaches the F[0.05];4,5 = 3.1 criteria. Hence MA is less than them. The unfed group sits between MA and the other fed groups.

The above contrasts are called the Helmert orthogonal set.

Appendix 7B Analysis of Variance, Clam Larvae Size

ANOVA

Source	Sum of Squares	DF	Mean-Square
Diet	2980.878	4	745.220
Error	831.889	5	166.378

where F[0.05]; 4,5 = 3.1 (Rodger's F)

	Group 1 MA	Group 2 AP	Group 3 50%AP	Group 4 MAAP	Group 5 Unfed
m_j=	271.617	281.137	300.256	276.15	246.675

Contrasts evaluated by $F = \dfrac{N\left(\sum c_j m_j\right)^2}{v_1 s^2 \sum c_j^2}$

where N is number of replicates

v_1 is numerator degrees of freedom

s^2 is mean square

$$F_{3-5} = \frac{(300.256 - 246.675)^2}{4 \times 166.378 \times 2} = 4.314$$

Hence 50% AP is larger than Unfed.

$$F_{2-1} = \frac{(281.137 - 271.617)^2}{4 \times 166.378 \times 2} = 0.136$$

$$F_{1+2vs4} = \frac{(271.617 + 281.137 - 2 \times 276.15)^2}{4 \times 166.378 \times 6} = 0.000$$

Groups MA, AP, and MAAP do not appear to differ.

$$F_{3+5-1-2-4} = \frac{(3 \times 300.256 + 3 \times 246.675 - 2 \times 271.617 - 2 \times 281.137 - 2 \times 276.15)^2}{4 \times 166.378} = 0.029$$

Three of the treatments (1,2 and 4) sit somewhere in the middle between the large 50%AP and the small Unfed group.

Appendix 7C Statistics on Clam and Oyster Survival

Testing $\varphi_1 = \varphi_2 = \varphi_3 = \varphi_4 = \varphi_5$ for the five treatment groups.

From Rodgers (1974) F table, we know F[0.05];4,∞=1.551 and for a contrast

$$F = \frac{N\left(\sum c_j p_j\right)^2}{v_1 p_\bullet (1 - p_\bullet) \sum c_j^2}$$

for example: $\varphi_1 - \varphi_2 = 0$ where $\varphi_1 = \text{VA}, \varphi_2 = \text{AP}$; $p_1 - p_2 = \frac{3}{1040}$

$$F = \frac{N(p_1 - p_2)^2}{v_1 p_\bullet (1 - p_\bullet) \sum c_j^2} = \frac{1040(\frac{3}{1040})^2}{4 \times 0.2239 \times 2} = 0.005$$

There appears to be no difference between diets MA and AP.

Clam Survivors						
	Diet					
	MA	AP	50% AP	MAAP	Unfed	Σ
Success	762	759	474	674	771	3440
Failure	278	281	566	366	269	1760
N	1040	1040	1040	1040	1040	5200
Survivors						
Rep 1	326	381	62	237	277	
Rep 2	436	378	412	437	494	

Oyster Survivors Diet	MA	AP	50% AP	MAAP	Unfed	Σ
Success	1349	1622	1651	1621	1859	8102
Failure	2151	1878	1849	1879	1641	9398
N	3500	3500	3500	3500	3500	17500
Survivors						
Rep 1	279	886	610	948	1012	
Rep 2	1070	736	1041	673	847	

Appendix 7D One Way ANOVA on *Placopecten magellanicus* Shell Height Data (Juveniles)

	Row mean	Where
M1	0.1052	M1 - Microparticulate diet 1
M2	0.1017	M2 - Microparticulate diet 2
M3	0.1321	M3 - Microparticulate diet 3
AL	0.2080	AL - Algal diet
AS	0.1805	AS - Algae and spray dried
MA	0.1295	MA - Micro 1 and algae
U	0.1380	U - Unfed

Groups	6	0.03636	0.00606	2.61098
Error	21	0.04874	0.00232	
Total	27	0.08510		

Where F[0.05]; 6, 21 = 1.42

For contrasts, $F = \dfrac{N\left(\sum c_j m_j\right)^2}{v_1 s^2 \sum c_j^2}$

1. Null hypothesis $\mu_1 - \mu_2 = 0$

$$F_1 = \frac{4(m_1 - m_2)^2}{6 \times 0.00232 \times 2} = 0.002$$

2. Null hypothesis $\mu_U - \mu_{MA} = 0$

$$F_2 = \frac{4(m_U - m_{MA})^2}{6 \times 0.00232 \times 2} = 0.010$$

3. Null hypothesis $\mu_U + \mu_{MA} - \mu_1 - \mu_2 = 0$

$$F_3 = \frac{4(m_U + m_{MA} - m_1 - m_2)^2}{6 \times 0.00232 \times 4} = 0.264$$

4. Null hypothesis $4\mu_3 - \mu_U - \mu_{MA} - \mu_1 - \mu_2 = 0$

$$F_4 = \frac{4(4m_3 - m_U - m_{MA} - m_1 - m_2)^2}{6 \times 0.00232 \times 20} = 0.042$$

At this point there appears to be no difference among the diets. The two left to be tested are AL (algal diets) and AS (Algae plus spray dried Tetra).

5. Null hypothesis $5\mu_{AL} - \mu_3 - \mu_U - \mu_{MA} - \mu_1 - \mu_2 = 0$

$$F_5 = \frac{4(5m_{AL} - m_3 - m_U - m_{MA} - m_1 - m_2)^2}{6 \times 0.00232 \times 30} = 1.800$$

Reject the null hypothesis.

Therefore $\mu_{AI} > \mu_1 = \mu_2 = \mu_3 = \mu_U = \mu_{MA}$

6. Null hypothesis $6\mu_{AS} - \mu_{AL} - \mu_3 - \mu_U - \mu_{MA} - \mu_1 - \mu_2 = 0$

$$F_6 = \frac{4(6m_{AS} - m_{AL} - m_3 - m_U - m_{MA} - m_1 - m_2)^2}{6 \times 0.00232 \times 42} = 0.493$$

Hence μ_{AS} sits in the middle between the large μ_{AL} and the smaller set $\mu_3 = \mu_U = \mu_{MA} = \mu_1 = \mu_2$.

Appendix 7E One Way ANOVA on *Argopecten* Juveniles

	Row Means
M1	0.0301
M2	0.0259
M3	0.0252
AL	0.1694
AS	0.1930
MA	0.1556

Groups	5	0.1301	0.0260	7.1397
Error	18	0.0656	0.00364	
Total	23	0.1957		

Where F[0.05]; 5,18 = 1.597

Five treatments, not 6, because the unfed treatment had no survivors. As previously described for *Placopecten*, contrasts were evaluated.

1. Null hypothesis $\mu_{AS} - \mu_{MA} = 0$

$$F = \frac{4(0.1930 - 0.1556)^2}{5 \times 0.00364 \times 2} = 0.154$$

2. Similarly for $\mu_{AS} + \mu_{MA} - 2\mu_{AL} = 0$, F=0.004

3. Then $\mu_1 - \mu_2 = 0$, F=0.002.

4. and $\mu_1 + \mu_2 - 2\mu_3 = 0$; F=0.001

5. Then $\mu_{AS} + \mu_{MA} + \mu_{AL} - \mu_1 - \mu_2 - \mu_3 = 0$, F=6.989

Rejecting only the last null hypothesis implies the survivors of the three microparticulate diets (M1, M2, M3) diets are smaller than the three others (AL, AS, MA).

Appendix 7F Survival Data: *Argopecten* Juveniles

ANOVA Results

Source	Sum of Squares	DF	Mean-Square
Diet	1825.914	6	304.286
Error	777.00	21	37.000

where F[0.05]; 6,21 = 1.412

Null hypothesis $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6$

$$F = \frac{4 \times 195.17709}{6 \times 37.00} = 3.517$$

Therefore, reject null hypothesis.

Null hypothesis $\mu_1 = \mu_3 = \mu_4 = \mu_5 = \mu_6$

$$F = \frac{4 \times 70.124995}{6 \times 37.00} = 1.264$$

Therefore, accept null hypothesis.

Null hypothesis $\mu_3 = \mu_4 = \mu_5 = \mu_6$

$$F = \frac{4 \times 17.3125}{6 \times 37.00} = 0.312$$

Therefore, accept null hypothesis.

Null hypothesis $\mu_1 = \mu_2 = \mu_U$

$$F = \frac{4 \times 84.875}{6 \times 37.00} = 0.312$$

Therefore, reject null hypothesis

Final result: $\mu_1 = \mu_3 = \mu_4 = \mu_5 = \mu_6 > \mu_2 > \mu_U$

Appendix 7G Survival Data: *Placopecten* Juveniles

ANOVA Results

Source	Sum of Squares	DF	Mean-Square
Diet	434.214	6	72.369
Error	278.750	21	13.274

where F[0.05]; 6,21 = 1.412

Null hypothesis $\mu_3 = \mu_5 = \mu_6$

$$F = \frac{4 \times 0.2916}{6 \times 13.274} = 0.015$$

Therefore. accept null hypothesis.

Null hypothesis $\mu_3 = \mu_4 = \mu_5 = \mu_6$

$$F = \frac{4 \times 9.046875}{6 \times 13.274} = 0.454$$

Therefore, accept null hypothesis.

Null hypothesis $\mu_1 = \mu_2 = \mu_7$

$$F = \frac{4 \times 2.5416}{6 \times 13.274} = 0.128$$

Therefore, accept null hypothesis.

Null hypothesis $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7$

$$F = \frac{4 \times (90.25)^2}{6 \times 13.274 \times 84} = 4.870$$

Therefore, reject null hypothesis

Final result: $\mu_3 = \mu_4 = \mu_5 = \mu_6 > \mu_1 = \mu_2 = \mu_7$

Appendix 8 Analysis of Variance, Feeding Behavior

Source	Sum-of-Squares	DF	Mean-Square	F-Ratio	P
Flow	853.792	2	426.896	3.274	0.052
Feed	1555.028	4	388.757	2.982	0.035
Flow * Feed	1059.476	8	132.435	1.016	0.445
Error	3911.120	30	130.371		

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