

EFFECT OF ALGAL CELL DENSITY, DIETARY  
COMPOSITION, GROWTH PHASE AND MACRONUTRIENT  
CONCENTRATION ON GROWTH AND SURVIVAL OF  
GIANT SCALLOP *Placopecten magellanicus*  
(GMELIN, 1791) LARVAE AND SPAT IN A  
COMMERCIAL HATCHERY

CENTRE FOR NEWFOUNDLAND STUDIES

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Effect of algal cell density, dietary composition, growth phase and macronutrient concentration on growth and survival of giant scallop *Placopecten magellanicus* (Gmelin, 1791) larvae and spat in a commercial hatchery.

by

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## Abstract

This study was aimed at optimizing the growth and survival of *Placopecten magellanicus* larvae and spat by manipulation of the hatchery algal diet with respect to cell density, species composition, phase of harvest, and macronutrient concentration of the algal growth medium. Algae were cultured in a chemostat system in which variations in cell density and growth rate could be controlled.

This study indicated that an algal cell density of 40 cells/ $\mu$ l resulted in most efficient use of the algal ration. This ration was then fed to spat in subsequent growth trials. Spat were stained with calcein (0.15 g/L seawater) prior to experimentation, for a duration of 72 hours, to introduce fluorescent marker bands which could be used to measure new shell growth in these trials.

Attempts to improve the standard hatchery diet by the addition of algal species showed that *Tetraselmis suecica*, *Chaetoceros calcitrans* and *Chroomonas salina* were not beneficial as a supplement to a mixed algal diet for *Placopecten* larvae and spat.

Scallop larvae were found to grow better when fed logarithmic phase algae or algae cultured under high macronutrient concentration, suggesting a higher requirement for protein during early development. Spat were found to grow better overall when fed stationary phase algae or algae cultured under low macronutrient concentration, which were found to be high in lipid and carbohydrate content important for metamorphosis and postlarval (spat) development. Spat shell growth and total lipid and carbohydrate of the diet were positively correlated.

The results of the study also indicated that *Thalassiosira weissflogii* was beneficial in a mixed algal diet for giant scallop spat, and that spat selectively ingest significantly more *Thalassiosira weissflogii* by volume in comparison to a species of similar cell size, *Tetraselmis suecica*.

This study indicates where improvements can be made to traditionally used mixed algal diets fed to larvae and spat in a commercial hatchery giving higher growth and survival prior to transfer to the natural environment at nursery or grow-out sites.

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## Chapter 1

### Introduction

#### 1.1 General

The giant scallop or sea scallop (*Placopecten magellanicus* Gmelin, 1791) ranges from the north shore of the Gulf of St. Lawrence to Cape Hatteras, North Carolina (Posgay, 1957). It is a benthic, subtidal, active suspension feeder ingesting phytoplankton, small zooplankton, spores, and detrital particles (Shumway et al., 1987).

In the giant scallop, the sexes are separate, gametes are released into the surrounding water, and fertilization occurs externally (Levitan, 1991). Fertilized eggs undergo development into planktonic D-shaped veliger larvae approximately 4 days after spawning. When larvae reach about 220 $\mu$ m, approximately 28 days after spawning, they develop paired eyespots and a foot (pediveliger stage), settle on the bottom, undergo metamorphosis (spat stage) (approximately 35 days after spawning), and attach to a variety of substrates (Culliney, 1974).

*Placopecten magellanicus* once supported a valuable fishery in eastern Canada and the northeastern United States, and still does in certain regions. Between 1976 and 1987 giant scallop harvests represented about 30% of the world production of all scallop species, but overfishing has depleted some natural stocks in recent years (Naidu, 1991). This has stimulated renewed interest in giant scallop aquaculture.

*Placopecten* has a number of features that make it an excellent candidate for culture in Atlantic Canada (Dadswell and Parsons, 1991). Scallops of commercial size (80-100 mm) contain a large amount of meat which commands a high market price (Wildish et al., 1988). The species is tolerant of the low winter temperatures found in Atlantic Canada, and it is hardy when handled. Scallop culture can be divided into three stages (Claereboudt et al., 1994): a breeding or hatchery stage, where adults are conditioned and spawned and larvae raised to maturity, a nursery or intermediate stage where juveniles are raised to an outplanting size, and a growout stage where juveniles are

grown to commercial size. Numerous studies have examined methods of improving intermediate and grow-out techniques (Dadswell et al., 1988; Parsons and Dadswell, 1992; Couturier et al., 1995), but spat (juvenile scallop) supply remains critical to the economic viability and success of a scallop aquaculture or enhancement venture. The major problem facing the industry today is unpredictability in the availability of spat (Helm, 1994). If spat could be reliably grown in a hatchery, a reliable source of seed for the farmers would be ensured, eliminating the seed supply problem. When hatcheries are used to produce juvenile bivalves, maximum survival of mature larvae must be realized to make bivalve culture economically viable. Low survival of larvae and juveniles results in very high costs of hatchery operations. In order to maximize production, it is necessary to study the biology of larval and juvenile scallops, and determine the optimum conditions for their growth and survival.

## **1.2 Algal culture**

The production of unicellular algae is a very important step in the rearing of marine bivalves on both laboratory and commercial scales. A variety of batch and continuous culture systems has been used. Batch culture can be defined as a culture of constant volume confined within a vessel with no replacement of culture medium (Eppley, 1991). The great advantage of batch culture is its simplicity; its main disadvantage is that conditions are constantly changing in the culture vessel. Growth rate changes with time, as does the physical and chemical environment of the cells. It is possible to stabilize the cells' chemical environment by using cage cultures, whereby cells are grown inside dialysis bags exposed to a reservoir of medium. Nutrients diffuse into the bags and exudates diffuse out (Parrish and Wangersky, 1990). The use of cage cultures does not, however, overcome the problem of varying growth rates and an increasing degree of self-shading by the algae; this can be done only by using continuous culture methods.

Continuous cultures can be defined as constant volume cultures provided with a continuous inflow of new medium and a corresponding outflow of culture. Two types of continuous cultures can be identified : turbidostat and chemostat. The standing stock



of cells is regulated in turbidostat cultures, while the flow rate of new medium is regulated in chemostat cultures (Eppley, 1991). Continuous cultures are particularly attractive in laboratory work and mass culture work because they provide a continuous means of sampling or harvesting and because they are amenable to automation (Wangersky et al., 1989).

The algal production unit at the Belleoram Sea Scallop Hatchery, located in Fortune Bay, Newfoundland, obtained from Seasalter Shellfisheries is a large chemostat in which many algal culture vessels are supplied continuously with nutrients and continuously harvested at the same rate as the nutrient supply. The rate of nutrient delivery is controlled by a valve which regulates the pressure in the nutrient delivery line. When the cell density of algae in the culture vessels is constant, the growth rate of the algae is determined by the pressure of the nutrient line. Manipulation of the concentrations of the major chemical nutrients supplied to the culture vessels changes the cell density in the vessels, with an increase in the concentration of limiting nutrient leading to an increase in cell density (Dabinett et al., 1998). Adjustments in the rate of nutrient delivery to the algal chemostat culture system and concentration of nutrients may give better growth and survival of *Placopecten* larvae and spat.

### **1.3 Algal cell density**

Algal cell density affects the clearance rate, ingestion rate and absorption efficiency of *Placopecten magellanicus*, and thus growth and survival. Growth of bivalves is a function of suspension feeding, which can be determined from the measurement of clearance rate (the volume of water swept free of particles per unit time per animal). Essentially, ciliated gills generate water currents, filter particles from the water, trap these particles in mucus, and transport them to the labial palps for further sorting and either ingestion or rejection as pseudofaeces (particles which are removed from the water but are not ingested) (Thompson and Bayne, 1972).

Two general approaches to the measurement of clearance rate have been identified, a direct and an indirect method. The direct method has been used where the water from the animal's exhalant siphon can be sampled directly, and can be used to determine pumping rate. The indirect method measures the removal of particles per unit time from a known volume of water which contains the animal (s). This is the more commonly used method. The apparatus used to measure clearance may be a simple static system composed of a container containing the animal (s) and suspension of cultured algae, in which the decrease in particle density is measured over time, or a flow-through system where water with a constant algal density flows through a chamber containing the animal (s), and clearance is measured by determining the particle concentration in the inflowing and outflowing water (Thompson and Bayne, 1972).

The general relationship between algal density and particle concentration is that clearance rate decreases with increasing algal density. Many bivalves have the ability to regulate feeding within a range of particle densities in order to obtain a constant ingestion ration (Thompson and Bayne, 1972; Winter, 1973; Palmer and Williams, 1980; Seiderer et al., 1984). Food uptake in a dense algal culture is limited by the capacity of the gut and the digestibility of the algae (McMahon and Rigler, 1965). At low algal densities, food uptake by *Placopecten magellanicus* is characterized by a constant clearance rate and ingestion rate increases with increasing algal densities (Manning, 1986). In aquaculture it is important to determine the maintenance and optimum rations for a given species. Maintenance ration is the amount of food which supports basal metabolism but results in zero growth. Optimum ration is that which maximizes gross growth efficiency. The algal ration fed to larvae and juveniles should be sufficient to cover maintenance costs, but should not exceed the optimum ration because of the high cost of growing the algae (Manning, 1986).

Hollett (1989) investigated the effect of ration (algal cell density) on the growth of juvenile *Placopecten magellanicus* to determine the most efficient use of algal cultures to promote optimum growth in a hatchery. The diet consisted of a mixture of three cultured algae in a 1:1:1 ratio based on cell count. The mixed algal diet was batch fed daily to

provide initial mean cell densities of 12, 22, 45, 68, and 87 cells/ $\mu$ l. Clearance and ingestion rates were monitored. The results indicated that clearance rate was inversely related to algal cell density. Ingestion rate increased at low cell density to a maximum at 22 cells/ $\mu$ l. Between cell densities of 22 and 45 cells/ $\mu$ l, ingestion rate remained constant. Further increases in algal cell density caused a decrease in ingestion rate. The algal cell density of 45 cells/ $\mu$ l resulted in superior growth and most efficient use of the algal ration.

#### **1.4 Algal dietary composition**

For hatchery purposes, it is important to know the algal diet supporting growth and survival of larvae through metamorphosis, and resulting in optimum growth of the juveniles. A number of studies have examined the nutritive value of a variety of phytoplankton species for molluscs. From the several thousands of phytoplankton species existing in nature, only 40 (De Pauw, 1981), distributed over eight taxonomic classes, have been tested as food for juvenile bivalves belonging to the genera *Ostrea*, *Crassostrea*, *Mercenaria*, *Mytilus*, and *Venerupis*. From the results of these studies it can be concluded that there are considerable differences in food value between different algal species used as food for juvenile bivalves (De Pauw, 1981). Only 16 species out of 40 supported excellent growth in juveniles : *Dicrateria inornata*, *Isochrysis galbana*, *Pavlova lutheri*, *Pseudoisochrysis paradoxa*, *Tetraselmis suecica*, *T. chui*, *T. maculata*, *T. tetrahele*, *T. inconspicua*, *Chaetoceros calcitrans*, *C. curvisetus*, *C. simplex*, *Bellerochea polymorpha*, *B. spinifera*, *Skeletonema costatum*, and *Thalassiosira pseudonana*. Eighteen had very poor nutritive value and some were even toxic. In general, representatives of the Chlorophyceae, Cyanophyceae, Dinophyceae, and Xanthophyceae were less suitable as food for bivalves because of a thick cell wall or the production of toxic metabolites (Davis and Guillard, 1958; Guillard, 1958; Ukeles, 1971, 1980). On the contrary, small chrysomonads, having no thick cell wall and producing no toxic metabolites, were good food as were cryptomonads, green flagellates, and diatoms. However, even related species within the same class, family, or genus, may be nutritionally

good or poor. Furthermore, not all the bivalves considered reacted in the same way towards given algal species; for example, *Dunaliella tertiolecta* was a poor food source for *O. edulis* and *M. mercenaria*, but good for *C. gigas* (De Pauw, 1981). Algal diets that promote rapid growth of the bivalve genera examined are not, however, necessarily among the best scallop diets, so information on nutritional requirements of these genera cannot be applied directly to scallops.

During the last three decades, considerable efforts have been made to determine the value of mixed diets of monospecific algae versus that of single algal diets as food for juvenile bivalves. The results showed that with suitable algal species, far better growth of juveniles is obtained with mixtures of two or more species than with either species alone (Helm et al., 1973; Dupuy et al., 1977; Ukeles et al., 1984; Lucas et al., 1986; Gillis, 1993).

In addition to considering whether or not an algal species possesses a thick cell wall or produces toxic metabolites, several other properties must be taken into account when determining which algal species will provide the best food, including: (1) cost of growing the algae (2) ease with which the algae can be cultured in a hatchery and (3) biochemical composition of the algae (Wikfors et al., 1984; Enright et al., 1986; Gillis, 1993).

## **1.5 Algal growth phase**

Many researchers have compared algal diets in an attempt to determine the optimal diet for bivalves. It is important to consider why some diets result in increased growth. One enigma is the relationship between the food value of an alga and its chemical composition (Epifanio, 1979). Phytoplankton can vary in their nutritional value to a variety of planktivorous animals. During early experimentation in aquaculture, variation in nutritional value of phytoplankton did not appear to be related to their general biochemical composition (Walne, 1963). These early findings suggested that palatability and digestibility were significant factors in determining the nutritional value of phytoplankton. However, the availability of more sophisticated analytical techniques provided superior

tools for investigating the biochemical composition of phytoplankton, e.g., the Chromarod-Iatroscan (TLC/FID) system used for lipid class measurements (Ackman, 1981), and the analysis of the composition of individual fatty acids have shown that the biochemical composition of the algae is highly significant in affecting molluscan growth.

Phytoplankton as food must supply both energy and essential nutrients. Although little is known of specific nutrient requirements for bivalves (Langdon and Waldock, 1981), lipid, carbohydrate, and protein remain the major dietary sources of energy for growth and development. In most marine invertebrate larvae lipid is the major reserve material (Holland, 1978), whereas adults use glycogen as their major energy source (Giese, 1969). During the pelagic stage, neutral lipid is accumulated by larvae and reaches a peak just before metamorphosis. Most bivalve larvae are unable to feed on particulate matter during metamorphosis and settlement, as a result of the larval feeding organ (the velum) being cast off, and the adult feeding organ (the gill) not yet being functional (Hickman and Gruffydd, 1971). Lipid reserves are rapidly depleted during this period to meet the costs of metabolism and morphogenesis (Holland and Hannant, 1974). Therefore, the correct diet is required not only to ensure good growth of pelagic larvae, but to allow them to accumulate enough energy reserves to sustain them through metamorphosis.

The conditions under which phytoplankton grow determine their biochemical composition and thereby affect their energy content and nutrient value (Whyte, 1987). One factor influencing algal biochemical composition is the growth phase of algae harvested for food. In the logarithmic phase, synthesis of metabolites and cell division is rapid and cell numbers increase in a geometric progression, while in the stationary phase cell numbers remain constant and their viability is maintained by stored energy reserves. Studies on changes in biochemical composition of phytoplankton during the different growth phases are not very common, and published data usually refer to a single phase of the culture (Fernandez-Reiriz et al., 1989).

Brown et al. (1996) investigated the effect of harvest stage (logarithmic and stationary phases) on the biochemical composition of the diatom *Thalassiosira pseudonana*, and found that with the onset of stationary phase, carbohydrate and to a lesser extent lipid content increased, and protein decreased, whereas cells in logarithmic phase contained the most protein. This was a result of metabolism being shunted from protein synthesis (cell division) during the logarithmic phase to energy (carbon) storage during the stationary phase.

Other studies of the nutritive value of phytoplankton species during different growth phases have shown that high levels of carbohydrate, obtained from algae harvested during stationary phase, produce the best growth in juvenile oysters (*Ostrea edulis*) (Enright et al., 1986). High dietary protein, obtained from algae harvested during logarithmic phase, produces optimum growth in juvenile mussels (*Mytilus trossulus*) (Kreeger and Langdon, 1993). Flaak and Epifanio (1978) also demonstrated that algae in the stationary phase contained more carbohydrate and resulted in oysters with more glycogen; algae in the exponential phase contained more protein but supported less growth.

#### **1.6 Algal macronutrient concentration**

Biochemical studies on microalgae are also required to determine how organic composition may be affected by the concentrations of dissolved macronutrients in the surrounding medium. Changes in the chemical composition of algal species cultured at different macronutrient (nitrate, phosphate and silicate) concentrations have long been observed (Ketchum and Redfield, 1949; Spoehr and Milner, 1949). Most such studies have shown repeatable trends when the nitrogen source is manipulated: nitrogen deficiency results in an increase in carbohydrate and decrease in protein, whereas nitrogen enrichment results in cells rich in protein and low in carbohydrate (Thomas et al., 1984; Wikfors, 1986).

Harrison et al. (1990), measured changes in protein, carbohydrate, and lipid of three algal species (*Isochrysis galbana*, *Chaetoceros calcitrans*, and *Thalassiosira*

*pseudonana*) (harvested during mid-logarithmic growth phase) as growth became limited by nitrogen (N) or silicon (Si). Under N starvation (2 days) % lipid remained relatively constant, while % carbohydrate increased and % protein decreased in all three species, compared with cells growing under no nutrient limitation. Under Si starvation (6 hours) there was no change in lipid, protein or carbohydrate. No changes in lipid were observed, probably because cell composition was determined after only 6 hours of Si starvation. An increase in lipids due to Si limitation in *Cyclotella cryptica* and *Chaetoceros muelleri* has been observed by several workers (Vaulot et al., 1987; Roessler, 1988).

Wikfors et al. (1984) analysed two algal flagellates (*Dunaliella tertiolecta* and *Tetraselmis maculata*) for total carbohydrate, protein, and lipid. The two algal species were cultured in three different growth media and harvested in stationary phase. When cultured in the enriched-nutrient reduced seawater medium X, both species contained more carbohydrate and less protein than they did when cultured in E (standard formulation/enriched seawater growth medium). The third medium (N/P) (all medium components decreased except nitrate and phosphate) produced algae with decreased carbohydrate and increased protein when compared with E. The total lipid content of *D. tertiolecta* was significantly less than that of *T. maculata* regardless of culture medium. *Tetraselmis maculata* was a consistently better food source than *D. tertiolecta*, indicating a relationship between algal lipid content and oyster growth. Growth of oysters fed algae cultured in medium X was higher than growth of oysters fed algae cultured in E or N/P, suggesting a nutritional requirement for more carbohydrate than protein.

Several authors have pointed out the importance of lipid (Dunstan et al., 1993; Parrish et al., 1998) and carbohydrate (Enright et al., 1986) for growth of mollusc larvae and spat, and Brown et al. (1997) reported that the amino acid composition of the 40 species of microalgae examined during their study was strikingly similar, suggesting that protein quality was also similar. The protein quality of microalgae also remains high under different culture conditions (Brown et al., 1993). For these reasons the protein content of

the microalgae was not determined during the present study and only lipid and carbohydrate analyses were performed.

The importance of providing larvae and bivalve spat with a suitable algal diet in the hatchery is very clear; they will grow faster and more efficiently, reach a higher quality, and perform better when transferred to the natural environment.

### **1.7 Selection of algal species by filter-feeding bivalves**

The choice of algal species is partially dependent on the particular morphological and hydrodynamic characteristics of the ctenidia of a given bivalve species, which can lead to different filtering and particle retention capabilities based on size, electrical charge, and flow rates (Rubenstein and Koehl, 1977; Jørgensen, 1983; LaBarbera, 1984; Riisgard, 1988).

Experiments conducted by Lesser et al. (1991) with *Placopecten magellanicus*, using different sizes of phytoplankton at densities that did not produce pseudofaeces, revealed that the selection of algae was not based on size alone. The total number of cells cleared was not significantly different between any of the experiments. Particle selection in these cases was apparently based on characteristics other than size, or by preingestive sorting of algae by juvenile scallops. This could reflect the ability of juvenile scallops to regulate their feeding rates in order to obtain a constant ration. If maintaining a constant ration is partially regulated by factors other than size, other mechanisms must be invoked to explain the results. These mechanisms might include chemosensory cues, the physiological state and biochemical composition of the algal cells determining which cells are cleared and ingested (Lesser et al., 1991).

### **1.8 Calcein staining**

Chemical tagging of organisms to provide identification, time, or growth marks has been used successfully in many studies (eg. Bevelander, 1965). The chemical calcein (3,6-Dihydroxy-2,4-bis-[N,N'-di (carboxymethyl)-aminomethyl fluoran),  $C_{30}H_{26}N_2O_{13}$ , is one such marker which has received relatively little attention and which offers several



benefits over others. Calcein was designed as an indicator for calcium (Diehl and Ellingboe, 1956). It can be incorporated into the skeletons of a wide variety of taxa, providing a fluorescent mark which can be used for identification, for studies of calcification, and as a benchmark from which to measure growth since marking. It fluoresces bright yellow-green under blue or ultraviolet light, shows very little toxicity, and has a good shelf life in solution. Applied through bath or injection, it appears to be permanently incorporated into any calcified structure laid down while the chemical is present, while not modifying the growth rate of marked individuals. As a marker, calcein resembles tetracycline, but fluoresces more brightly, is less toxic, is more easily absorbed in a bath, and has a much longer shelf life in solution (Rowley and Mackinnon, 1995). It has been used previously to mark fish otoliths (Wilson et al., 1987) and mammal skeletons (Suzuki and Matthews, 1966), and is presently being used to study sea-urchin growth and to mark juvenile razor clams (*Siliqua* spp.) in a study of transplant mortality and growth, and to mark scallop larvae (Crocker, 1998).

## **1.9 Objectives**

Experience at the sea scallop hatchery in Belleoram since it opened in 1995 has shown that survival and growth of larvae prior to settlement is predictable and consistent. In contrast survival and growth of larvae through settlement to 1 mm in shell height (spat) has been unpredictable. Growth and survival rates have fluctuated, indicating that physical conditions and conditions such as diet and ration have not been optimal.

This study was undertaken to investigate the effect of manipulation of the algal diet with respect to cell density, species composition, growth stage of harvest, and macronutrient concentration on growth of sea scallop larvae and spat. Based on studies previously cited in this chapter, the hypotheses tested were that:

- 1) calcein staining of *Placopecten* spat would provide a fluorescent mark which could be used as a benchmark from which to measure new shell growth since staining.

2) different algal densities of a standard diet fed to *Placopecten* spat would result in different growth rates of spat.

3) there are differences in food value among different algal species.

4) *Placopecten* larvae and spat fed algal diets harvested during the stationary phase show faster growth than those fed algal diets harvested during logarithmic phase.

5) the biochemical composition of algae depends on the macronutrient (nitrate/ phosphate/silicate) concentration in the chemostat medium. Macronutrient deficiency results in an increase in absolute carbohydrate content and/or lipid in algal cells, whereas macronutrient enrichment results in algal cells low in carbohydrate and/or lipid.

*Placopecten* larvae and spat fed macronutrient deficient diets show higher growth than those fed macronutrient enriched diets.

6) *Placopecten* spat preferentially select one algal species over another when fed two species in equal proportion based on cell count or cell size.

## Chapter 2

### Materials and Methods

#### 2.1 Study site

Research was conducted at a commercial sea scallop hatchery located in Belleoram, Fortune Bay, Newfoundland.

#### 2.2 Algal culture

Stock cultures of various algal species (Table 1) were illuminated under constant light from fluorescent tubes at about 20°C. The cultures were maintained in glass test tubes containing seawater enriched with commercially available *f/2* medium (Guillard, 1975), and were subcultured every two weeks under aseptic conditions. Algal cultures used for feeding larvae and spat were inoculated first into 100 mL volumes and then to 2 L volumes in 4 L flasks. These cultures were used to inoculate 500 L polyethylene bags, which comprised the algal production unit chemostat at the hatchery, designed by and purchased from Seasalter Shellfisheries, England. Each 500 L culture bag was supplied with a continuously pumped inflow of pasteurised medium and continuously harvested at the same rate through an overflow tube. The rate of medium (nutrient) delivery or harvest was controlled by a valve which regulated the pressure in the nutrient delivery line. When the cell density of algae in the culture bags was constant, the growth rate of the algae could be determined by the nutrient line pressure (Eppley, 1991).

The effect of algae harvested from different growth phases on the growth of scallop larvae and spat was investigated by adjusting the nutrient line pressure (5.2 psi) to control the harvest rate, and by adding a second nutrient delivery line to those cultures maintained in logarithmic phase at high growth rates. Dividing the volume of algae (diatoms and flagellates) harvested per day by the volume of the culture bags provided an estimation of instantaneous growth rate ( $\mu$ ). Growth rate in divisions per day (d) was obtained by dividing  $\mu$  by the natural log (ln) of 2 (Eppley, 1991). The growth rates

(divisions/day) were approximately 0.70 for logarithmic phase diatoms, 0.52 for logarithmic phase flagellates, and 0.14 for near-stationary phase diatoms and flagellates.

The concentrations of the major chemical macronutrients (nitrate, phosphate and silicate) supplied to the culture bags were varied to modify the lipid and carbohydrate concentration of the algae in order to investigate the effect of algal macronutrient concentration on the subsequent growth of scallop larvae and spat which were fed these cultures. Replete cultures were supplied macronutrient concentrations of 883  $\mu\text{M}$  nitrate, 36.3  $\mu\text{M}$  phosphate, and 107  $\mu\text{M}$  silicate (Guillard, 1975) based on *f/2* formulation. Deplete cultures were supplied concentrations of 294  $\mu\text{M}$  nitrate, 12  $\mu\text{M}$  phosphate, and 107  $\mu\text{M}$  silicate by decreasing the nitrate and phosphate concentrations of the *f/2* medium by 1/3. Diatoms were found to be silicate and phosphate limited in both replete and deplete cultures, while flagellates were limited only by phosphate in deplete cultures. Nitrate was not limiting in either replete or deplete cultures for diatoms and flagellates (S. Andrews, BSc. Hons. Thesis, Memorial University of Newfoundland, pers. com.).

To investigate temperature effects on the quality of algae fed to scallop larvae, the temperature of the macronutrient cultures was varied by adjusting the cold water flow in heat exchangers attached to each culture.

Daily estimates of culture cell densities were used to determine the amount required for feeding larvae and spat. A Model  $Z_r$  Coulter Counter with a 100  $\mu\text{m}$  aperture tube was used for algal cell counting.

Table 1. Algal cultures used for feeding giant scallop larvae and spat.

Type	Algal Species	Cell length ( $\mu\text{m}$ )	Source
flagellate	<i>Isochrysis</i> (clone T-Iso)	3-6	CCMP
flagellate	<i>Isochrysis galbana</i>	5-6	CCMP
flagellate	<i>Tetraselmis suecica</i>	10-15	Seasalter Shellfisheries
flagellate	<i>Pavlova lutheri</i>	3-6	CCMP
flagellate	<i>Chroomonas salina</i>	6-9	CCMP
diatom	<i>Chaetoceros calcitrans</i>	3-7	CCMP
diatom	<i>Chaetoceros ceratosporum</i>	3-16	Seasalter Shellfisheries
diatom	<i>Chaetoceros muelleri</i>	4-9	Seasalter Shellfisheries
diatom	<i>Thalassiosira pseudonana</i> (clone 3H)	4-6	CCMP
diatom	<i>Thalassiosira weissflogii</i>	10-15	CCMP

CCMP = Provasoli-Guillard Center for Culture of Marine Phytoplankton, Maine, USA

### **2.3 Algal dry weight**

Algal dry weight determinations were performed as follows : two counts were performed on three separate aliquots of each algal culture to determine cell density. Subsamples (100 mL) of the algal cultures were filtered through pre-weighed, pre-combusted Whatman GF/C filters using gentle vacuum. The cells on the filter were rinsed with distilled water and the filters were placed in aluminum weighing pans, dried at 70°C for 24 hours, cooled in a desiccator and reweighed.

### **2.4 Larval and spat experimental rearing tanks**

Three types of experimental tanks were used throughout this research. Plastic cylindrical tanks (20 L) containing 16 L of seawater (salinity 30-31 ppt, temperature 15°C)

were used for small-scale spat experimentation. The tanks were partly submerged in a water bath for temperature control. Air was bubbled into the tanks to maintain oxygen levels and circulate the algal cells. Spat were removed temporarily every 2 or 3 days while the tanks were cleaned, refilled and algae added.

Conical fibreglass tanks (250 L) filled with 200 L of seawater were used for intermediate-scale larval and spat experimentation. Air was bubbled into the tanks to maintain oxygen levels and algal distribution. Larvae and spat were removed temporarily twice a week while the tanks were cleaned, refilled and food added. Spat were held in downwellers hooked onto the sides of the conical tanks. Downwellers were constructed from 16 L plastic cylindrical tanks with the bottoms cut out and replaced with 650  $\mu\text{m}$  mesh screens. An airlift was placed on the side of each downweller to recirculate water to the spat at a flow rate of 1.2 L/min.

Fibreglass hatchery production tanks (7000 L) filled with 6000 L of seawater were used for large-scale larval experimentation. Air was gently bubbled into the tanks to provide gentle circulation. Larvae were removed temporarily twice a week while the tanks were cleaned, and refilled and food added.

## **2.5 Spat staining with calcein**

Scallop spat shells were stained with calcein to introduce fluorescent marker bands which could be used to measure new shell growth in subsequent growth trials. One hundred spat, ranging in shell height from 0.5 mm - 2.0 mm, were placed in each of two 10 L plastic buckets containing either 6.6 L of calcein solution or 6.6 L of seawater, partly submerged in a water bath as a temperature control. Air was bubbled into the buckets to maintain oxygen levels and algal distribution. Calcein solution was prepared by dissolving 0.15 g calcein/litre seawater, then adjusting the pH with NaOH to equal that of ambient seawater (pH ~ 8.10) (Crocker, 1998). The calcein, in powdered form, was obtained from Sigma Chemical (Lot 123H0594). The spat were batch fed approximately 50 cells/ $\mu\text{l}$  of a standard diet, consisting of equal numbers of cells of six unicellular algal

species, namely, *Thalassiosira pseudonana*, *Chaetoceros muelleri*, *Chaetoceros calcitrans*, *Pavlova lutheri*, *Isochrysis galbana*, and T-Iso.

Scallop spat were left to stain for 24 hours, when 25 were removed from the calcein bath and examined by epifluorescence microscopy. Fluorescent banding was weak, so the spat were put back in the calcein bath and left for an additional 24 hours. Examination after 48 hours of immersion illustrated that banding was not sufficiently strong, so the spat were once again replaced in the calcein bath and left for another 24 hours. Banding was strong after 72 hours of immersion, so the spat were removed from both the calcein and seawater baths, and mortality determined using low power light microscopy. This method was then used to stain spat in subsequent growth trials.

## **2.6 Duration of growth trials**

All subsequent growth trials were conducted for a minimum of 12 days. At the end of the trials shell height (from the hinge to the periphery of the shell ) was measured by low power light microscopy for at least 30 larvae. New shell growth (from the fluorescent band to the periphery of the shell) was measured by epifluorescence microscopy for at least 20 spat. Mortality was assessed in some growth trials by low power light microscopy.

## **2.7 Algal cell density and spat growth**

The effect of algal cell density on growth of spat was investigated to determine the optimum density at which to feed spat in subsequent growth trials. Scallop spat ranging in shell height from 0.3 mm - 1.7 mm were calcein stained, and 200 were placed in each of ten 20 L cylindrical tanks. Each tank contained a different cell density of a standard diet (2 control tanks/no cultured food added, two tanks of 10 cells/ $\mu$ L, two of 20 cells/ $\mu$ L, two of 40 cells/ $\mu$ L, and two of 80 cells/ $\mu$ L). The standard diet consisted of equal numbers of cells, of six unicellular algal species, namely *Isochrysis galbana*, T-Iso, *Chroomonas salina*, *Tetraselmis suecica*, *Thalassiosira pseudonana*, and *Chaetoceros muelleri*. The

experiment ran for 14 days, during which the algal cell densities were restored to their initial values if they dropped below 20% of that value before the water was changed. Clearance rates were determined for spat in all tanks daily from cell counts and the equation of Coughlan (1969):

$$F = ((M \cdot t^{-1} \cdot \ln C_o/C_t) - (M \cdot t^{-1} \cdot \ln C_o'/C_t')) \cdot n^{-1} \quad \text{where:}$$

F = clearance rate (mL/h/animal), M = volume of water with suspended algae (mL),  $C_o$  = initial particle concentration (cells/ $\mu$ L),  $C_t$  = concentration at time t (cells/ $\mu$ L), t = time (hours),  $C_o'$  = initial concentration in control chamber (cells/ $\mu$ L),  $C_t'$  = concentration at time t in control chamber (cells/ $\mu$ L), and n = number of animals per tank.

Ingestion rate was determined as the decrease in the number of cells with time per scallop. The following equation was used (Hollett, 1989):

$$IR = (C_i - C_f) V \cdot t^{-1} \cdot n^{-1} \quad \text{where:}$$

IR = ingestion rate (cells/scallop/hour),  $C_i$  = initial cell concentration (cells/ $\mu$ L),  $C_f$  = final cell concentration (cells/ $\mu$ L), V = volume of seawater (mL), t = time (hours), and n = number of scallops.

Five control tanks (1 for each algal cell density) were set up for three days, under the same conditions as the 10 treatment tanks, except they contained no spat. Clearance rates were monitored for the control tanks to determine if there was settling out of algae.

## **2.8 Attempted improvement of standard hatchery diet by addition of algal species**

The effect of species composition of algal diets on larval and spat growth was investigated by performing several growth trials in which algal species (*Chaetoceros calcitrans*, *Tetraselmis suecica*, and *Chroomonas salina*) were added to the standard hatchery diet of 5 species of algae (*Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana*, and T-Iso) to look for possible improvements in the diet.



### 2.8.1 *Chaetoceros calcitrans* in larval diets

Larvae were suspended in 10 L of seawater by gentle agitation with a plastic plunger. One millilitre aliquots were removed and the larvae killed with ethanol and counted by inverted microscopy. Approximately 2 million scallop larvae were placed in each of two 7000 L hatchery tanks and batch fed 2 diets in logarithmic phase. One diet consisted of equal numbers of cells of 5 unicellular algal species, namely *Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana* and T-Iso, while the other diet consisted of the same 5 algal species in addition to *Chaetoceros calcitrans*. Larvae were fed 16 cells/ $\mu$ L at the beginning of the experiment and the food density was increased as the larvae grew, to a final density of 30 cells/ $\mu$ L.

### 2.8.2 *Chaetoceros calcitrans* in spat diets

Scallop spat ranging in shell height from 0.5 mm - 0.75 mm were stained with calcein, and 200 placed in each of four 20 L cylindrical tanks (2 replicates of each diet). Spat were batch fed 40 cells/ $\mu$ L of 2 logarithmic phase diets. One diet consisted of equal numbers of cells of 5 unicellular algal species, namely *Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana* and T-Iso while the other diet consisted of the same 5 algal species in addition to *Chaetoceros calcitrans*.

### 2.8.3 *Tetraselmis suecica* and *Chroomonas salina* in spat diets

Scallop spat ranging in size from 1.7 mm - 2.0 mm were stained with calcein, and 200 placed into each of eight 20 L cylindrical tanks. Each tank contained the same algal cell density (40 cells/ $\mu$ L), but different algal dietary compositions. Four different diets were examined, namely a standard diet, a standard + *Tetraselmis suecica* + *Chroomonas salina* diet, a standard + *Tetraselmis suecica* diet, and a standard + *Chroomonas salina* diet (2 replicates of each diet). The standard diet consisted of 4 unicellular algal species, namely *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana*, and T-Iso. *Chaetoceros ceratosporum* was excluded from the standard diet because the culture

crashed. The diets consisted of equal numbers of cells of the unicellular algal species listed for each treatment.

## **2.9 Algal growth phase**

The effect of algae harvested from logarithmic and near-stationary growth phases on the subsequent growth of scallop larvae and spat was investigated.

### **2.9.1 Larvae**

Four experiments were conducted to examine the effect of algae harvested from different growth phases on growth of scallop larvae. In the first experiment approximately 3.5 million larvae were placed in each of two 7000 L hatchery tanks and batch fed a standard diet of algae harvested from logarithmic and near-stationary growth phases. The diet consisted of equal numbers of cells of 7 unicellular algal species, namely *Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana*, T-Iso, *Chaetoceros calcitrans*, and *Pavlova lutheri*. Larvae were fed 16 cells/ $\mu$ L at the beginning of the experiment and the algal cell density was increased to a density of 30 cells/ $\mu$ L as the larvae grew.

The second experiment followed the same protocol as the first, except that approximately 3.9 million larvae were placed in each of 2 tanks.

The third and fourth experiments followed the same protocol as the first two, except that approximately 3 million larvae were placed in each of 2 tanks. In contrast to the first 2 experiments, the larvae were fed only 6 unicellular algal species, with *Chaetoceros calcitrans* being excluded from the diet.

### **2.9.2 Spat**

Four experiments were conducted to investigate the effect of algae harvested from different growth phases on growth of calcein-stained scallop spat. In the first experiment, 400 spat ranging in shell height from 0.3 mm - 0.5 mm and 1.4 mm - 1.7 mm were placed in each of eight 20 L cylindrical tanks. Each tank contained 40 cells/ $\mu$ L of a standard

diet harvested from logarithmic and near-stationary phase growth. Spat in 4 tanks were batch fed algae harvested during the logarithmic phase, while spat in the other 4 tanks were fed algae harvested during the near-stationary phase. There were 2 tanks containing spat of each size range for each harvest phase. The standard diet consisted of equal numbers of cells of four unicellular algal species, namely, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana*, and T-Iso. Clearance rates were determined daily from cell counts using a Coulter Counter and the equation of Coughlan (1969).

The second experiment followed the same protocol as the first, with the exception that spat ranged in shell height from 0.5 mm - 0.75 mm and 0.75 mm - 1.4 mm, and 300 spat were placed in each of 8 tanks. The diet in this experiment consisted of 6 unicellular algal species, namely *Thalassiosira pseudonana*, *Chaetoceros muelleri*, *Chaetoceros ceratosporum*, *Pavlova lutheri*, *Isochrysis galbana*, and T-Iso.

The third experiment followed the same protocol as the second with the exception that 200 spat were placed in each of 8 tanks.

In the fourth experiment only one size range was examined (1.4 mm - 1.7 mm), and there were no replicate tanks. Two hundred spat were placed in each of 2 tanks, and fed the same diets as spat in the first experiment. Clearance rates were not monitored.

## **2.10 Algal macronutrient concentration**

The effect of algae grown under conditions of high and low macronutrient concentration on the subsequent growth of scallop larvae and spat was investigated.

### **2.10.1 Larval diets of algae harvested from continuous culture at high and low macronutrient concentrations**

Approximately 200,000 larvae were placed in each of twelve 250 L conical tanks. Larvae were batch fed four logarithmic phase diets harvested from algae grown under high and low macronutrient concentrations (3 replicates of each treatment), namely a

100% low macronutrient diet, a 100% high macronutrient diet, a 50/50 ratio low/high macronutrient diet, and a 80/20 ratio low/high macronutrient diet. The diets were fed to larvae at an initial algal cell density of 16 cells/ $\mu\text{L}$ , and the density was increased as the larvae grew, up to a density of 30 cells/ $\mu\text{L}$ . The diets consisted of equal numbers of cells of 4 unicellular algal species, namely *Isochrysis galbana*, T-Iso, *Chaetoceros muelleri*, and *Thalassiosira pseudonana*.

#### 2.10.2 Spat diets of algae harvested from continuous culture at high and low macronutrient concentrations

Approximately 3300 calcein-stained spat, ranging in shell height from 0.75 mm - 1.4 mm, were placed in each of 9 downwellers hung in 250 L conical tanks. Flow rates were adjusted to 1.2 L/min for each downweller. Spat were batch fed three logarithmic phase diets harvested from algae grown under high and low macronutrient concentrations (3 replicates of each treatment), namely a 100% low macronutrient diet, a 100% high macronutrient diet, and a 50/50 ratio of low/high macronutrient diet, at an algal cell density of 40 cells/ $\mu\text{L}$ . The diets consisted of equal numbers of cells of diatoms to flagellates of 3 unicellular algal species, namely T-Iso, *Chaetoceros muelleri*, and *Thalassiosira weissflogii*. Controls with no spat were set up for each treatment at the beginning of the experiment. Two samples of each algal species grown under high and low macronutrient concentrations were taken at the end of the experiment for lipid and carbohydrate analysis. Clearance rates were calculated daily using previously described methods.

#### 2.10.3 Larval diets of algae harvested from continuous culture at high and low macronutrient concentrations and high and low temperature regimes

The effect of algae grown under conditions of high and low macronutrient concentrations and high and low temperature regimes on the subsequent growth of larvae was investigated. Approximately 200,000 larvae were placed in each of twelve 250 L conical tanks. Larvae were batch fed four logarithmic phase diets grown under conditions

of high and low macronutrient concentrations and high and low temperature regimes (3 replicates of each treatment), namely a 50/50 ratio of low macronutrient-high temperature /high macronutrient-high temperature diet, a 50/50 ratio of low macronutrient-high temperature/high macronutrient-low temperature diet, a 50/50 ratio of low macronutrient -low temperature/high macronutrient-high temperature diet, and a 50/50 ratio of low macronutrient-low temperature /high macronutrient-low temperature diet. The diets were fed at an initial algal cell density of 16 cells/ $\mu\text{L}$  and the density was increased as the larvae grew, up to a density of 30 cells/ $\mu\text{L}$ . The diets consisted of equal numbers of cells of diatoms to flagellates of 3 unicellular algal species, namely T-Iso, *Chaetoceros muelleri*, and *Thalassiosira pseudonana*.

The temperature of the algal culture bags was recorded twice weekly. One sample of each algal species grown under conditions of high and low macronutrient concentrations and high and low temperature regimes was taken at the end of the experiment for lipid analysis.

#### **2.11 *Tetraselmis suecica*, *Thalassiosira weissflogii* and T-Iso fed to spat singly and in combination**

Based on recent hatchery experience, where improved growth of spat was obtained by the addition of *Thalassiosira weissflogii* to the standard diet, a growth trial was conducted with *Tetraselmis suecica*, *Thalassiosira weissflogii*, and T-Iso fed to spat singly and in combination to determine the effect on subsequent growth of spat and seek evidence for possible selectivity. Spat ranging in shell height from 0.75 mm - 1.4 mm were calcein-stained and 150 spat were placed in each of 15 (3 replicates of 5 treatments) 4 L plastic buckets each containing 4 L of seawater and air stones, and partly submerged in a water bath. The 5 treatments consisted of spat being batch fed 40 cells/ $\mu\text{L}$  of logarithmic phase algae grown under conditions of low macronutrient concentration. The five diets consisted of a 100% T-Iso diet, a 100% *Tetraselmis suecica* diet, a 100% *Thalassiosira weissflogii* diet, a 50/50 ratio of T-Iso/*Tetraselmis suecica* diet, and a 50/50

ratio T-Iso/*Thalassiosira weissflogii* diet, based on cell count. A control bucket for each treatment was set up containing no spat. Algal cell densities were calculated daily using a Coulter Multisizer II to determine clearance and ingestion rates and seek evidence for selectivity, i.e. the preferential selection of an algal species from mixed diets. Plots obtained using a Coulter Multisizer II provided information pertaining to cell densities of each algal species in the 50/50 ratio diets. By overlaying plots from each successive time interval and subtracting algal cell densities while allowing for settling of the algal cells in the control bucket over the same interval, clearance rate, ingestion rate and selectivity (number and volume of each species selected) were determined. Clearance rate was calculated using the equation of Coughlan (1969) and ingestion rate using the equation of Hollett (1989).

Samples of each algal species were taken at the end of the experiment for lipid and carbohydrate analysis (2 samples of each species). Total lipid and total carbohydrate actually ingested from each algal species of the 50/50 ratio diets by spat per hour were obtained by multiplying the number of cells of each species ingested by the corresponding amount of lipid and carbohydrate per cell.

## **2.12 Lipid analysis of algae**

Extraction and lipid analytical procedures were based on Parrish (1987), and conducted at the Ocean Sciences Centre, Logy Bay, Newfoundland. Algae were sampled from 100 mL volumes of each culture by filtering onto precombusted 47 mm diameter glass-fibre filters (Whatman GF/C). The filters were stored in chloroform at  $-20^{\circ}\text{C}$  under nitrogen in glass vials until analysed. Total lipids were extracted with a mixture of chloroform and methanol (2:1 v/v). Lipid classes were measured by analysing an aliquot of the total lipid extract on Chromarod S-III silica rods with an Iatroscan MK III TH-10 TLC-FID analyser. The solvent system used for the lipid separation was hexane-diethyl ether-acetic acid (60/17/0.5, v/v/v) which resolves triacylglycerols and free fatty acids from the common neutral lipid classes. Peaks were identified by comparison with chromatograms of standards.

### **2.13 Carbohydrate analysis of algae**

Dissolved carbohydrate was measured using the phenol-sulphuric acid method (Strickland and Parsons, 1972). Algae were sampled from 100 mL volumes of each culture by filtering onto 47 mm diameter glass-fibre filters (Whatman GF/C). The filters were stored at -20°C in glass vials until analysed. Five millilitres of boiling distilled water were added to each vial. Filters were ground with a glass rod. The rod was rinsed with 1 mL boiling distilled water. Vials were centrifuged for 2 minutes at 500 rpm. One millilitre of sample was removed from each vial and diluted to 50 mL distilled water in an Erlenmeyer flask. Two millilitres of sample were removed from each flask and placed in an additional Erlenmeyer flask. Two millilitres of phenol reagent (25 g of phenol dissolved in 500 mL of distilled water) were added to each flask containing the 2 mL samples using an automatic pipette. Ten millilitres of sulphuric acid reagent (2.5 g of hydrazine sulphate dissolved in 500 mL concentrated sulphuric acid) were added rapidly to each flask using a pipette with the tip cut off. The flasks were cooled by allowing to stand for one hour at room temperature. The absorbances of the solutions were measured in a spectrophotometer using a 1 cm cell, at 485 nm. The concentration of unknown carbohydrate was determined graphically using a standard curve generated by plotting D-glucose standard solutions against corresponding absorbances.

### **2.14 Statistical analysis**

Variability between replicate tanks of the same treatment and between different treatments for each experiment was analysed using nested one-way Anova followed by Tukey's analysis if a significant difference existed between treatments.

Student t-tests were conducted for the larval experiments carried out in 7000 L hatchery production tanks. No replicate tanks were used during these experiments. A t-test was conducted for the algal growth phase spat experiment where only one size range was available with no replication. Student t-tests were also conducted on the proportion of each species ingested by number and volume, during the selectivity experiment.

The criterion for statistical significance in all analyses was  $p \leq 0.05$ .

Correlation coefficients of lipid class composition of the diet and shell growth rate and total carbohydrate and shell growth rate were obtained using Pearson correlation analysis. The criterion for statistical significance was  $p \leq 0.01$ .

All analyses were performed using the SPSS statistical package.



## Chapter 3

### Results

#### **3.1 Spat staining with calcein**

Seventy-two hours were required for immersion of spat in calcein solution to obtain a strong fluorescent band (Figure 1). Survival of the calcein stained spat and control spat was 99% and 100% respectively.

#### **3.2 Algal cell density and spat growth**

Nested one-way analysis of variance showed that growth rates of spat did not differ significantly at different algal cell densities ( $F_{(4,5)} = 3.824$ ,  $p > 0.05$ ) (Figure 2).

Clearance rates of spat differed significantly at different algal cell densities (Nested Anova,  $F_{(4,5)} = 45.500$ ,  $p < 0.05$ ), with replicate tanks at the same cell density not differing significantly (Nested Anova,  $F_{(5,190)} = 0.155$ ,  $p > 0.05$ ). Data from replicates were pooled and Tukey's analysis showed that clearance rate was higher at 0 cells/ $\mu$ L than at the other cell concentrations (Figure 3).

Ingestion rates of spat differed significantly when spat were fed different algal cell densities (Nested Anova,  $F_{(4,5)} = 68.281$ ,  $p < 0.05$ ) with replicate tanks at the same cell density not differing significantly (Nested Anova,  $F_{(5,190)} = 0.367$ ,  $p > 0.05$ ). Data from replicates were pooled and Tukey's analysis showed that ingestion rate increased as the algal cell density increased (Figure 4).

#### **3.3 Attempted improvement of standard hatchery diet by addition of algal species**

##### **3.3.1 *Chaetoceros calcitrans* in larval and spat diets**

Neither larval (t-test,  $p > 0.05$ ) nor spat. (Nested Anova,  $F_{(1,2)} = 17.430$ ,  $p > 0.05$ ) growth were significantly affected by diets including and excluding the algal species

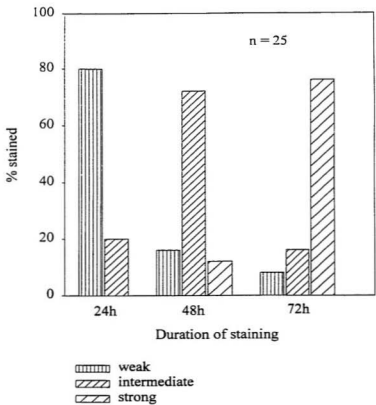


Figure 1. Staining of scallop spat (0.5 mm - 2 mm shell height) with calcein.

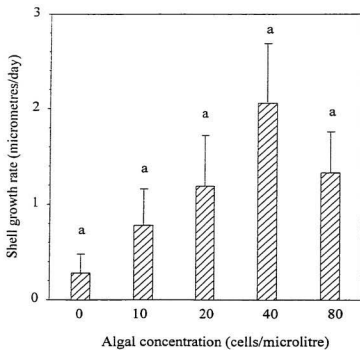


Figure 2. Growth of *Placopecten* spat fed a diet of *Isochrysis galbana*, T-Iso, *Chroomonas salina*, *Tetraselmis suecica*, *Thalassiosira pseudonana* and *Chaetoceros muelleri* at various cell densities. [Mean + SE, n = 30] Common letter denotes no significant difference at  $p > 0.05$ .

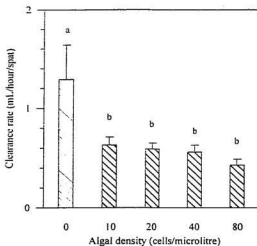


Figure 3. Clearance rate of *Placopecten* spat fed a diet of *Thalassiosira pseudonana*, *Chaetoceros muelleri*, *Tetraselmis suecica*, *Chroomonas salina*, *Isochrysis galbana* and T-Iso at various cell densities.

Common letter denotes no significant difference at  $p > 0.05$ . [Mean + SE,  $n = 20$ ]

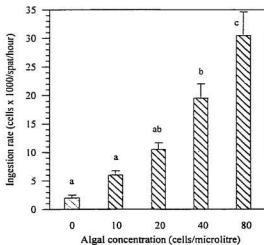


Figure 4. Ingestion rate of *Placopecten* spat fed a diet of *Thalassiosira pseudonana*, *Chaetoceros muelleri*, *Tetraselmis suecica*, *Chroomonas salina*, *Isochrysis galbana* and T-Iso at various cell densities.

Common letter denotes no significant difference at  $p > 0.05$ . [Mean + SE,  $n = 20$ ]

*Chaetoceros calcitrans* (Figures 5 and 6). Survival of spat did not significantly differ between the two diets in each case.

### 3.3.2 *Tetraselmis suecica* and *Chroomonas salina* in spat diets

Nested one-way analysis of variance showed that growth rates of spat did not significantly differ when fed four different diets ( $F_{(3,4)} = 0.090$ ,  $p > 0.05$ ) (Figure 7). Survival of spat did not significantly differ between the four diets examined, and ranged from 98% to 100%.

## **3.4 Algal growth phase**

### 3.4.1 Larvae

In three out of four experiments conducted, larvae fed logarithmic phase algae were significantly larger at the end of the experiments than those fed near-stationary phase algae (t-test,  $p < 0.05$ ) (Figure 8).

### 3.4.2 Spat

Results of the first experiment showed that for the size range 0.3 mm - 0.5 mm, growth rates of spat differed significantly between replicate tanks (Nested Anova,  $F_{(2,156)} = 4.650$ ,  $p < 0.05$ ). Data from replicates were not pooled and Tukey's analysis showed that spat fed logarithmic phase algae grew faster than those fed near-stationary phase algae, with growth rates differing significantly between replicates 1 of both diets ( $p < 0.05$ ). For the size range 1.4 mm - 1.7 mm, growth rates did not differ significantly between diets (Nested Anova,  $F_{(1,2)} = 7.209$ ,  $p > 0.05$ ) or between replicate tanks of the same diet (Nested Anova,  $F_{(2,156)} = 0.069$ ,  $p > 0.05$ ) (Figure 9a).

Results of the second experiment showed that for the size range 0.5 mm - 0.75 mm, growth rates did not significantly differ between diets (Nested Anova,  $F_{(1,2)} = 0.426$ ,  $p > 0.05$ ) or between replicate tanks of the same diet (Nested Anova,  $F_{(2,196)} = 1.855$ ,  $p > 0.05$ ). For the size range 0.75 mm - 1.4 mm, growth rates did differ significantly between

replicate tanks of the same diet (Nested Anova,  $F_{(2,196)} = 24.580$ ,  $p < 0.05$ ). Data from replicates were not pooled and Tukey's analysis showed that spat fed near-stationary phase algae grew significantly faster than those fed logarithmic phase algae ( $p < 0.05$ ) (Figure 9b).

Results of the third experiment showed that for the size range 0.5 mm -0.75 mm, growth rates differed significantly between replicate tanks of the same diet (Nested Anova,  $F_{(2,196)} = 62.580$ ,  $p < 0.05$ ). Data from replicates were not pooled and Tukey's analysis showed that spat fed logarithmic phase algae grew faster than those fed near-stationary phase algae, with both replicates of the near-stationary diet being significantly different from the second replicate of the logarithmic diet. For the size range 0.75 mm - 1.4 mm, growth rates did not significantly differ between replicate tanks of the same diet (Nested Anova,  $F_{(2,196)} = 0.690$ ,  $p > 0.05$ ), but did differ significantly between diets, with spat fed logarithmic phase algae growing faster than those fed near-stationary phase algae (Nested Anova,  $F_{(1,2)} = 283.570$ ,  $p < 0.05$ ) (Figure 9c). The fourth experiment illustrated that spat fed logarithmic phase algae grew significantly faster than those fed near-stationary phase algae (one size range 1.4 mm -1.7 mm) (t-test,  $p < 0.05$ ) (Figure 9d).

Clearance rates of spat did not significantly differ between diets or between replicate tanks of the same diet, for each size range, during all three experiments examined (Nested Anova,  $p > 0.05$ ) (Figure 10). Survival did not significantly differ between diets, for each size range, during all four experiments. Survival ranged from 97% to 100%. The different size ranges examined during three of the experiments were not compared to one another.

### **3.5 Algal macronutrient concentration**

#### **3.5.1 Larval diets of algae harvested from continuous culture at high and low macronutrient concentrations**

Nested one-way analysis of variance showed that final larval shell heights differed significantly when larvae were fed four different diets ( $F_{(3,8)} = 46.620$ ,  $p < 0.05$ ) and that shell heights of larvae in replicate tanks fed the same diet did not significantly differ

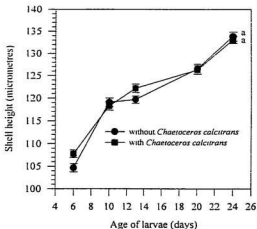


Figure 5. The effect of adding *Chaetoceros calcitrans* to a standard diet (*Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana* and T-Iso) on growth of *Placopecten* larvae.

Common letter denotes no significant difference at  $p > 0.05$ . [Mean  $\pm$  SE,  $n = 50$ ]

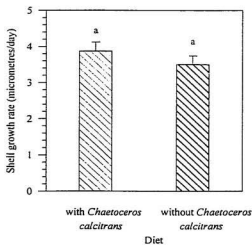


Figure 6. The effect of adding *Chaetoceros calcitrans* to a standard diet (*Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana* and T-Iso) on growth of *Placopecten* spat.

Common letter denotes no significant difference at  $p > 0.05$ . [Mean  $\pm$  SE,  $n = 50$ ]

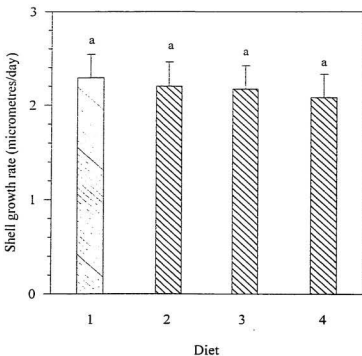


Figure 7. The effect of adding *Tetraselmis suecica* and *Chroomonas salina* to a standard diet (*Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana* and T-Iso) on growth of *Placopecten spat.*  
Common letter denotes no significant difference at  $p > 0.05$ . [Mean + SE, n = 30]

- 1 = standard diet
- 2 = standard + *Tetraselmis suecica* + *Chroomonas salina* diet
- 3 = standard + *Tetraselmis suecica* diet
- 4 = standard + *Chroomonas salina* diet



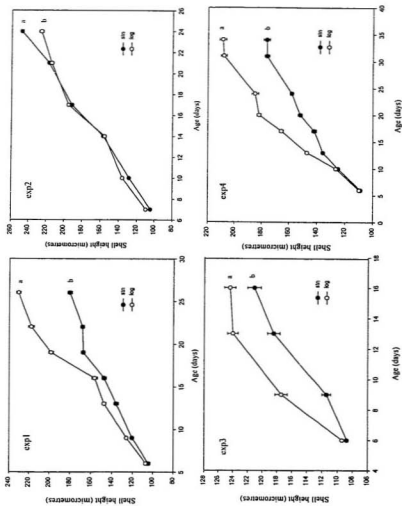


Figure 8. Growth of *P. pacificus* larvae on diets of mixed algae harvested from cultures maintained in log and near stationary phase. [Mean  $\pm$  SE,  $n=50$ ]. Common letter denotes no significant difference at  $p > 0.05$ .

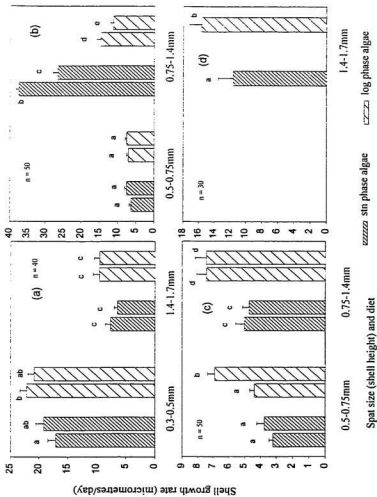


Figure 9. Growth of *Placopecten* spat on diets of mixed algae harvested from cultures maintained in log and near stationary phase. [Mean + SE]  
 Common letter denotes no significant difference at  $p > 0.05$ .

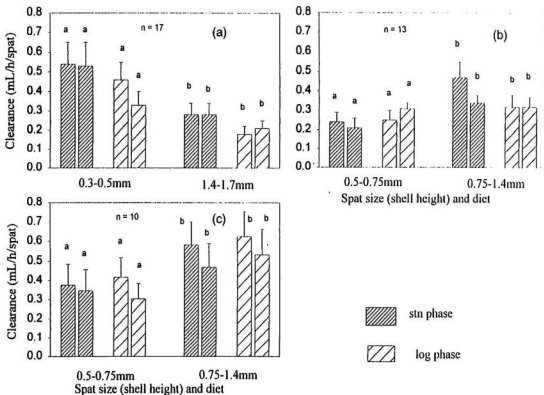


Figure 10. Clearance rate of *Placopecten* spat grown on diets of mixed algae harvested from cultures maintained in log and near stationary phase. [Mean + SE] Common letter denotes no significant difference at  $p > 0.05$ .

(Nested Anova,  $F_{(8,348)} = 1.892$ ,  $p > 0.05$ ). Data from replicate tanks were pooled and Tukey's analysis showed that larvae fed the 100% low macronutrient diet were significantly smaller at the end of the experiment than those fed the other three treatments (Figure 11). A significant difference was found between the 100% low macronutrient diet and the other three diets (Tukey,  $p < 0.05$ ). Shell heights of larvae fed the other three diets did not significantly differ from one another.

### 3.5.2 Spat diets of algae harvested from continuous culture at high and low macronutrient concentrations

Growth rates of spat differed significantly when fed three different diets (Nested One-Way Anova,  $F_{(2,6)} = 20.310$ ,  $p < 0.05$ ). Growth rates of spat in replicate tanks fed the same diet did not differ significantly (Nested One-Way Anova,  $F_{(6,171)} = 0.870$ ,  $p > 0.05$ ). Data from replicate tanks were pooled and Tukey's analysis showed that spat fed the 100% low macronutrient diet grew significantly faster than those fed the other 2 treatments (Figure 12). Growth rates of spat fed the three different diets significantly differed from one another ( $p < 0.05$ ).

There were no significant differences between clearance rates of spat in replicate tanks or between clearance rates of spat fed different diets (Nested One-Way Anova,  $p > 0.05$ ) (Figure 13).

### 3.5.3 Larval diets of algae harvested from continuous culture at high and low macronutrient concentrations and high and low temperature regimes

Shell heights differed significantly in larvae fed four different diets (Nested One-Way Anova,  $F_{(3,8)} = 127.225$ ,  $p < 0.05$ ). Shell heights of larvae in replicate tanks fed the same diet did not differ significantly (Nested Anova,  $F_{(8,348)} = 1.183$ ,  $p > 0.05$ ). Data from replicate tanks were pooled and Tukey's analysis showed that larvae fed the 50/50 ratio of low macronutrient/high temperature:high macronutrient/low temperature diet were larger at the end of the experiment than those fed the other three treatments (Figure 14). A significant difference was found between larvae fed the 50/50 ratio of low

macronutrient/high temperature:high macronutrient/low temperature diet and the other three diets (Tukey,  $p < 0.05$ ). Shell heights of larvae fed the other three diets did not significantly differ from one another.

Low temperature algal cultures averaged approximately 22°C throughout the experiment, and high temperature algal cultures approximately 26°C.

### **3.6 *Tetraselmis suecica*, *Thalassiosira weissflogii* and T-Iso fed to spat singly and in combination**

Growth rates of spat differed significantly when fed five different diets (Nested One-Way Anova,  $F_{(4,10)} = 338.220$ ,  $p < 0.05$ ). Growth rates of spat in replicate tanks fed the same diet did not differ significantly (Nested Anova,  $F_{(10,435)} = 1.038$ ,  $p > 0.05$ ). Data from replicate tanks were pooled and Tukey's analysis showed that spat fed the 100% *Thalassiosira weissflogii* diet, and the 50:50 ratio of *Thalassiosira weissflogii* : T-Iso diet, grew significantly faster than those fed the other 3 treatments (Figure 15). Spat fed the 100% *Thalassiosira weissflogii* diet and the 50:50 ratio *Thalassiosira weissflogii*/T-Iso diet did not significantly differ from each other, but significantly differed from the other 3 treatments. Spat fed the diet 100% T-Iso differed significantly from those fed the 100% *Tetraselmis suecica* diet and the 50:50 ratio *Tetraselmis suecica*/T-Iso diet ( $p < 0.05$ ).

There was a significant difference between clearance rates of spat fed five different diets (Nested Anova,  $p < 0.05$ ), but there was no significant difference between clearance rates of spat from replicate tanks. Data from replicate tanks were pooled and Tukey's analysis showed that clearance rate was highest for spat fed the diets of 100% T-Iso and the 50:50 ratio *Thalassiosira weissflogii*/T-Iso. Clearance rate was lowest for spat fed diets of 100% *Tetraselmis suecica* and the 50:50 ratio of *Tetraselmis suecica*/T-Iso (Figure 16). Clearance rate did not differ significantly for spat fed the diets 100% *Thalassiosira weissflogii*, 100% T-Iso and the 50:50 ratio of *Thalassiosira weissflogii*/T-

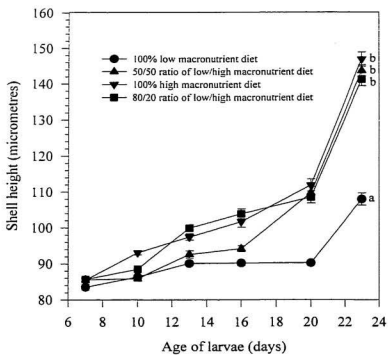


Figure 11. Effect of diets of algae cultured under conditions of high and low macronutrient concentrations on growth of *Placopecten* larvae. Common letter denotes no significant difference at  $p > 0.05$ . [Mean  $\pm$  SE,  $n = 30$ ]

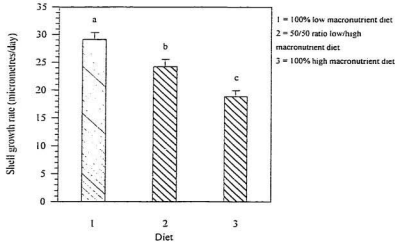


Figure 12. Effect of diets of algae cultured under high and low macronutrient concentrations on growth of *Placopecten* spat. Common letter denotes no significant difference at  $p > 0.05$ . [Mean + SE,  $n = 20$ ]

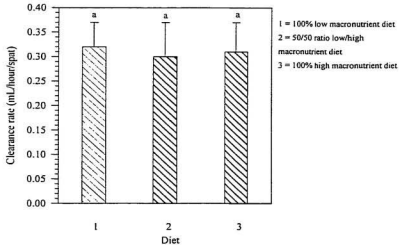


Figure 13. Clearance rate of spat fed diets of algae cultured under conditions of high and low macronutrient concentrations. Common letter denotes no significant difference at  $p > 0.05$ . [Mean + SE,  $n = 6$ ]

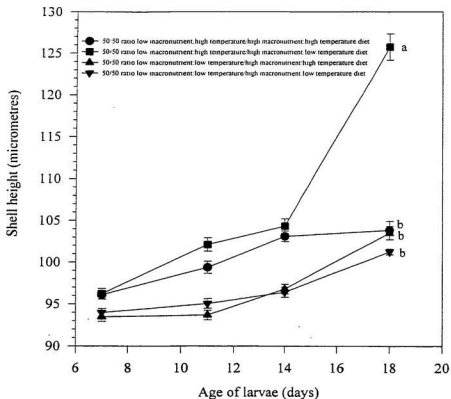


Figure 14. Effect of diets of algae cultured under conditions of high and low macronutrient concentrations and high and low temperature regimes on growth of *Placopecten* larvae. Common letter denotes no significant difference at  $p > 0.05$ . [Mean $\pm$ SE, n=30]



Iso ( $p > 0.05$ ). Clearance rate for spat fed the diet 100% T-Iso did significantly differ from that of spat fed the diet 100% *Tetraselmis suecica*, and clearance rate for spat fed the 100% *Tetraselmis suecica* diet and the 50:50 ratio of *Tetraselmis suecica*/T-Iso, in turn, significantly differed from the clearance rate of spat fed the 50:50 ratio of *Thalassiosira weissflogii*/T-Iso. Survival of spat did not significantly differ between the five treatments examined, ranging from 97% to 100%.

The proportion of each species ingested (by number and volume) when fed the 50:50 ratio diet of *Tetraselmis suecica*/T-Iso and the 50:50 ratio diet of *Thalassiosira weissflogii*/T-Iso was determined from Coulter plots of particle number and volume, an example of which is given in Figure 17 (a) and (b). Results showed that no significant difference existed between replicate tanks (Nested Anova,  $p > 0.05$ ). Spat ingested significantly ( $p < 0.05$ ) more T-Iso than either *Tetraselmis suecica* or *Thalassiosira weissflogii* by cell number (Figures 18 and 19). By cell volume, spat did not ingest significantly more ( $p > 0.05$ ) T-Iso than *Tetraselmis suecica* (Figure 20). Ingestion did significantly differ by volume between T-Iso and *Thalassiosira weissflogii*, with spat ingesting more *Thalassiosira weissflogii* (Figure 21).

Ingestion rates of *Placopecten* spat of each algal species of the 50/50 ratio T-Iso /*Tetraselmis suecica* and T-Iso/*Thalassiosira weissflogii* diets over the entire duration of the feeding trial, obtained using Multisizer plots, are illustrated in Figure 22. Spat ingested relatively little *Tetraselmis suecica* over the duration of the feeding trial, whereas a sharp increase in ingestion of *Thalassiosira weissflogii* was observed between days 8 and 10 of the trial.

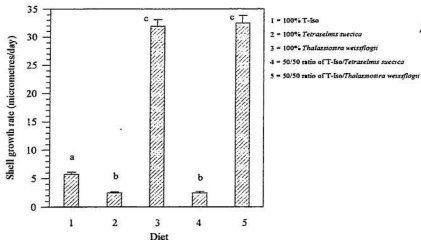


Figure 15. Effect of feeding T-Iso, *Tetraselmis suecica* and *Thalassiosira weissflogii* singly and in combination on growth of *Placopecten* spat.

Common letter denotes no significant difference at  $p > 0.05$ .

[Mean + SE, n = 30]

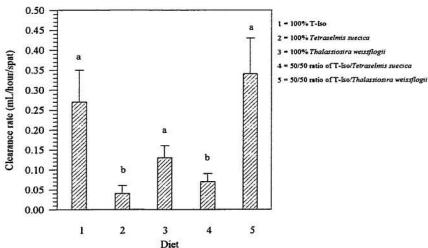


Figure 16. Clearance rate of *Placopecten* spat fed T-Iso, *Tetraselmis suecica* and *Thalassiosira weissflogii* singly and in combination.

Common letter denotes no significant difference at  $p > 0.05$ .

[Mean + SE, n = 5]

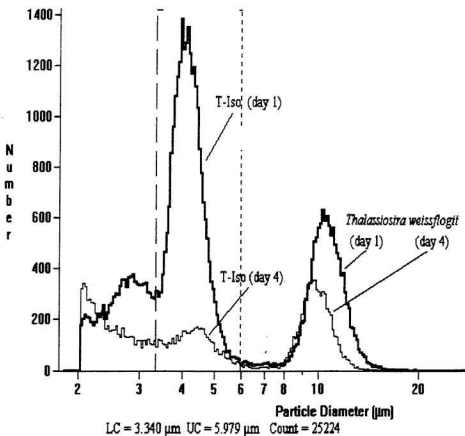


Figure 17 (a). Coulter Multisizer plots showing the initial number of cells in a 50/50 ratio diet of T-Iso/*Thalassiostraea weissflogii* and after 4 days in a feeding trial. (LC = lower limit, UC = upper limit of range counted)

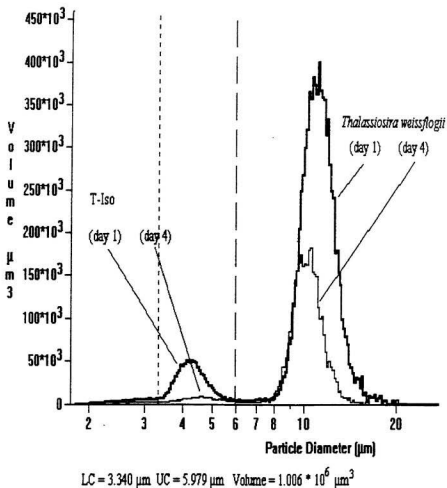


Figure 17 (b). Coulter Multisizer plots showing the initial volume of cells in a 50/50 ratio diet of T-Iso/*Thalassiosira weissflogii* and after 4 days in a feeding trial. (LC = lower limit, UC = upper limit of range for volume determination)

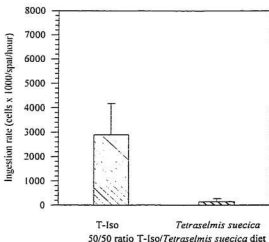


Figure 18. Number of algal cells of each species in the 50/50 ratio of T-Iso/*Tetraselmis suecica* diet ingested by *Placopecten* spat per hour. Data from multisizer plots. [Mean + SE, n = 5]

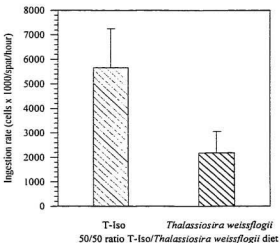


Figure 19. Number of algal cells of each species in the 50/50 ratio of T-Iso/*Thalassiosira weissflogii* diet ingested by *Placopecten* spat per hour. Data from multisizer plots. [Mean + SE, n = 5]

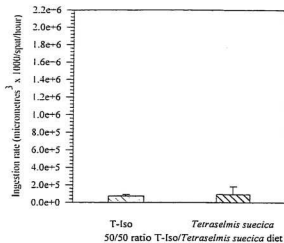


Figure 20. Volume of algal cells of each species in the 50/50 ratio of T-Iso/*Tetraselmis suecica* diet ingested by *Placopecten* spat per hour. Data from multisizer plots. [Mean + SE, n = 5]

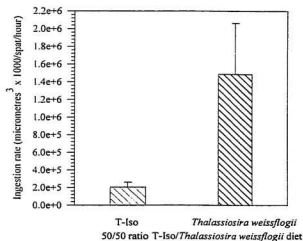


Figure 21. Volume of algal cells of each species in the 50/50 ratio of T-Iso/*Thalassiosira weissflogii* diet ingested by *Placopecten* spat per hour. Data from multisizer plots. [Mean + SE, n = 5]

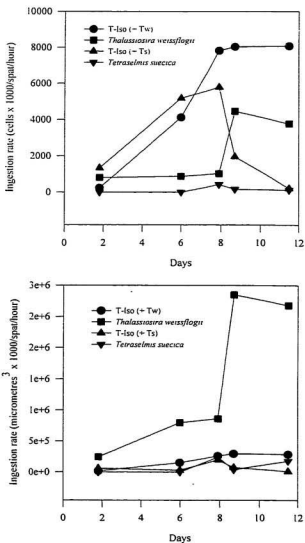


Figure 22. Ingestion rates of *Placopecten* spat fed 50/50 ratio T-Iso/*Tetraselmis suecica* and T-Iso/*Thalassiosira weissflogii* diets over the duration of the feeding trial.

Tw= *Thalassiosira weissflogii* and Ts= *Tetraselmis suecica*

### **3.7 Lipid analysis of algae**

#### **3.7.1 Larval diets of algae harvested from continuous culture at high and low macronutrient concentrations and high and low temperature regimes**

Lipid class composition of the diets is illustrated in Figure 23. Concentrations of lipid classes in each algal species used in each diet are shown in Table 2. Correlation of lipid class composition of the diets and shell height illustrated that hydrocarbons, sterol/wax esters, glyceryl ethers, alcohols, sterols, acetone mobile polar lipids, phospholipids, total lipids, total lipids without hydrocarbons and total acyl lipids were positively correlated with shell height ( $p < 0.01$ ). Methyl esters, ethyl ketones, methyl ketones, free fatty acids and diacylglycerols were negatively correlated with shell height ( $p < 0.01$ ). Triacylglycerols were not significantly correlated with shell height ( $p > 0.01$ ) (Table 3).

#### **3.7.2 Spat diets of algae harvested from continuous culture at high and low macronutrient concentrations**

The lipid class compositions of the diets are illustrated in Figure 24. Concentrations of lipid classes in each algal species used in each diet are shown in Table 4. Correlation of lipid class composition of the diets and shell growth rate illustrated that hydrocarbons, methyl esters, methyl ketones, glyceryl ethers, triacylglycerols, free fatty acids, alcohols, sterols, acetone mobile polar lipids, phospholipids, total lipids, diacylglycerols, total acyl lipids and total lipids without hydrocarbons were positively correlated with shell growth rate ( $p < 0.01$ ). Sterol/wax esters and ethyl ketones were negatively correlated with shell growth rate ( $p < 0.01$ ) (Table 3).

#### **3.7.3 *Tetraselmis suecica*, *Thalassiosira weissflogii* and T-Iso fed to spat singly and in combination**

The lipid class compositions of the diets are illustrated in Figure 25. The concentrations of lipid classes in each algal species used in each diet are shown in Table 5.



Correlation of lipid class composition of the diets and shell growth rate illustrated that methyl esters, triacylglycerols, free fatty acids, alcohols, phospholipids, acetone mobile polar lipids, total acyl lipids, total lipids and total lipids without hydrocarbons were positively correlated with shell growth rate ( $p < 0.01$ ). Hydrocarbons, sterol/wax esters, ethyl ketones and diacylglycerols were negatively correlated with shell growth rate ( $p < 0.01$ ). Sterols were not significantly correlated with shell growth rate ( $p > 0.01$ ) (Table 3).

Total lipid, without hydrocarbon, actually ingested from each algal species of the 50/50 ratio T-Iso/*Tetraselmis suecica* and T-Iso/*Thalassiosira weissflogii* diets by spat per hour is illustrated in Figures 26 and 27. Spat ingested relatively the same amount of lipid from T-Iso and *Tetraselmis suecica* in the 50/50 ratio diet (t-test,  $p > 0.05$ ). However, seven times more lipid was ingested from *Thalassiosira weissflogii* than T-Iso in the 50/50 ratio diet. Total lipid ingested was positively correlated with shell growth rate ( $p < 0.05$ ).

### **3.8 Carbohydrate analysis of algae**

#### **3.8.1 Spat diets of algae harvested from continuous culture at high and low macronutrient concentrations**

Total carbohydrate in each diet is shown in Figure 2.8. The concentration of carbohydrate in each algal species used in each diet is shown in Table 6. The correlation of carbohydrate in the diets and shell growth rate illustrated that total carbohydrate and shell growth rate were positively correlated ( $p < 0.01$ ).

#### **3.8.2 *Tetraselmis suecica*, *Thalassiosira weissflogii* and T-Iso fed to spat singly and in combination**

Total carbohydrate in each diet is shown in Figure 2.9. The carbohydrate concentration in each algal species used in each diet is shown in Table 7. Total

carbohydrate in the diets and shell growth rate were found to be negatively correlated ( $p < 0.01$ ).

Total carbohydrate actually ingested from each algal species of the 50/50 ratio T-Iso/*Tetraselmis suecica* and T-Iso/*Thalassiosira weissflogii* diets by spat per hour is illustrated in Figures 30 and 31. Relatively the same amount of carbohydrate was ingested by spat, from T-Iso and *Tetraselmis suecica* in the 50/50 ratio diet (t-test,  $p > 0.05$ ). Whereas, five times more carbohydrate was ingested from *Thalassiosira weissflogii* than T-Iso in the 50/50 ratio diet. Total carbohydrate ingested was found to be positively correlated with shell growth rate ( $p < 0.01$ ).

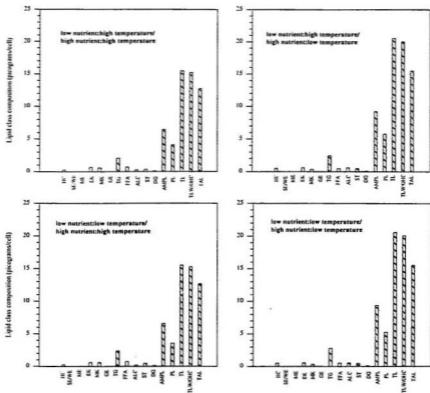


Figure 23. Lipid class composition of algal diets grown at low and high macronutrient concentrations and temperature regimes. Total diet compositions represent a combination of lipid class analyses of single algal species fed to spat.

HC = hydrocarbons  
 SE/WE = sterol/wax esters  
 ME = methyl esters  
 EK = ethyl ketones  
 MK = methyl ketones  
 GE = glyceryl ether  
 TG = triacylglycerols  
 FFA = free fatty acids

ALC = alcohols  
 ST = sterols  
 DG = diacylglycerols  
 AMPL = acetone mobile polar lipids  
 PL = phospholipids  
 TL = total lipids  
 TLW/O HC = total lipids without hydrocarbons  
 TAL = total acyl lipids

Table 2. Concentration of lipid classes in algal species cultured at low and high macronutrient concentrations and temperature regimes and fed to *Placopecten* spat.

Lipid class	Concentration (picograms/cell)					
	LN/HT 7l	LN/HT Gr	LN/HT Th	LN/LT 7l	LN/LT Gr	LN/LT Th
Hydrocarbons	0.0	0.0	0.2	0.0	0.0	0.2
Methyl esters	0.0	0.0	0.0	0.0	0.0	0.0
Methyl ketones	0.0	0.0	0.0	0.0	0.0	0.0
Glycerol ethers	0.0	0.0	0.0	0.0	0.0	0.0
Triacylglycerols	0.7	1.4	0.5	1.0	1.5	0.5
Free fatty acids	0.1	0.1	0.1	0.1	0.4	0.0
Alcohols	0.0	0.0	0.2	0.0	0.1	0.0
Sterols	0.0	0.1	0.1	0.0	0.2	0.1
Acetone mobile polar lipids	1.8	0.9	2.4	1.4	2.0	2.2
Phospholipids	1.4	1.1	0.9	0.9	1.0	1.0
Total lipids	4.3	3.7	4.5	3.8	5.3	4.1
Total lipids without hydrocarbons	4.3	3.7	4.3	3.7	5.3	4.0
Diacylglycerols	0.0	0.0	0.0	0.0	0.1	0.0
Total acyl lipids	3.9	3.3	3.8	3.3	4.5	3.7
Sterol/wax esters	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl ketones	0.2	0.0	0.0	0.2	0.0	0.0

Lipid class	Concentration (picograms/cell)					
	HN/HT 7l	HN/HT Gr	HN/HT Th	HN/LT 7l	HN/LT Gr	HN/LT Th
Hydrocarbons	0.1	0.1	0.1	0.3	0.0	0.3
Methyl esters	0.0	0.0	0.0	0.0	0.0	0.0
Methyl ketones	0.6	0.1	0.0	0.2	0.1	0.1

Glycerol ethers	0.0	0.0	0.0	0.0	0.0	0.0
Triacylglycerols	0.1	0.7	0.0	0.1	0.5	0.8
Free fatty acids	0.2	0.5	0.0	0.0	0.3	0.2
Alcohols	0.0	0.1	0.0	0.1	0.1	0.6
Sterols	0.0	0.2	0.1	0.1	0.4	0.2
Acetone mobile polar lipids	1.5	2.0	1.1	2.3	3.3	3.9
Phospholipids	0.9	1.3	0.3	1.7	1.9	1.5
Total lipids	3.8	4.8	1.6	5.0	6.7	7.6
Total lipids without hydrocarbons	3.8	4.8	1.6	4.7	6.7	7.3
Diacylglycerols	0.0	0.0	0.0	0.0	0.0	0.0
Total acyl lipids	2.5	3.9	1.4	4.0	5.8	6.3
Sterol/wax esters	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl ketones	0.4	0.0	0.0	0.3	0.0	0.0

\*Value from one analysis

LN/HT = low nutrient/high temperature

LN/LT = low nutrient/low temperature

HN/HT = high nutrient/high temperature

HN/LT = high nutrient/low temperature

71 = T-Iso

Gr = *Chaetoceros muelleri*

Th = *Thalassiostra pseudonana*

Table 3. Pearson correlation coefficients: larval shell height and spat shell growth rate vs. lipid class composition of the diets.

Lipid class	Pearson correlation coefficient		
	Exp 1	Exp 2	Exp 3
Hydrocarbons	0.361**	0.408**	-0.437**
Methyl esters	-0.578**	0.408**	0.668**
Methyl ketones	-0.406**	0.408**	a
Glyceryl ethers	0.523**	0.408**	a
Triacylglycerols	-0.004	0.408**	0.775**
Free fatty acids	-0.473**	0.408**	0.780**
Alcohols	0.441**	0.408**	0.340**
Sterols	0.220**	0.408**	-0.074
Acetone mobile polar lipids	0.353**	0.408**	0.280**
Phospholipids	0.497**	0.408**	0.309**
Total lipids	0.375**	0.408**	0.380**
Total lipids without hydrocarbons	0.375**	0.408**	0.668**
Diacylglycerols	-0.308**	0.408**	-0.644**
Total acyl lipids	0.383**	0.408**	0.669**
Sterol/wax esters	0.428**	-0.408**	-0.419**
Ethyl ketones	-0.286**	-0.408**	-0.656**

\*\*correlation is significant at the 0.01 level (2-tailed)

a = cannot be computed because at least one of the variables is constant

Exp 1 = Algae cultured under high and low macronutrient concentrations and temperature regimes and fed to scallop larvae

Exp 2 = Algae cultured under high and low macronutrient concentrations and fed to scallop spat

Exp 3 = T-Iso, *Tetraselmis suecica* and *Thalassiosira weissflogii* fed singly and in combination to scallop spat

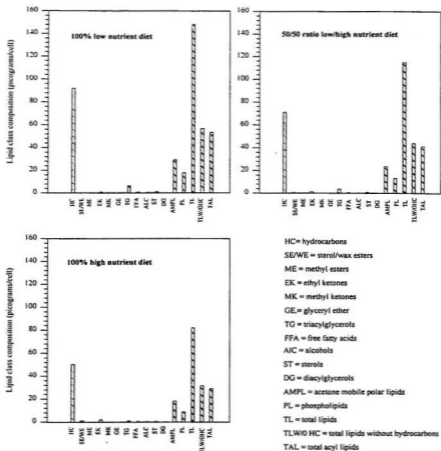


Figure 24. Lipid class composition of algal diets grown at low and high macronutrient concentrations. The total diet compositions represent a combination of lipid class analyses of single algal species fed to *Placocysten* spat.

Table 4. Concentration of lipid classes in algal species cultured at low and high macronutrient concentrations and fed to *Placopecten* spat.

Lipid class	Concentration (picograms/cell)					
	LN T-Iso	HN T-Iso	LN Cm	HN Cm	LN Tw	HN Tw
Hydrocarbons	5.6	1.6	8.2	7.1	72.5	40.1
Methyl esters	0.1	0.0	0.0	0.0	0.0	0.0
Methyl ketones	0.0	0.0	0.0	0.0	0.1	0.0
Glycerol ethers	0.0	0.0	0.0	0.0	0.0	0.0
Triacylglycerols	0.5	0.4	0.8	0.1	4.5	0.0
Free fatty acids	0.0	0.2	0.1	0.2	0.5	0.0
Alcohols	0.1	0.0	0.1	0.1	0.2	0.2
Sterols	0.0	0.0	0.1	0.2	0.9	0.4
Acetone mobile polar lipids	1.9	1.3	3.1	2.3	22.2	13.4
Phospholipids	1.6	0.9	2.3	1.0	12.2	6.1
Total lipids	9.9	4.6	14.9	10.9	113.5	61.9
Total lipids without hydrocarbons	4.4	3.0	6.8	3.8	41.1	21.8
Diacylglycerols	0.0	0.0	0.1	0.0	0.0	0.0
Total acyl lipids	4.0	2.6	6.3	3.4	38.9	20.7
Sterol/wax esters	0.0	0.0	0.0	0.0	0.0	1.2
Ethyl ketones	0.1	0.2	0.1	0.0	0.5	1.6

\*Mean values from duplicate analyses.

LN = low macronutrient concentration

HN = high macronutrient concentration

Cm = *Chaetoceros muelleri*

Tw = *Thalassiosira weissflogii*



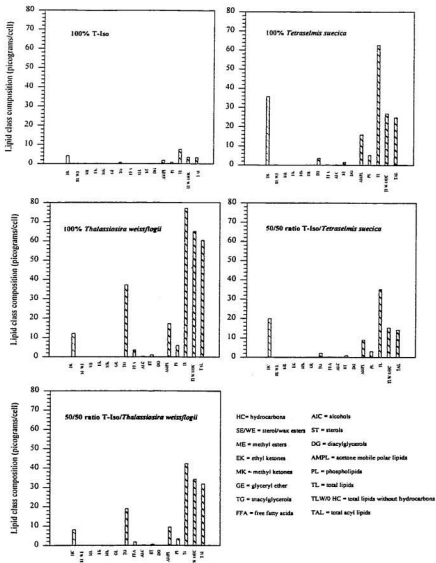


Figure 25. Lipid class composition of diets consisting of T-Iso, *Tetraselmis suecica* and *Thalassiosira weissflogii* fed to *Placocpecten* spat. Composition of 50/50 ratio diets represent a combination of lipid class analyses of single algal species.

Table 5. \*Concentration of lipid classes in algal species cultured at low macronutrient concentrations and fed to *Placopecten* spat singly and in combination.

Lipid class	*Concentration (picograms/cell)		
	T-Iso	<i>Tetraselmis suecica</i>	<i>Thalassiosira weissflogii</i>
Hydrocarbons	4.1	35.7	12.0
Methyl esters	0.0	0.0	0.1
Methyl ketones	0.0	0.0	0.0
Glyceryl ethers	0.0	0.0	0.0
Triacylglycerols	0.6	3.5	37.2
Free fatty acids	0.0	0.2	3.4
Alcohols	0.0	0.2	0.2
Sterols	0.1	1.5	1.0
Acetone mobile polar lipids	1.7	16.0	17.2
Phospholipids	0.8	5.2	5.8
Total lipids	7.5	62.6	77.0
Total lipids without hydrocarbons	3.4	26.9	65.0
Diacylglycerols	0.0	0.1	0.0
Total acyl lipids	3.2	24.9	60.3
Sterol/wax esters	0.0	0.2	0.1
Ethyl ketones	0.1	0.1	0.0

\*Mean values from duplicate analyses.

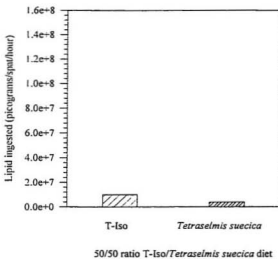


Figure 26. Total lipid (without hydrocarbon) ingested per hour from each algal species of the T-Iso/*Tetraselmis suecica* diet by *Placoepecten spat*.

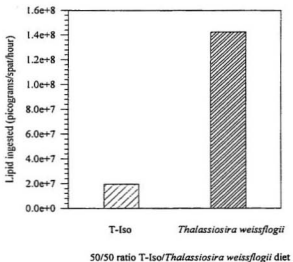


Figure 27. Total lipid (without hydrocarbon) ingested per hour from each algal species of the 50/50 ratio of T-Iso/*Thalassiosira weissflogii* diet by *Placoepecten spat*.

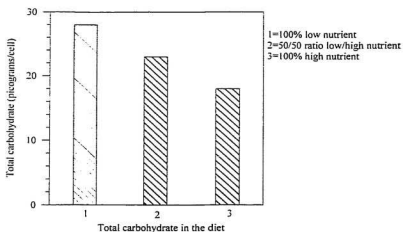


Figure 28. Total carbohydrate in algae cultured at low and high macronutrient concentration and fed to *Placopecten* spat. Total diets represent a combination of carbohydrate analyses of single algal species.

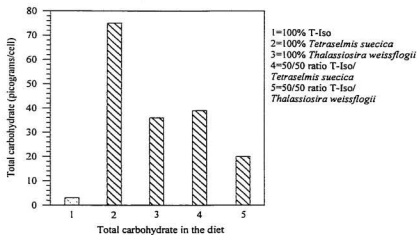


Figure 29. Total carbohydrate of T-Iso, *Tetraselmis suecica* and *Thalassiosira weissflogii* fed to *Placopecten* spat singly and in combination. 50/50 ratio diets represent a combination of carbohydrate analyses of single algal species.

Table 6. \*Concentration of total lipid and total carbohydrate in microalgae cultured at high and low macronutrient concentrations and fed to *Placopecten spat*.

Diets Species	Dry weight (picograms /cell)	Lipid (picograms /cell) (without hydrocarbon)	Lipid (% of dry weight) (without hydrocarbon)	Carbohydrate (picograms /cell)	Carbohydrate (% of dry weight) (without hydrocarbon)
<b>Low nutrient</b>					
T-Iso	69.0	4.4	6.4	4.0	5.8
<i>Thalassiosira weissflogii</i>	461.0	41.1	8.9	21.0	4.6
<i>Chaetoceros muelleri</i>	72.0	6.8	9.4	3.0	4.2
<b>High nutrient</b>					
T-Iso	41.0	3.0	7.3	2.0	4.9
<i>Thalassiosira weissflogii</i>	600.0	21.8	3.6	14.0	2.3
<i>Chaetoceros muelleri</i>	45.0	3.8	8.4	2.0	4.4

\*Mean values from duplicate analyses.

Table 7. \*Concentration of total lipid and total carbohydrate in microalgae cultured at low macronutrient concentrations and fed to *Placopecten* spat singly and in combination.

Algal species	Dry weight (picograms /cell)	Lipid (picograms /cell) (without hydrocarbon)	Lipid (% of dry weight) (without hydrocarbon)	Carbohydrate (picograms /cell)	Carbohydrate (% of dry weight)
T-Iso	69.0	3.4	5.0	3.0	4.4
<i>Tetraselmis suecica</i>	129.0	26.9	20.9	75.0	58.1
<i>Thalassiosira weissflogii</i>	461.0	65.0	14.1	36.0	7.8

\*Mean values from duplicate analyses.

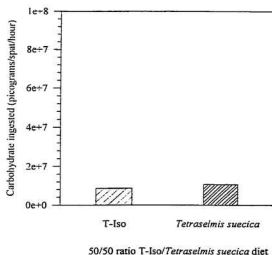


Figure 30. Total carbohydrate ingested by *Placopecten spat* per hour from each algal species of the 50/50 ratio of T-Iso/*Tetraselmis suecica* diet.

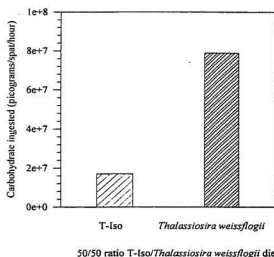


Figure 31. Total carbohydrate ingested by *Placopecten spat* per hour from each algal species of the 50/50 ratio of T-Iso/*Thalassiosira weissflogii* diet.

## Chapter 4

### Discussion

#### 4.1 Spat staining with calcein

Several chemical marking methods involve chelation or replacement of calcium during calcium carbonate deposition. The fundamental assumption of chemical marking is that neither the marking process nor the introduced chemical changes the behaviour, physiology, or biochemistry of the animals (Nielsen, 1992). High concentrations of, or long exposure to, any chemical can be harmful (Tsukamoto, 1985), so every proposed chemical marker and marking process should be carefully evaluated. It is also required that chemical marks remain identifiable on the marked animals throughout the desired interval.

Calcein has been employed in marking bones and scales of fish (Wilson et al., 1987) and in other calcified structures of invertebrate taxa such as brachiopods, bryozoans, gastropods, cephalopods, echinoids, ophiuroids, asteroids, holothuroids, polychaetes, crustaceans and bivalves (Rowley and Mackinnon, 1995). Calcein has recently been used for marking the newly-forming shell of the larvae of the giant scallop (Crocker, 1998), and it was shown that giant scallop veligers could be satisfactorily marked through immersion in the calcein stain for a minimum of 16 h at a concentration between 0.1-0.2 g calcein/L. Based on this research, a concentration of 0.15 g calcein/L was used to stain the shells of giant scallop spat during the present study and make more efficient use of the calcein. A staining trial was undertaken to determine the required duration of immersion of spat in calcein solution to obtain a strong fluorescent band under the belief that spat would require longer immersion in the calcein solution than larvae. It was found that 72 h was the required duration of immersion of spat in calcein solution to obtain strong fluorescence in comparison to 16 h for larvae. Although stronger fluorescence may have been observed after a longer immersion time, the shorter the length



of time the spat are handled and remain in the calcein solution the less chance of mortalities.

Calcein is useful in its ability to stain giant scallop spat to introduce fluorescent marker bands which can be used as benchmarks from which to measure new shell growth in subsequent short term growth trials. Calcein stained shells of scallop spat retrieved from the natural environment after over a year of being released, at shell heights of approximately 50 mm, were found to still strongly fluoresce when viewed under epifluorescence. This illustrates the potential of calcein to be used during mark and recapture experiments involving not only giant scallop larvae (Crocker, 1998) but also juveniles and possibly adults.

#### **4.2 Algal cell density and spat growth**

Within hatcheries, the cost of algal production may be as great as 40-50% of total running costs (Cropp and Frankish, 1989), emphasising the importance of optimizing the use of available food. One of the goals of this study was to determine the best cell density to feed spat in subsequent growth trials and relate cell density to clearance and ingestion rates of scallop spat. The relationship between algal cell density, clearance rate and ingestion rate observed in this study is similar to that of other filter feeding bivalves (Winter, 1978). A general trend of decreasing clearance rate with increasing algal cell density was observed during the present study. This relationship has been observed for *Mytilus edulis* larvae (Sprung, 1984), post-larval *Mytilus edulis* (Winter, 1973; Shulte, 1975), *Hiatella arctica* (Ali, 1970) and *Placopecten magellanicus* juveniles (Hollett, 1989). Owen (1974) suggested that this decrease in filtering activity at high algal densities is often due to overloading of the sorting and filtering mechanisms of the gill. Several other authors, including Sprung (1984) working with *M. edulis* larvae, and Riisgard and Randlov (1981) working with 1.6 cm - 2.0 cm *Mytilus edulis*, found that at very low algal cell densities clearance may rise sharply with increasing cell densities until a maximum rate is reached. Manning (1986) found evidence that this may occur in juvenile

*P. magellanicus*, ranging in size from 9 mm to 45 mm in height, at cell densities between 5 and 10 cells/ $\mu\text{L}$  of *Isochrysis galbana*. The same phenomenon may have been observed in this study if cell densities between 0 and 10 cells/ $\mu\text{L}$  had been examined, since clearance rate declined sharply from 0 to 10 cells/ $\mu\text{L}$  and then remained relatively constant from 10 to 80 cells/ $\mu\text{L}$ . The ability of bivalves to reduce clearance rate at very low cell densities is seen as an energy saving adaptation where the energetically expensive process of feeding is decreased at cell densities not energetically profitable to the animal (Riisgard and Randlov, 1981). This ability to reduce clearance rate at low cell density is consistent with Lehman's (1976) energy optimization model for filter feeders. Certainly this ability could potentially be advantageous to *P. magellanicus* if periodic low phytoplankton densities are encountered in its natural environment. In contrast with Lehman's energy optimization model, clearance rate was highest at 0 cells/ $\mu\text{L}$  during the present study and decreased sharply from 0 to 10 cells/ $\mu\text{L}$ . This may be due to suppression of clearance rate as a result of toxin production by one of the supplementary species. Possible toxin production may explain why clearance rate did not consistently decrease with increasing algal cell density, which is often the case in other filter feeding bivalves (Winter, 1978).

Ingestion rates generally increase with increasing algal density to a maximum value above which a further increase in algal density only serves to block the filtering mechanism and induce pseudofeces production (Palmer and Williams, 1980; Gillis, 1993). The threshold density has been reported at approximately 2 mg dry weight algae per litre which corresponds to 60-100 cells/ $\mu\text{L}$  for algae of 30-20 pg/cell respectively (Palmer and Williams, 1980). The range of densities in this study, 0-80 cells/ $\mu\text{L}$ , is thus at or below the critical threshold, possibly accounting for the reason no decrease in ingestion rate due to increased algal density was observed. Ingestion rates were found to increase with increasing algal cell density, but growth rates remained constant. This is likely due to the fact that absorption efficiency (efficiency with which the ingested ration is absorbed) typically decreases with increasing cell density (Bricelj and Shumway, 1991).

In aquaculture, the algal density fed to larvae and juveniles should be the minimum particle density which will result in maximum growth because of the high cost of growing

the algae. This study showed that growth rates of spat did not differ significantly at the five different algal densities examined. A study conducted by Hollett (1989), however, found that 45 cells/ $\mu\text{L}$  gave best growth of 2 mm *P. magellanicus* juveniles. Based on this study, and the fact that 40 cells/ $\mu\text{L}$  gave equivalent growth in comparison to the other four algal densities during the present study, spat were fed an algal cell density of 40 cells/ $\mu\text{L}$  in subsequent growth trials.

#### **4.3 Attempted improvement of standard hatchery diet by addition of algal species**

Many algal species have been evaluated as food sources for pectinid larvae and juveniles. Researchers have reported that feeding larvae and juveniles on a mixed algal diet may enhance growth as compared to feeding them unialgal diets (Helm et al., 1973; Dupuy et al., 1977; Gillis, 1993). The greater success of multialgal diets might be due to minimizing the detrimental effects of any one alga, while any nutrient deficiencies in one algal species may also be corrected by the others (Manning, 1986). Certain algae are known to provide a better diet for bivalve larvae and juveniles than others. *Isochrysis galbana*, *Tetraselmis suecica*, *Thalassiosira pseudonana*, and *Chaetoceros calcitrans* have long been recognized as good food for certain bivalve larvae and juveniles (Davis and Guillard, 1958; Walne, 1974; Helm and Millican, 1977; Ewart and Epifanio, 1981). Based on the literature, this study attempted to improve the standard hatchery diet consisting of *Chaetoceros ceratosporum*, *Chaetoceros muelleri*, *Thalassiosira pseudonana*, *Isochrysis galbana* and T-Iso by the addition of *Chaetoceros calcitrans*, *Tetraselmis suecica* and *Chroomonas salina*. However, it was found that *Tetraselmis suecica*, *Chaetoceros calcitrans*, and *Chroomonas salina* proved not to be beneficial as constituent species of a mixed algal diet. This is in contrast to other studies, e.g. Albentosa et al. (1996), which demonstrated that *Tetraselmis suecica* showed a high nutritional quality for seed of *Ruditapes decussatus*, and Walne (1970), who reported that *C. calcitrans* was a good algal species for larval oysters. Enright (1984) also found *C. calcitrans* to be the best

algal species, of those tested, for juvenile oysters, and Shumway et al. (1985) showed that the cryptomonad flagellate, *Chroomonas salina*, was preferentially absorbed by the majority of bivalves tested, including *Placopecten magellanicus*. The present study is in agreement with Epifanio (1979), who reported that *T. suecica* was a poor food for *Crassostrea virginica* juveniles, and Enright et al. (1986), who showed it to be of limited value as food for juvenile *Ostrea edulis*.

Certain factors associated with algal cells have been suggested as reasons why some algal species are better than others as a food source of molluscs, for example, cell size, cell wall composition, digestibility, toxic metabolites, gross biochemical composition and composition of polyunsaturated fatty acids (Webb and Chu, 1982). Many bivalve species have been shown to retain particles above 3 $\mu$ m diameter (Riisgard et al., 1980; Sprung, 1984), and since the three algal species examined during the present study are all larger than this, it can be assumed that all can be retained, and thus cell size cannot explain why the species was not beneficial. *Chaetoceros calcitrans* and *Tetraselmis suecica* both have rigid cell walls, possibly causing digestibility problems, which may explain why neither was beneficial as constituent species of a mixed algal diet (De Pauw, 1981). However, this does not explain why other *Chaetoceros* species are good foods for many bivalves. The inadequacy of *Tetraselmis suecica* as a food source may be due to flavour components that make these cells unpalatable to *Placopecten* spat. *Chroomonas salina*, although proving to be a good food for *Ostrea edulis*, *Crassostrea gigas*, *Crassostrea virginica*, and *Mercenaria mercenaria* juveniles (De Pauw, 1981), also failed to support growth during the present study. Because *C. salina* has no thick cell wall and produces few or no toxic metabolites (De Pauw, 1981), it would be expected to be a good food source for scallop spat, thus its biochemical composition may need to be examined to explain why it was not beneficial as a constituent species of a mixed algal diet.

#### **4.4 Algal growth phase**

Once a satisfactory ration and diet have been determined for a given bivalve species, other factors such as growth rate or phase of the harvested algae, which have

been reported to effect algal biochemical composition, can be optimized (Kreeger and Langdon, 1993; Brown et al., 1996). The biochemical composition of microalgae changes when culture parameters are changed. One such parameter is the growth phase during which the algae are harvested (Whyte, 1987). The importance of algal gross biochemical composition in the nutrition of grazing invertebrates has received some attention (Webb and Chu, 1983). The much cited paper of Parsons et al. (1961) introduced the idea that phytoplankton species are similar in gross biochemical composition when cultured under similar conditions based upon measurements of logarithmic phase algal populations. However, the preferred strategy of obtaining dense algal cultures for feeding invertebrates so that maximal yields can be achieved in limited space (Ukeles, 1980) usually results in the harvest of cultures in the stationary phase of the growth cycle. Algal populations enter the stationary phase when the limiting nutrient is depleted from the medium and two closely coupled processes occur: (1) cells demonstrate characteristics of nutrient deficiency, i.e., those processes that require the depleted nutrient slow down or stop altogether; (2) cells remain metabolically active and, although not dividing, often continue to fix carbon, accumulating carbohydrate and/or lipid (Spoehr and Milner, 1949).

Whyte (1987) compared the chemical composition in both the stationary and the exponential (logarithmic) phases of growth of the following species of algae: (1) *Thalassiosira pseudonana*, (2) *Chaetoceros calcitrans*, (3) *Chaetoceros* spp., (4) *Isochrysis galbana*, (5) T-Iso and (6) *Tetraselmis suecica*. Both species of *Isochrysis* at both stages of growth had the highest lipid levels. The diatoms examined had higher levels of total carbohydrates than did phytoflagellates. The phytoflagellates had higher total nitrogen concentrations than the diatoms, with a greater concentration observed during the stationary phase. Other studies examining the nutritive value of phytoplankton species during different growth phases show that high levels of carbohydrates, obtained from algae harvested during stationary phase, produce the best growth in juvenile oysters (*Ostrea edulis*) (Enright et al., 1986). In contrast, high dietary protein obtained from algae harvested during logarithmic phase produces best growth in juvenile mussels

(*Mytilus trossulus*) (Kreeger and Langdon, 1993). During the present study larvae were found to grow better on exponentially growing algal cultures in three out of four experiments conducted. The results of the spat experiments were inconclusive, with sometimes no significant differences found between diets, sometimes spat fed logarithmic cultures growing better than spat fed stationary cultures, and at other times spat fed stationary cultures growing better than spat fed logarithmic cultures. A very high shell growth rate was observed, however, for spat fed stationary cultures during the times they grew better than spat fed logarithmic cultures.

As in other bivalve larvae (Bayne, 1972; Holland and Spencer, 1973; Holland and Hannant, 1974), carbohydrate is a minor reserve material in the eggs, larvae and spat of scallops. In the eggs of the giant scallop, the protein:lipid:carbohydrate ratio remained constant (60:30:10) during a study conducted by Manning (1986), regardless of the conditioning treatment of the adults. When additional energy reserves were available to the adults these reserves were deposited in the eggs and partitioned equally between protein and lipid fractions. This is an indication of the importance of both protein and lipid in the successful development of scallop larvae. During the pelagic stage, scallop larvae accumulate protein very rapidly (Manning, 1986). Lipid levels, however, do not increase dramatically until the third or fourth week of development. This increase in lipid towards the end of larval life is an indication that lipid may be important in providing energy for metamorphosis in *Placopecten magellanicus* larvae (Manning, 1986).

The importance of both protein and lipid as energy sources in *Placopecten magellanicus* larvae may explain the difficulty in potentially improving the standard hatchery diet. According to Manning (1986), *Placopecten magellanicus* larvae have been observed to grow better on exponentially growing algal cultures, which generally possess high protein levels (Fogg, 1959). The present study is in agreement with this earlier study, since larvae also grew better on exponentially growing algal cultures. However, growth in the pediveliger stage and successful metamorphosis may require increased lipid levels, helping to explain the inconclusive results obtained from the spat experiment, which may illustrate a transition between the high protein requirement of early larval development and

the requirement for increased lipid during later development. A mixed diet of exponentially and stationary growing algal cultures may increase growth of scallop spat by meeting the requirements for both protein and lipid.

#### **4.5 Algal macronutrient concentration**

Changes in the biochemical composition of algal species cultured under different conditions of macronutrient concentration have long been observed (Ketchum and Redfield, 1949; Spoehr and Milner, 1949), and subsequent studies have demonstrated that microalgal species produce varying amounts of cell constituents, i.e., protein, carbohydrate, and lipid, when cultured under various conditions of macronutrient sufficiency, deficiency, and overabundance (Healey, 1975). Wikfors et al. (1984) investigated the gross chemical composition of *Dunaliella tertiolecta* and *Tetraselmis maculata*, cultured in three enriched natural seawater growth media and utilized as molluscan diets. Both species showed significant differences in carbohydrate and protein composition in the different media. The composition of these algal foods influenced growth rates of juvenile oysters, with high lipid-high carbohydrate cells yielding the most rapid oyster growth. Subsequently, Wikfors et al. (1992), cultured three algal species in different media to manipulate gross biochemical composition, and demonstrated that both dietary protein and lipid must be present in sufficient quantities to support rapid growth of hard clams. This relationship between gross chemical composition of algae and the nutrition of grazing invertebrates emphasizes one of the practical benefits of understanding how macronutrients affect the biochemistry of microalgae.

The present study investigated the effect of manipulation of algal macronutrient concentration on growth on scallop larvae. It was found that in order to obtain good growth of larvae the diet must consist of a minimum of 20% high macronutrient grown algae. Larvae fed a 100% low macronutrient diet were significantly smaller at the end of the experiment than those fed a 80/20 ratio low/high macronutrient diet, a 50/50 ratio low/high macronutrient diet, and a 100% high macronutrient diet. The last three diets did

not differ significantly with respect to larval growth. These results suggest a higher requirement for protein during early larval development, which is consistent with algae grown under high macronutrient conditions (Thomas et al., 1984; Wikfors, 1986). Protein analysis of algae was not conducted during the present study on the assumption that protein would not be limiting, although the results suggest that future investigation of how protein correlates to larval growth may be beneficial.

The effect of manipulation of algal macronutrient concentration on growth on scallop spat was also investigated, and the results illustrated that spat grew significantly faster on a 100% low macronutrient diet than a 50/50 ratio low/high macronutrient diet and a 100% high macronutrient diet. This may illustrate a higher requirement for lipid and/or carbohydrate during later postlarval development, which is consistent with results from algae grown under low macronutrient conditions (Thomas et al., 1984; Wikfors, 1986). A higher requirement for lipid and carbohydrate during later development was confirmed from lipid and carbohydrate analyses of algae. Lipid analysis showed that shell growth rate and total lipids of the diet were positively correlated, while carbohydrate analysis also illustrated that shell growth rate and total carbohydrate of the diet were positively correlated, demonstrating that spat would grow better on high lipid-high carbohydrate diets, which can be obtained by growing most algal species under low macronutrient conditions.

The combined effects of temperature and macronutrient concentration of algal cultures on larval growth were also investigated during the present study. As previously mentioned, many researchers have shown that microalgae undergo biochemical changes when the culture conditions are varied, the greatest changes being associated with low levels of nitrogen in the culture medium, causing a large decrease in microalgal protein and large increases in lipid and carbohydrate (Spoehr and Milner, 1949; Fabregas et al., 1986; Wikfors, 1986). The biochemical composition of microalgal species may also be modified by manipulating other environmental parameters such as temperature. Aaronson (1973) found that higher incubation temperatures caused an increase in lipid and protein concentrations in the chrysophyte *Onchomonas danica*, and Opute (1974) confirmed this



in the diatom *Nitzschia palea*. Temperature is also one of the more important factors influencing the fatty acids produced by microalgae (Ackman et al., 1968). Fatty acids are the fundamental structural components of practically all forms of lipids. Their long hydrocarbon chain confers on lipids their hydrophobicity. Moreover, the variability in both the number of carbon atoms and the number of double bonds determines to a large extent the physical properties of biological membranes (Spector and Yorek, 1985). It has been reported that some microalgae respond to a decrease in temperature by increasing the ratio of unsaturated fatty acids to saturated fatty acids, thereby increasing the fluidity of biomembranes (Ackman et al., 1968). Optimum nutritional value of microalgal species is associated with levels of some essential polyunsaturated fatty acids (PUFAs), in particular two members of the omega-3 group, eicosapentaenoic acid (EPA), 20:5(n-3) and docosahexaenoic acid (DHA) 22:6(n-3) (Parrish et al., 1998). The importance of these fatty acids has been demonstrated in the growth of oyster larvae (Langdon and Waldo, 1981), queen conch larvae (Pillsbury, 1985), and barramundi fingerlings, *Lates calcarifer* (Rimmer et al., 1988). A study conducted by Delaunay et al. (1993) also showed that C20 and C22 PUFAs are selectively incorporated in phospholipids of *Pecten maximus*, and indicate that larvae of this species also have an essential requirement for long chain (n-3) and (n-6) PUFAs.

It was found during the present study that scallop larvae fed a 50/50 ratio of low macronutrient/high temperature algae:high macronutrient/low temperature algae grew significantly better than those fed a 50/50 ratio of low macronutrient/high temperature algae:high macronutrient/high temperature algae, a 50/50 ratio of low macronutrient/low temperature algae:high macronutrient/high temperature algae, and a 50/50 ratio of low macronutrient/low temperature algae:high macronutrient/low temperature algae. These results may be explained in terms of the total lipid and protein supplied by each diet. Comparison of low macronutrient cultures showed that, overall, more lipid was found in those cultured under high temperature conditions. This is in agreement with the studies previously mentioned which found that higher temperatures caused increases in lipid and

protein concentrations in the algal species examined (Aaronson, 1973; Opute, 1974). Comparison of high macronutrient cultures showed more lipid was found in those cultured under low temperature conditions. Lower temperatures also increase the production of PUFAs in microalgae (Ackman et al., 1968). Thus, the superior low macronutrient/high temperature:high macronutrient/low temperature algal diet may provide a combination of increased lipid and protein obtained from high temperature cultures and an increase in PUFAs obtained from low temperature cultures, combined with an increased lipid concentration obtained from both the low macronutrient /high temperature cultures and high macronutrient/low temperature cultures. Lipid analysis of algae revealed that total lipid was positively correlated with larval shell height.

#### **4.6 Tetraselmis suecica, Thalassiosira weissflogii and T-Iso fed to spat singly and in combination**

Shumway et al. (1985) measured clearance rates of six bivalve species fed mixed cell suspensions of the algal species *Prorocentrum minimum*, *Phaeodactylum tricornutum*, and *Chroomonas salina*. Use of flow cytometry also allowed estimation of the proportional occurrence of individual cell types in the pseudofaeces and faeces. It was recognized that at least three mechanisms of selecting suspended particles may have been present in isolation or in combination. These were: (a) preferential entrainment on the ctenidia; *Ostrea edulis* preferentially cleared *Prorocentrum minimum* compared to the other two algal species; (b) preingestive selection on the labial palps; *Phaeodactylum tricornutum* was preferentially rejected in the pseudofaeces of *Ensis directus*, *Placopecten magellanicus*, and *Arctica islandica*; (c) post-ingestive selection (differential absorption in the gut); of the mixed diet that was ingested, *Chroomonas salina* was preferentially absorbed in the majority of the bivalves from which faecal material was obtained.

Based on the previous literature, a selectivity experiment involving the algal species T-Iso, *Tetraselmis suecica*, and *Thalassiosira weissflogii* was conducted during the present study to determine if spat would preferentially select one algal species over another when fed a 50/50 ratio T-Iso/*Tetraselmis suecica* diet and a 50/50 ratio T-Iso

*Thalassiosira weissflogii* diet. The improvement of the standard hatchery diet by the addition of *Thalassiosira weissflogii* was also investigated. All algal species were grown under low macronutrient conditions on the basis of results obtained previously during the spat macronutrient concentration experiment, whereby low macronutrient grown algae, containing increased amounts of lipid and carbohydrate, gave better growth of spat. Total lipid in the diet was also positively correlated with shell growth rate during the selectivity experiment, whereas total carbohydrate was negatively correlated. The negative correlation witnessed between shell growth rate and total carbohydrate of the diet was a result of examining total carbohydrate of the diet presented to spat and not the proportion which was actually ingested by spat, which resulted in a positive correlation. With respect to the attempted improvement of the standard hatchery diet, it was found that the diets of 100% *Thalassiosira weissflogii* and the 50/50 ratio of T-Iso/*Thalassiosira weissflogii* resulted in the best growth of scallop spat, possibly due to the high lipid contribution of *Thalassiosira weissflogii*. However, since synergistic growth effects of diets incorporating several algal species have been shown (Whyte et al., 1989), it would be more appropriate to feed spat several species in comparison to a unialgal diet such as one consisting of 100% *Thalassiosira weissflogii*, so that if one species is lacking in a component (or components) vital to growth and development it may be balanced by the presence of another species. However, optimizing the use of available food due to the high cost of algal production may be accomplished by varying the relative proportion of each algal species of a mixed diet.

Clearance rate did not differ significantly for spat fed the diets 100% T-Iso, 100% *Thalassiosira weissflogii*, and the 50/50 ratio of T-Iso/*Thalassiosira weissflogii*, even though the 100% T-Iso diet gave significantly less growth. This suggests that T-Iso may be lacking in a component (or components) vital to growth of scallop spat. Pillsbury (1985) showed that T-Iso lacks the polyunsaturated fatty acid 20:5(n-3), but does contain 22:6(n-3), and Romberger and Epifanio (1981) showed it to have a low carbohydrate content. Laing and Millican (1986) reported that in the lowest food value diets for *O.*

*edulis*, clearance rates were higher, associated with lower organic growth of the animals. They suggested that this may represent an attempt by the spat to obtain the required amounts of essential nutrients from the low food value diets by increasing the clearance rate, leading to a greater metabolic demand on the assimilated ration, with less energy available for organic growth. The same situation may have occurred during the present study, with the clearance rate of the 100% T-Iso diet being comparable with clearance rates of the 100% *Thalassiosira weissflogii* diet and the 50/50 ratio of *Thalassiosira weissflogii*/T-Iso diet, even though the 100% T-Iso diet resulted in significantly less growth.

*Tetraselmis suecica*, although high in carbohydrate, lacks 22:6(n-3) (Pillsbury, 1985), which may explain why the 50/50 ratio diet of T-Iso/*Tetraselmis suecica* outperformed the 100% *Tetraselmis suecica* diet. *Tetraselmis suecica* may also be a poor food for scallop spat due to digestibility problems, as previously mentioned. This may explain why the 100% T-Iso diet gave better growth than the 50/50 ratio of T-Iso/*Tetraselmis suecica* diet, and why total carbohydrate was negatively correlated with shell growth rate. Even though *Tetraselmis suecica* is high in carbohydrate it may have caused poor growth of spat because it was not ingested. Spat were also found to clear less of the 100% *Tetraselmis suecica* and the 50/50 ratio T-Iso/*Tetraselmis suecica* diets, indicating that there may be some inhibitory flavour component, possibly related to properties of the cell surface, (Ward et al., 1997) in the *Tetraselmis* cells, and that total carbohydrate of the diet was negatively correlated with growth simply because *Tetraselmis suecica* was not being cleared much by the spat. A correlation of total carbohydrate of the diet actually ingested, found by multiplying the number of cells of each algal species in the 50/50 ratio diets ingested by the concentration of carbohydrate per cell, with shell growth rate illustrated that both were positively correlated. This is in contrast to the previous results due to the swamping effect of *Tetraselmis suecica* which though containing a high concentration of carbohydrate, was simply not being cleared.

Concentrations of lipid and carbohydrate (pg/cell) in the microalgae found in the present study (Tables 6 and 7) are comparable to those found in the literature (Table 8).

Table 8. Comparison of concentrations of total lipid and total carbohydrate found in microalgae under different culturing regimes.

Species	Author	Observations	Lipid (pg/cell)	Lipid (% of dry weight)	Carbohydrate (pg/cell)	Carbohydrate (% of dry weight)
<i>Isochrysis galbana</i>	Fernandez-Reiriz et al., 1989	log phase	5.3		3.5	
<i>I. galbana</i>	Fernandez-Reiriz et al., 1989	early stationary phase	10.6		15.5	
<i>I. galbana</i>	Fernandez-Reiriz et al., 1989	late stationary phase	17.0		22.7	
<i>I. galbana</i>	Webb and Chu, 1982		7.2			
<i>I. galbana</i>	Utting, 1985	.9.8mgN/l	5.9		5.5	
<i>I. galbana</i>	Utting, 1985	0.613mgN/l	7.5		8.2	
<i>I. galbana</i>	Fabregas et al., 1986	2mMNaNO <sub>3</sub> , log phase			2.3	
<i>I. galbana</i>	Fabregas et al., 1986	2mMNaNO <sub>3</sub> , stationary phase			2.3	
<i>I. galbana</i>	Albentosa et al., 1996	early stationary phase	4.8		3.7	
<i>I. galbana</i>	Brown, 1991	late log phase	7.0		3.9	
<i>I. galbana</i>	Whyte, 1987	log phase		21.2		
<i>I. galbana</i>	Whyte, 1987	stationary phase		23.5		
<i>I. galbana</i>	Parrish et al., 1998	semi-continuous culture			7.8-8.6	

<i>Isochrysis</i> spp.	Dunstan et al., 1993	batch culture log phase	4.8			
<i>Isochrysis</i> spp.	Dunstan et al., 1993	batch culture stationary phase	7.0			
T-Iso	Wikfors et al., 1996	nitrogen deficient stationary phase	3.7		0.0	
T-Iso	Brown, 1991	late log phase	5.9		1.8	
T-Iso	Whyte, 1987	log phase		25.1		
T-Iso	Whyte, 1987	stationary phase		24.9		
<i>Tetraselmis suecica</i>	Fernandez-Reiriz et al., 1989	log phase	61.9		19.7	
<i>T. suecica</i>	Fernandez-Reiriz et al., 1989	early stationary phase	51.7		61.1	
<i>T. suecica</i>	Fernandez-Reiriz et al., 1989	late stationary phase	44.6		98.3	
<i>T. suecica</i>	Parsons et al., 1961		6.6		34.1	
<i>T. suecica</i>	Wikfors et al., 1984		18.3		43.6	
<i>T. suecica</i>	Wikfors et al., 1984		9.2		26.5	
<i>T. suecica</i>	Wikfors et al., 1984		10.6		15.5	
<i>T. suecica</i>	Utting, 1985	9.8mgN/l	29.1			
<i>T. suecica</i>	Utting, 1985	0.613mgN/l	17.9			
<i>T. suecica</i>	Fidalgo et al., 1994	log phase		10.0		4.7

<i>T. suecica</i>	Albertosa et al., 1996	early stationary phase	22.1		97.1	
<i>T. suecica</i>	Brown, 1991	late log phase	16.8		20.2	
<i>T. suecica</i>	Whyte, 1987	log phase		7.0		
<i>T. suecica</i>	Whyte, 1987	stationary phase		8.1		
<i>Chaetoceros calcitrans</i>	Fernandez-Reiriz et al., 1989	log phase	0.9		0.2	
<i>C. calcitrans</i>	Fernandez-Reiriz et al., 1989	early stationary phase	1.5		1.6	
<i>C. calcitrans</i>	Fernandez-Reiriz et al., 1989	late stationary phase	2.0		1.9	
<i>C. calcitrans</i>	Parsons et al., 1961		15.7		15.0	
<i>C. calcitrans</i>	Utting, 1985	9.8mgN/l	2.1		3.0	
<i>C. calcitrans</i>	Utting, 1985	0.613mgN/l	2.6		7.4	
<i>C. calcitrans</i>	Brown, 1991	late log phase	1.8		0.7	
<i>C. calcitrans</i>	Whyte, 1987	log phase		15.8		
<i>C. calcitrans</i>	Whyte, 1987	stationary phase		16.9		
<i>C. calcitrans</i>	Thompson et al., 1992				3.5	
<i>C. muelleri</i>	Parrish and Wangersky, 1990	nutrient replete continuous culture	2.1			
<i>C. muelleri</i>	Brown, 1991	late log phase	5.2		2.0	

<i>C. muelleri</i>	Thompson et al., 1992				0.9	
<i>C. muelleri</i>	Harrison et al., 1990		1.3-2.8			
<i>C. muelleri</i>	Parrish et al., 1998	semi-continuous culture	5.4		27.0	
<i>Thalassiosira pseudonana</i>	Brown, 1996	100 μmol/24:0h L:D cycle log phase		21.0		19.0
<i>T. pseudonana</i>	Brown, 1996	100 μmol/24:0h L:D cycle stationary phase		31.0		36.0
<i>T. pseudonana</i>	Brown, 1991	late log phase	5.5		2.5	
<i>T. pseudonana</i>	Whyte, 1987	log phase		9.8		
<i>T. pseudonana</i>	Whyte, 1987	stationary phase		14.2		



However, concentrations of lipid and carbohydrate as percentages of dry weight vary considerably in comparison to the literature. This discrepancy may be a result of the algal dry weights (pg/cell) obtained during this study differing significantly from literature values (Gillis, 1993). The algal dry weights tended to be higher than those found in the literature, leading to lower concentrations of lipid and carbohydrate (% of dry weight) in the microalgae in comparison to literature values. A general trend of increased lipid and carbohydrate (pg/cell) in low macronutrient algal cultures in comparison to high macronutrient algal cultures was, however, observed during the present study, and clearly demonstrates that the algal production unit at the hatchery can be used to modify the biochemical composition of microalgae by manipulation of macronutrient concentration.

Comparison of correlation coefficients of larval shell height and spat shell growth rate versus lipid class composition of the diet for the three experiments conducted during the present study shows several contradictions. Overall, it was found that glyceryl ethers, alcohols, acetone mobile polar lipids, phospholipids, total lipids, total lipids without hydrocarbons and total acyl lipids were positively correlated with both larval shell height and spat shell growth rate during all three experiments. In contrast, ethyl ketones were found to be negatively correlated with both larval shell height and spat shell growth rate during all three experiments. Methyl esters, methyl ketones and free fatty acids were found to be positively correlated with growth rate of spat, but negatively correlated with shell height of larvae. In contrast, sterol/wax esters were positively correlated with larval shell height, but negatively correlated with spat shell growth rate. Triacylglycerols were positively correlated with spat shell growth rate, but showed no significant correlation with larval shell height. Correlations of sterols, diacylglycerols and hydrocarbons with larval shell height and spat shell growth rate were variable between experiments. This variability in hydrocarbon composition in the different diets of the three experiments conducted over the course of this research may explain why the y axes, lipid class composition (picograms/cell) (Figures 23, 24 and 25), were extremely variable for the three experiments. Excluding hydrocarbons and focusing on total lipids without

hydrocarbons illustrates that the y axes or lipid class compositions of the three experiments are actually similar. The source of the hydrocarbon levels in some diets remains unknown and requires future investigation. However, it is apparent that the high hydrocarbon composition did not affect the growth rate of *Placopecten* larvae and spat.

An analysis of the proportion of each species ingested (by number and volume) when fed the 50/50 ratio diet of *Tetraselmis suecica*/T-Iso and the 50/50 ratio diet of *Thalassiosira weissflogii*/T-Iso, obtained by using Multisizer plots, illustrated that by number, spat ingested significantly more T-Iso than either *Tetraselmis suecica* or *Thalassiosira weissflogii*. This is probably due to the small size of T-Iso in comparison to *Tetraselmis suecica* and *Thalassiosira weissflogii*. By volume, however, spat ingested the same proportion of T-Iso and *Tetraselmis suecica*, showing no preferential selection of either species. However, ingestion did significantly differ by volume between T-Iso and *Thalassiosira weissflogii*, with spat ingesting more *Thalassiosira weissflogii*, indicating a preferential selection of *Thalassiosira weissflogii* in comparison to T-Iso and *Tetraselmis suecica*. These results suggest that *Thalassiosira weissflogii*, which gave increased growth of scallop spat and was preferentially selected by the spat themselves, would be of great value in an algal diet.

#### **4.7 Growth rates of scallop larvae and spat**

Over the course of this research growth rates of scallop larvae and spat were found to be extremely variable, ranging from less than  $1 \mu\text{m}/\text{day}$  up to around  $38 \mu\text{m}/\text{day}$ . One possible explanation for this may be that larvae and spat were batch fed algal diets harvested from continuous cultures which were not axenic. Also, the larvae and spat used over the course of this research were not all from a single spawning batch of adult scallops. However, larvae and spat used for individual experiments were the offspring of the same adult scallops.

#### **4.8 Summary**

The results of the algal cell density experiment indicate that an algal cell density of 40 cells/ $\mu\text{L}$  be fed to giant scallop spat to obtain most efficient use of the algal ration. The algal cell density fed to juveniles should be sufficient to cover maintenance costs, but should not exceed the optimum density because of the high cost of growing the algae.

Results of this study also indicate that *Tetraselmis suecica*, *Chaetoceros calcitrans*, and *Chroomonas salina* are not beneficial as constituent species of a mixed algal diet for *Placopecten* larvae and spat. Therefore, the effort of culturing these species of algae may not be worthwhile.

In addition to investigating algal density and dietary composition, different algal culturing methods and how they affected growth of giant scallop larvae and spat were also explored. The results indicate that scallop larvae grew better when fed logarithmic phase algae which may indicate a requirement for increased protein during early development, while lipid and carbohydrate are important for metamorphosis and post larval (spat) development. The higher protein requirement can be obtained by feeding larvae algae harvested during logarithmic phase or cultured under high nutrient conditions, while the increased need of spat for lipid and carbohydrate can be met by feeding algae harvested during stationary phase or cultured under low nutrient conditions. In order to ensure that biochemical requirements are met throughout the entire life-cycle of the giant scallop, it would be beneficial to feed a mixture of both logarithmic and stationary phase algae, or high and low nutrient cultured algae, ensuring that all biochemical requirements are met during the transition from a increased need for protein to an increased need for lipid and carbohydrate.

The results of this study also indicate that *Thalassiosira weissflogii* is of great value in an algal diet for giant scallop spat. Although the ration of 40 cells/ $\mu\text{L}$  has been shown to be a good value for scallop spat, perhaps the equal ratios of each component species in mixed diets should be altered. A higher proportion of *Thalassiosira weissflogii* in a mixed diet for scallop spat may be more beneficial for growth.

In order to have a successful giant scallop aquaculture industry there must be a reliable source of spat, and often this is not the case with spat collected from the natural environment. If spat could be reliably grown in a hatchery situation, a reliable source of seed would be ensured, eliminating the seed supply problem. When hatcheries are used to produce juvenile bivalves, maximum survival of mature larvae and juveniles must be realized to make bivalve culture economically viable. Studies such as those discussed here are useful in determining optimal diets. The importance of providing bivalve larvae and spat with a suitable algal diet in the hatchery is very clear; they will grow faster and more efficiently, reach higher quality, and perform better when transferred to the natural environment.

In addition to those studies discussed here, further research of more algal species in terms of their food value for larval and juvenile *Placopecten magellanicus* should be undertaken. Further investigation of how different culturing methods affect growth of scallop larvae and spat, and why, with respect to algal biochemical composition should also be conducted. Although the present study showed that scallop larval growth was increased when fed logarithmic phase or high nutrient cultured algae, assuming a requirement for increased protein during early development, biochemical studies investigating the relationship between protein and scallop larval growth should be undertaken. Also, closer examination of algal characteristics such as cellular size, presence or absence of a rigid cell wall, cellular arrangement, the presence or absence of toxic metabolites and the composition of fatty acids is needed.

Such research as has been conducted here and the suggestions for further research will help ensure that a hatchery produces large yields of higher quality *Placopecten magellanicus* spat which will perform better when transferred to the natural environment, and help ensure the success of the giant scallop aquaculture industry.

#### **4.2 Conclusions**

- 1) Seventy-two hours is the required duration for immersion of spat in calcein solution to obtain a strong fluorescent band.

- 2) *Tetraselmis suecica*, *Chaetoceros calcitrans*, and *Chroomonas salina* are not beneficial as a supplement of a mixed algal diet.
- 3) Clearance rates decrease from 0 to 10 cells/ $\mu$ L and remain constant from 10 to 80 cells/ $\mu$ L, whereas ingestion rates increase with increasing algal density. An algal cell density of 40 cells/ $\mu$ L should be fed to scallop spat to obtain most efficient use of the algal ration.
- 4) Scallop larvae grew better when fed logarithmic phase algae or high macronutrient cultured algae. Scallop spat, overall, grew better when fed stationary phase algae or low macronutrient cultured algae.
- 5) *Thalassiosira weissflogii* would be of great value in an algal diet for scallop spat.
- 6) *Placopecten* spat selectively ingest significantly more *Thalassiosira weissflogii* by volume in comparison to *Tetraselmis suecica* and T-Iso.
- 7) The algal production unit at the hatchery can be used to modify the biochemical composition of microalgae by manipulation of macronutrient concentration. Low macronutrient grown algae exhibit higher concentrations of total lipid and carbohydrate in comparison to high macronutrient grown algae.

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