Use of tropical microalgae species as food for pearl oyster (*Pinctada margaritifera* L.) larvae

Thesis submitted by

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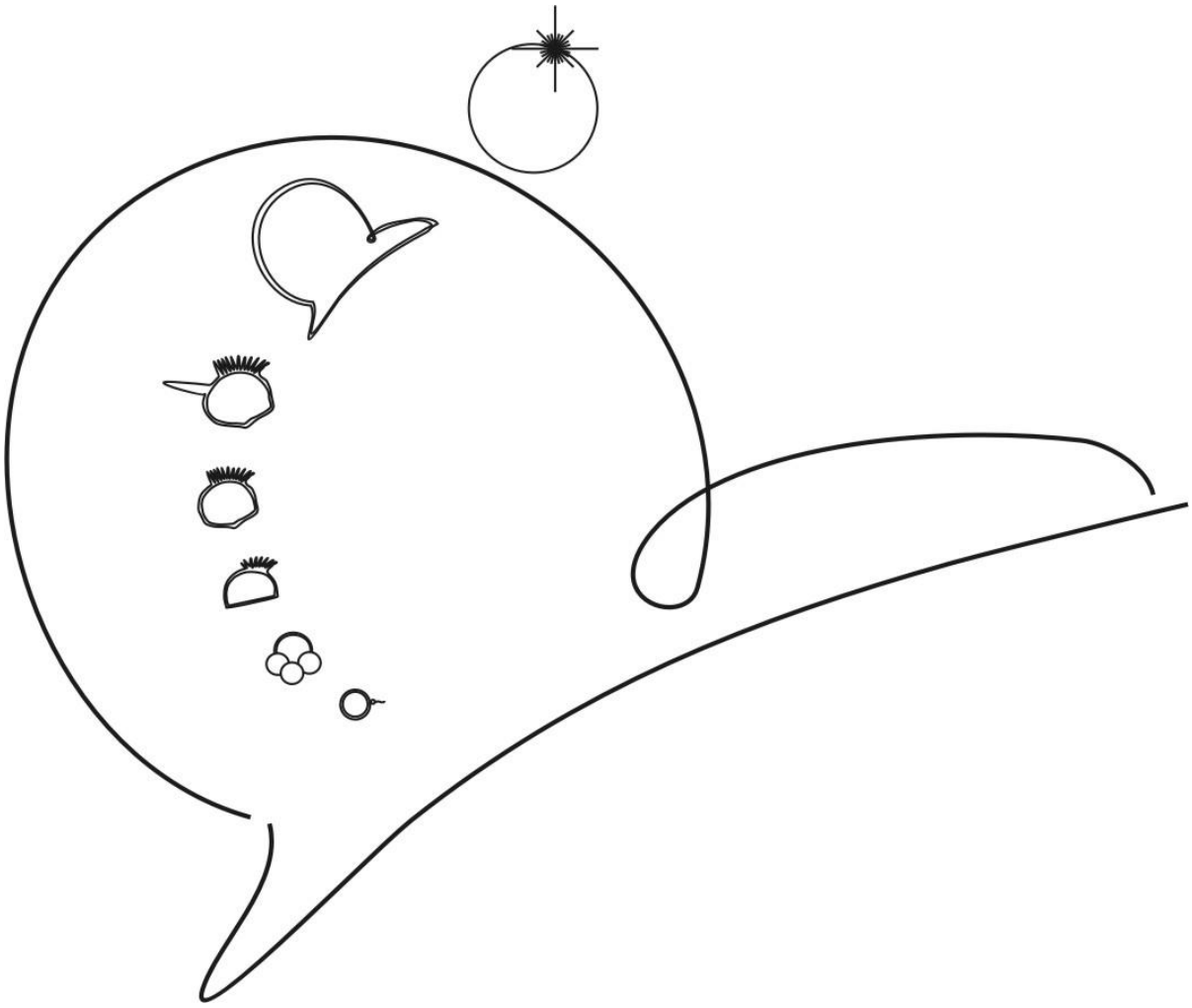

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Abstract

The most famous round pearls of the world come from pearl oysters of the genus *Pinctada*; 'south-sea pearls' are cultured using the silver- or gold-lip pearl oyster, *Pinctada maxima,* and 'black pearls' are cultured from the black-lip pearl oyster, *P. margaritifera*. Marine pearl culture is one of the most valuable aquaculture industries in the world with an estimated value of $US475 millions. *P. maxima* is the basis of Australian pearl industry while *P. margaritifera* supports cultured pearl industries in southern Japan and the Pacific island nations of French Polynesia and Cook Islands. In French Polynesia, pearl production has an annual value of US$170 millions and, not surprisingly, other nations across the Pacific have shown interest in developing similar industries. The usual way to obtain oysters for pearl farms in Polynesia is by collection of wild spat (or juveniles), activity that relies entirely on natural recruitment which can be unpredictable and unreliable. Hatchery production of pearl oyster is increasing in significance as a source of oyster stock for pearl production and offers many advantages over the collection of oysters from the wild. Hatchery production of pearl oysters is a relatively new field and, as such, techniques and protocols adopted are generally based on those developed for other bivalve species of commercial importance, usually of temperate origin. Much of the research on hatchery production of pearl oyster has focused on *P. margaritifera* and has covered aspects such as culture systems, feeding and nutrition of larvae and early nursery culture.

Microalgae culture is fundamentally important to commercial hatcheries rearing marine molluscs, since they are currently the only suitable food source. Microalgae provided to larvae during culture can affect their growth and survival. Only recently have significant numbers of tropical microalgae species become available to the aquaculture industry. However, many of these have not been assessed for their nutrient content or their nutritional value for culture animals. Microalgae of tropical origin are likely to be better suited to the culture conditions used for tropical species, such as *P. maxima* and *P. margaritifera*.

This study assessed the nutritional value of eight tropical microalgae species for *P. margaritifera* larvae. Each was analysed for carbohydrate, lipid and protein content as well as fatty acid and amino acid composition. Each species of microalgae was fed singly to early (D-stage veliger, Chapter 3 (*section 3.3.1*)) and later (umbo-stage veliger, *(section 3.3.2)*) *P. margaritifera* larvae. The results showed *Pavlova* sp. and *P. salina* to be the most nutritious of eight microalgae fed to both D-stage and umbone larvae. Relationships between the levels of various nutritional components of microalgae and resulting larval growth were determined. A significant correlation between the growth of D-stage larvae and total protein, lipid and carbohydrate contents of microalgae was found. However, for umbo-stage larvae only carbohydrate contents in microalgae was positively correlated to larval growth. Significant positive correlation between the saturated fatty acid (SFA) and polyunsaturated fatty acid (PUFA) contents of microalgae and larval growth were seen for both larval stages.

Bivalve larvae are generally fed a mixture of microalgae species in order to provide a better nutrient balance. This study assessed the more nutritious species of microalgae from Chapter 3 in binary and ternary combinations. D-stage *P. margaritifera* larvae were fed *Pav. salina*, *Pavlova* sp, TISO and *Micromonas pusilla* in binary and ternary combinations, Chapter 4 *(section 4.3.1)*. Umbo-stage larvae were fed the best binary algae combinations (flagellates) to which one diatom species (either *Chaetoceros muelleri*, *Chaetoceros* sp. or *Skeletonema* sp.) was added per combination (*section 4.3.2*). The best two ternary algae combinations from the first experiment in Chapter 4 with D-stage larvae (flagellates only), were also used to feed umbo-stage larvae in the second experiment in Chapter 4; these were *Pav. salina/ Pavlova* sp/TISO and TISO/*M. pusilla*/*Pavlova* sp. Greater growth rate was shown by D-stage *P. margaritifera* larvae fed the ternary combination of *Pavlova sp/Pav.salina/*TISO followed by the binary combination of *Pavlova* sp./*M. pusilla*; however, larvae fed *Pavlova* sp. as a mono-specific diet performed as well as those fed the combination of *Pavlova* sp/*Pav. salina/M. pusilla.* Umbo-stage larvae fed ternary combinations containing a diatom showed noticeably greater growth rates than larvae fed combinations without diatoms. The best binary and ternary microalgae combinations for umbo-stage larvae were *Pavlova* sp./*C. muelleri* and *Pavlova* sp./*M. pusilla/Skeletonema* sp., respectively. However, the growth rate of larvae fed the binary combinations of *Pavlova* sp./*C. muelleri* did not differ significantly from those of larvae fed ternary diet combinations.

Experiments in Chapters 3 and 4 identified microalgae supporting the greatest growth rates of D-stage and umbo-stage larvae of *P. margaritifera*. Biochemical analysis of these

microalgae (Chapter 2) allowed identification of nutrients within the microalgae that correlate to good growth rates. Chapter 5 investigated the possibility that manipulation of culture conditions could be used as a means of improving (increasing) the levels of these key nutrients in microalgae. Such a development would have clear benefits in the culture of *P. margaritifera* larvae. TISO, *Pav. salina, Pavlova* sp., *M. pusilla* and *C. muelleri* were cultured under different light regimes. All microalgae tested were easy to grow. Under the culture conditions used during this experiment, photoperiods of 18 hours light and 6 hours dark (18L:6D) and continuous light resulted in greater productivity of algae cultures. Differences in proximate compositions of microalgae were not significant in terms of growth phase; however, general increases in lipid contents of microalgae between the logarithmic and stationary phase growth phases were observed as reported in similar studies. Harvesting microalgae during the stationary growth phase will provide microalgae with high lipid and protein values. Changes in fatty acid contents were species specific; however contents were not significantly different when algae were culture under each light regime.

In summary, this study is the first comprehensive assessment of the nutritional value of tropical microalgae species for pearl oyster larvae and the first to relate larval growth rates and survival to the nutrient composition of microalgae. This study has identified species of microalgae that are highly nutritious for *P. margaritifera* larvae, and the nutrients that impart high nutritional value to them. Assessment of growth rates and changes in the biochemical compositions of microalgae cultures of different ages and under varying culture conditions will allow tropical microalgae to be cultured according

to a regime which not only maximizes their productivity, but optimizes the nutritional composition for *P. margaritifera* larvae. On this basis, the results of this study provide a basis for development of more effective larval culture techniques for *P. margaritifera* and larvae of different ages.

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Chapter 1

General Introduction

The most famous round pearls in the world come from the genus *Pinctada*; the so-called 'South sea pearls' which are produced by the silver- or gold-lip pearl oyster, *Pinctada maxima*, and 'Black pearls' which are produced by the black-lip pearl oyster, *P. margaritifera.* Marine pearl culture is one of the most profitable aquaculture industries in the world with an estimated value of $US475 millions (Anon., 2006). The principal pearl producing countries are Japan, French Polynesia, China and Australia (Fassler, 1997). Pearling is Australia's second most valuable aquaculture industry with an annual farm-gate value of more than AU$250 million (O'Sullivan and Savage, 2003). The silver-lip pearl oyster, *P. maxima* is the basis of the Australian pearling industry with approximately 80% of production from Western Australia. The industry is founded on a sustainable (quota managed) wild fishery for *P. maxima* (Ryan and O'Sullivan, 2001). The black-lip pearl oyster, *P. margaritifera,* is widely distributed throughout the Indo-Pacific region from the Pacific coast of Central America to the Red Sea and Persian Gulf (Gervis and Sims, 1992). It supports cultured pearl industries in southern Japan and in the Pacific island nations of French Polynesia and the Cook Islands (Fassler, 1997). In French Polynesia, black-pearl aquaculture has played an increasingly important role since 1980 with production now exceeding 6 tonnes of pearls with a value in excess of US$170 million per annum (Pouvreau et al., 2000; Arnaud-Haond et al., 2003). Based on the success of this industry, it is not surprising that nations across the Pacific, including the

Federate States of Micronesia (Fassler, 1997), the Republic of the Marshall Islands (Ellis and Haws, 1999) and Kiribati (Southgate and Beer, 1996) have shown interest in developing similar cultured pearl industries.

There are three ways to obtain oysters for pearl farms: (1) collection of wild juveniles and adults; (2) collection of wild spat; and (3) hatchery-culture. The second option is the easiest method (Ellis and Haws, 1999), and is widely used in Polynesia (Arnaud-Haond et al., 2003); however, depletion of pearl oyster stocks may make natural recruitment unpredictable and unreliable. Hatchery production of pearl oysters is of particular significance as it offers many advantages over the collection of oysters from the wild. These include greater control over the genetic aspects that influence pearl quality (e.g. nacre colour, shell length, shell depth) and the possibility of year-round production of oysters. However, hatchery production of pearl oysters is a relatively new field, and the techniques used are based on those developed for other, usually temperate, bivalve species (Ryan and O'Sullivan, 2001). Much of the recent research on hatchery production of pearl oysters has focused on *P. margaritifera*, and has covered aspects such as culture systems (Southgate and Beer, 1997; Southgate and Ito, 1998), biochemical parameters (Doroudi et al., 1999b), eggs and larval culture density (Southgate et al., 1998a; Doroudi and Southgate, 2000), feeding and nutrition of larvae (Southgate et al., 1998b; Doroudi et al., 2002; Doroudi et al., 2003) and early nursery culture (Pit and Southgate, 2000).

This chapter deals briefly with important aspects of hatchery culture of *P. margaritifera* pertinent to this study and with the use of microalgae as the main source of nutrition for bivalve larvae.

1.1 The black-lip pearl oyster *Pinctada margaritifera*

1.1.1 Life cycle

Pinctada margaritifera is a protandrous hermaphrodite (Gervis and Sims, 1992) that is taxonomically classified as follows:

Phylum: Mollusca

Class: Bivalvia (= Lamellibranchia)

Order: Pterioida

Family: Pteriidae

Genus: *Pinctada*

Species: *Pinctada margaritifera* (Linnaeus, 1758).

This species is found attached to hard substrates as deep as 40 m, usually in association with reef habitats. Spawning season for this species has been reported to occur within the wet season (summer) when water temperatures are rising (Tranter, 1958; Acosta-Salmon and Southgate, 2004). Females may release millions of eggs into the water column with more than 60 million being recorded from a single spawning female (Southgate, unpublished data). Fertilization takes place externally by sperm released from nearby males. Eggs hatch and the free-swimming larvae pass through a number of

morphologically distinct phases until, approximately 3-4 weeks after fertilization, they metamorphose into juvenile pearl oysters (or spat) that attaches to the substrate (Ellis and Haws, 1999) (Fig. 1.1).

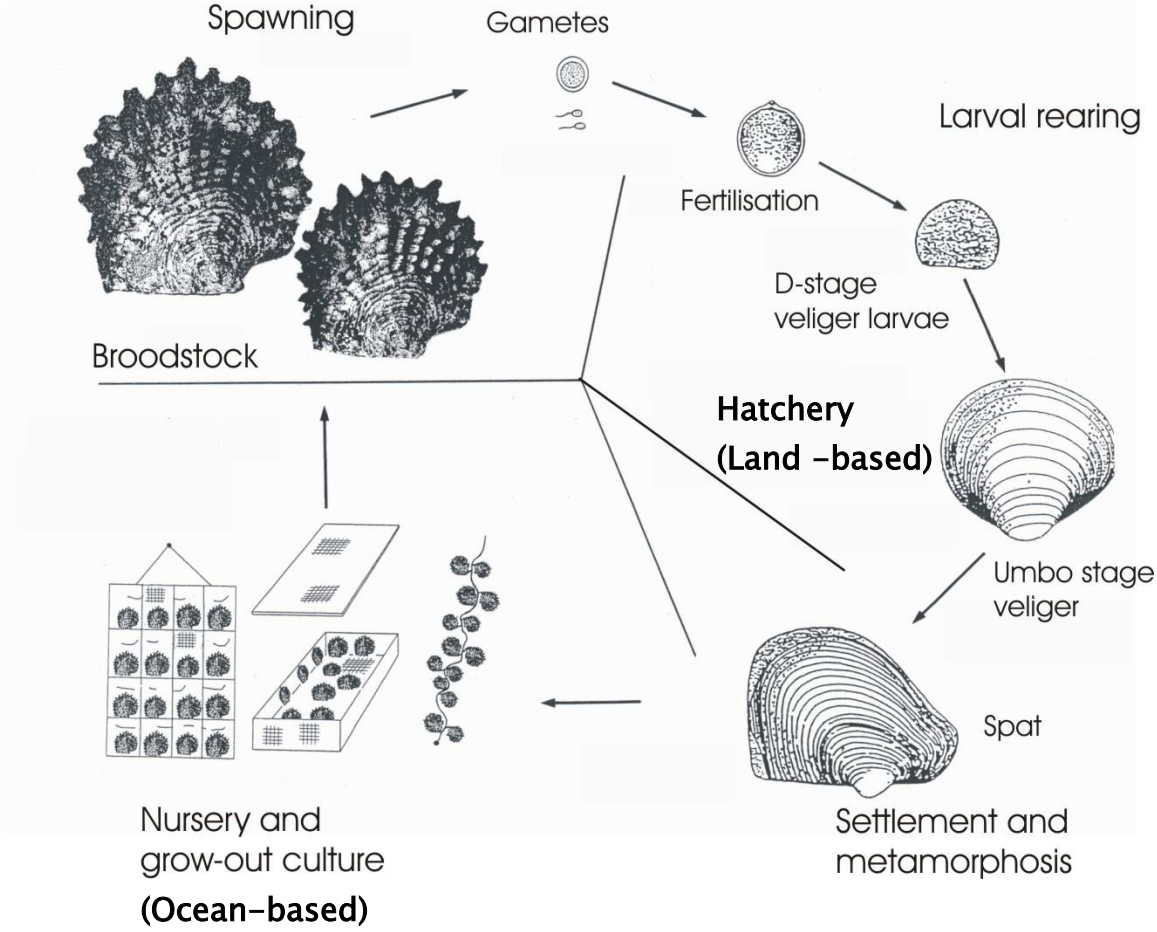


Fig. 1.1 Generalised life-cycle of cultured pearl oysters (modified from Southgate and Lucas, 2003).

1.1.2 Hatchery production

Much of the information relating to the hatchery culture of *P. margaritifera* has resulted from research at James Cook University that has investigated the development of simplified methods for hatchery and nursery culture, more appropriate for use in small Pacific island nations (Southgate and Beer, 1996). Hatchery culture methods for *P. margaritifera*, based on the results of this research, are shown schematically in Fig. 1.2. Cleaned broodstock are held in a minimum volume of seawater in an air-conditioned room over-night at 21ºC. Spawning is induce by increasing the water temperature to approximately 32ºC and spawning individuals are placed in separate containers and allowed to complete spawning. Fertilized eggs are incubated at densities of between 30-50 mL^{-1} in gently aerated 1-µm filtered seawater at 28 ºC (Southgate et al., 1998a). After 20-24 h, D-stage veliger larvae are removed from the incubation tanks, washed and placed into larval rearing tanks at a density of 1-2 larvae mL^{-1} (Doroudi & Southgate, 2000). Larvae are fed a combination of cultured microalgae consisting of *Isochrysis* sp. (TISO), *Pavlova salina* and *Chaetoceros muelleri* (Southgate and Beer, 1997) and food rations are varied according to the age of the larvae and are increased as the larvae develop (Doroudi and Southgate, 2000). Umbo-stage larvae are usually seen around 10 days after fertilization while 'eyed' umbone veliger larvae are usually present at 15-18 days after fertilization (Southgate and Beer, 1997).

Once larvae are large enough to be retained on a 150-µm sieve, they are removed from larval culture tanks and placed into settlement tanks which contain collectors bag (shade cloth in mesh bags) suspended into the tanks to provided substrate.

Approximately 100% of the water in settlement tanks is exchanged every 2 days using a flow-through water exchange system and microalgae are added at a density of approximately 20-25 cells μL^{-1} (Doroudi and Southgate, 2000). Spat are transferred to ocean-based nursery culture 3-5 weeks after settlement (Pit and Southgate, 2000) and then graded for nursery culture at 3 months of age (Southgate and Beer, 1997) (Fig.1.2).

Feeding is a very important aspect of the hatchery culture process. Cultured microalgae are the exclusive food source for pearl oyster larvae and the choice of microalgae species fed to the larvae may greatly influence larval growth and survival (Southgate et al., 1998b). However, much of the knowledge of the nutritional value of microalgae and their nutrient compositions relates to temperate species (Delaunay et al., 1993; Leonardos and Lucas, 2000).

1.2 Microalgae and phytoplankton

Phytoplankton is composed of all micro-organisms that contain chlorophyll and other photosynthetic pigments, which are able to transform light energy into chemical energy and organic components via photosynthesis (Dawes, 1991). Phytoplankton is the basis of the ocean food chain and a significant portion is made up of microalgae which are now

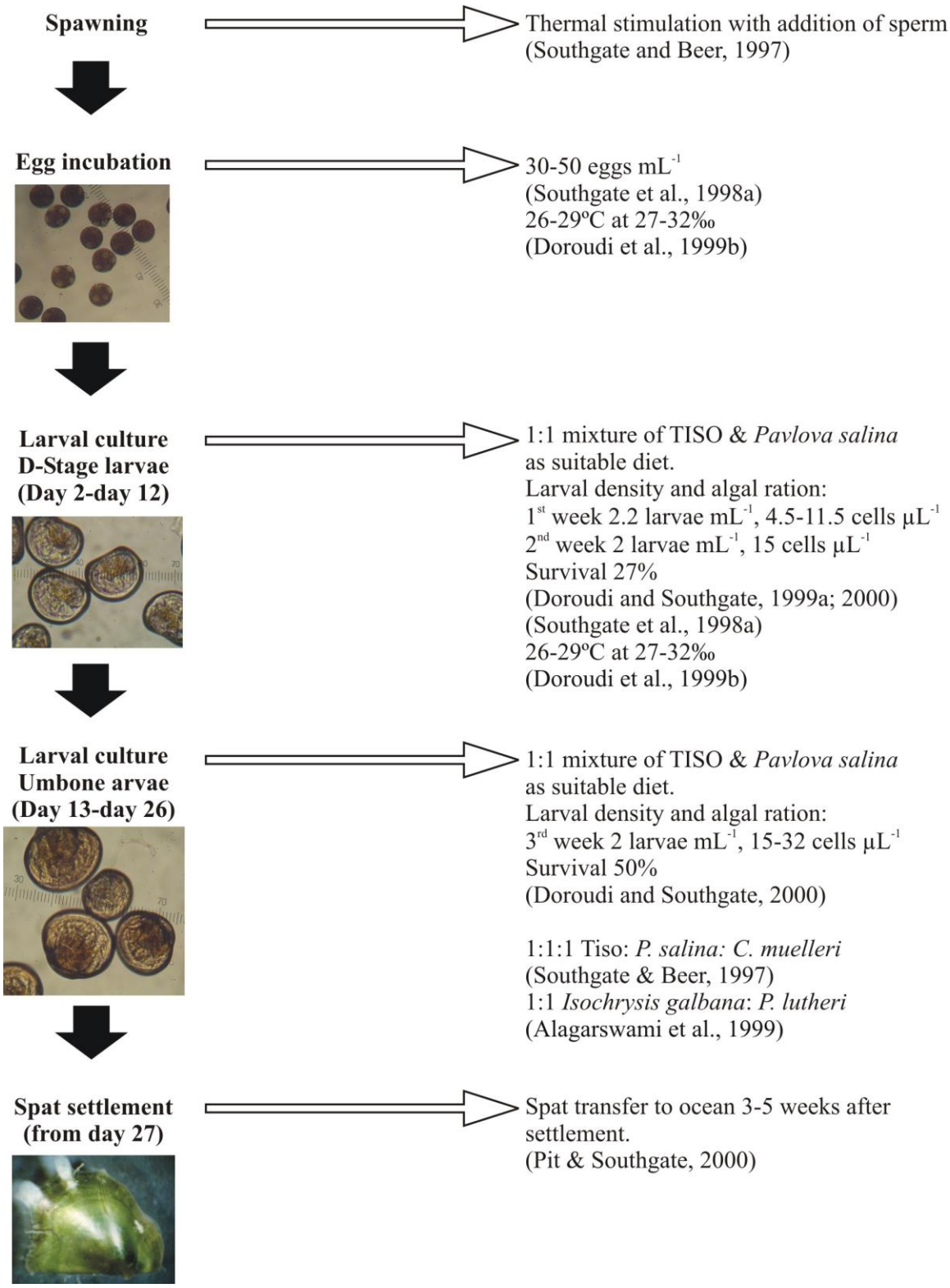


Fig. 1.2 General scheme for hatchery culture of *P. margaritifera* based on results of previous research.

widely used as a food source for the larvae of marine organisms in aquaculture hatcheries. Microalgal reproduction is primarily asexual, although gamete-like forms have been reported (Van den Hoek et al., 1995). Vegetative reproduction consists of equal division of the mother cell into two daughter cells, each exactly half the size of the mother cell. Microalgae metabolism involves numerous biochemical compounds of interest in nutrition, cosmetics and pharmaceutical industry. Their metabolic plasticity allows species adaptation to a wide range of environmental conditions (Müller et al., 2003), and this facilitates their use in aquaculture (Jeffrey et al., 1992).

1.3 Microalgae in aquaculture

Microalgae are the biological starting point of the energy flow through the aquatic food chain and, on this basis, microalgae production is an integral part of many aquacultural operations. Microalgae species used in aquaculture have been generally selected by trial and error with emphasis being placed initially on ease of culture and use. Subsequent research has guided the selection of species based on nutritional quality. When rearing commercial species of marine animals, microalgae are still the only suitable food source for the whole life-cycle of bivalve molluscs (Knauer and Southgate, 1999), larval stages of some crustacean species (Sanchez, 1986) and very early growth stages of some fish species (Papandroulakis et al., 2002).

Furthermore, mass production of zooplankton (rotifers, copepods, brine shrimp) which is used as food for larval and early-juvenile stages of cultured crustaceans and fish species,

also depends on microalgae cultivation (Müeller-Feuga et al., 2003; Southgate, 2003). Apart from their use as a food source, microalgae are also used in "green water" culture techniques for fish (and sometimes crustacean) larvae where they stabilize water quality, aid in nutrition of the larvae and modify microbial activity within culture tanks (Lavens and Sorgeloos, 1996). Of the numerous species of microalgae tested for their suitability in aquaculture, only a handful are widely used today; most of these are species of temperate origin that have been used primarily for culture of temperate organisms (Delaunay et al., 1993; Thompson et al., 1993; O'Connor and Heasman, 1997). Table 1.1 summarizes the major classes, genera and species of microalgae most commonly grown as an aquaculture food source.

Previous research has shown that many species of microalgae have high nutritional value for bivalves either as mono-specific diets or as part of a mixed-species diet; these include: *Chaetoceros calcitrans, C. muelleri, Pavlova lutheri, Isochrysis* sp. (TISO), *Tetraselmis suecica, Skeletonema costatum* and *Thalassiosira pseudonana* (O'Connor and Heasman, 1997, Leonardos and Lucas, 2000). Most of these species are of temperate origin (with exception of *C. muelleri* and TISO), and most of the research in this field has been based on the description of their biochemical composition and their nutritional value for temperate bivalve molluscs. It is generally accepted that multi-species combinations of microalgae may increase the chances of achieving a balanced diet and, on this basis, most cultured bivalves are fed diets composed of two or more microalgae species. The response of the larvae to mono-specific or plurispecific diets will depend, however, on the cultured organism.

Table 1.1. Classes, genera and species of commonly used microalgae currently used as a food source in aquaculture (modified from Lavens and Sorgeloos, 1996 and Müeller et al., 2003).

Class	**Genus**	**Species**	**Main use**
Bacillariophyceae (diatoms)	*Skeletonema*	*costatum, pseudocostatum*	PL, BL, BP
	Thalassiosira	*pseudonana*	PL, BL, BP
	Phaeodactylum	*tricornutum*	PL, BL, BP, BS
	Chaetoceros	*calcitrans, muelleri*	PL, BL, BP, BS
Chlorophyceae (green algae)	*Chlorella*	*minutissima, grossi, virginica,*	LPF
	Dunaliella	*tertiolecta, salina*	LPF, PL
	Nannochloris	*atomus*	BL
Prasinophyceae (scaled green algae)	*Tetraselmis*	*suecica, triata, chuii*	PL, BL, BP
	Pyramimonas	*virginica*	BL, BP
	Micromonas		BP
Chryptophyceae	*Rhodomonas*	*salina, baltica, reticulata*	PL, BL, BP
Eustigmatophyceae	*Nannochloropsis*	*oculata*	LPF
Prymnesiophyceae (golden-brown flagellates)	*Isochrysis*	*galbana,* clone TISO	PL, BL, BP, BS
	Pavlova	*lutheri, salina*	BL, BP, BS, LPF
Dinophyceae (dinoflagellates)	*Crypthecodinium*	*cohnii*	LPF
Thraustochytriidae	*Schizochytrium*	Sp.	LPF
Cyanophyceae (blue-green algae)	*Arthrospira* (*Spirulina*)	*platensis, maxima*	LPF

LPF, food for live prey of fish larvae; PL, penaid shrimp larvae; BL, bivalve mollusc larvae; BP, bivalve mollusc post-larvae.

1.4 Microalgae as food for molluscs

Molluscs, unlike fish and crustaceans, are fed microalgae directly for their entire life cycle. Both quality and quantity of microalgae production is closely related to mollusc development and culture success. Research in this field has focused primarily on determining which microalgae species provide the best food value in terms of optimizing growth and survival of molluscs. Despite numerous attempts to develop non-living diets for bivalve molluscs to replace live microalgae (Knauer and Southgate, 1999), such diets commonly result in lower growth rates and higher mortalities than recorded for controls fed live microalgae (Langdon, 1983; Laing and Millican, 1991; Nell and O'Connor, 1991; Curatolo et al., 1993). Some promising results have, however, been reported when concentrated and dried microalgae have been used as a food source for larvae and juvenile bivalves (Robert and Trintignac, 1997) including pearl oyster larvae (Doroudi et al., 2002).

Much of the research in this field has been based on temperate microalgae species, however, recent years have seen the introduction of tropical microalgae species to standard feeding protocols for bivalves. For example, in many culture facilities, the temperate flagellate species, *Isochrysis galbana*, has been replaced by *Isochrysis* aff. *galbana* "Tahiti" (clone T-ISO) which has shown to be more tolerant to higher water temperatures (Ewart and Prouder, 1981), have good nutritional value (Helm et al., 1987; Brown et al., 1993) and is easy to grow with good response to alternative cultured media (Valenzuela-Espinoza, 2002). It is now a very widely used microalga as a food for

bivalve molluscs. Tropical microalgae species are an attractive option to explore for aquaculture due their versatility and adaptation to culture conditions (Renaud et al., 1994 a, b). This potential has been strengthened by the increasing availability of tropical microalgae species over recent years (Brown et al., 1997) in response to the increasing importance of hatchery-based aquaculture of tropical species. It has been suggested that the most important criteria for microalgae as a potential food source for hatchery culture of bivalves are: cell size, digestibility, good nutritional values and ease of mass production (Robert and Trintignac, 1997; Doroudi et al., 2003; Martínez-Fernández et al., 2004).

1.4.1 Size: microalgae cell dimensions and shapes

Selecting the size of microalgae to be used as a larval food source depends primarily on larval mouth and oesophagus diameters (Fritz et al., 1984). It has been reported that bivalve larvae are capable of ingesting particles ranging in size from 0.2 to 30-µm, with a preferred size range of 2 to 4-µm (Knauer and Southgate, 1999). Selection by size must also consider microalgae shape because the shape of some microalgae (Fig. 1.3) makes them impossible to ingest by early larval stages of bivalves. Good examples of this are the diatom species that have long spines (Robert and Trintignac, 1997). For *Crassostrea virginica* larvae fed on natural plankton it was reported that larvae ingested cells ranging in size from 1 to 20-µm; moreover, when total ingested cell volume was considered, larvae were able to ingest up to 80% of cells sized from 3 to 10-µm and only 13% of cells sizes from 10 to 20-µm (Fritz et al., 1984). When oyster larvae were fed a mixture of four

cultured microalgae ranging in size between 1 to 11-µm, small larvae preferred 1-µm algae while large larvae preferred 11-µm algae; this study also suggested that feeding patterns of *C. virginica* larvae depended not only on the size, but growth rate and chemical quality of the food particles (Balwin, 1991, 1995).

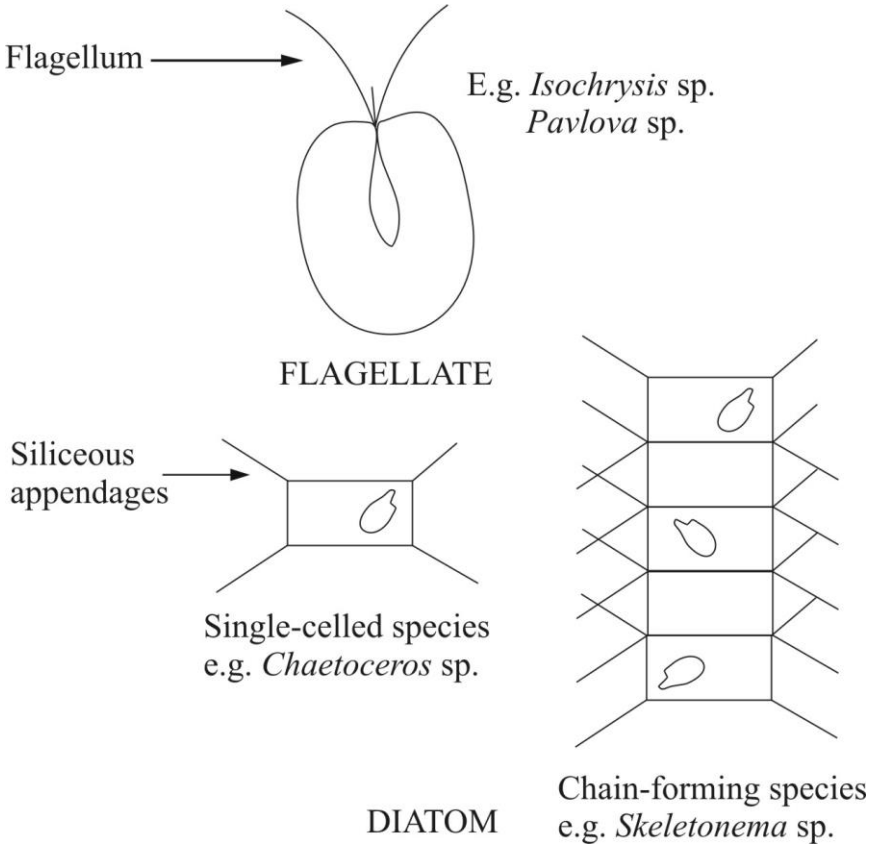


Fig. 1.3. Diagram showing general morphology of a flagellate and diatoms (modified from Southgate, 2003).

1.4.2 Digestibility: larval acceptance of different microalgae species

Digestion of microalgae is an important issue particularly for early-stage larvae, since some research has shown that low larval growth rates obtained with some microalgae species could be explained by the thickness of their 'cell wall' that make digestion more difficult (Müller et al., 2003). A typical example of this is the use of green-flagellates from the class Chlorophyceae which, when fed to early-stage bivalve larvae, are ingested but not digested resulting in poor larval growth in *Pecten maximus* and *Mytilus edulis* (Lucas and Rangel-Dávalos, 1981; Le Pennec and Rangel-Dávalos, 1985). Since some of the microalgae that are not well accepted by larvae are able to be used as food for adult stages, there is a recent a hypothesis that early-stage larvae may lack appropriate digestive enzymes to break-down the algae (Robert and Trintignac, 1997).

1.4.3 Nutritional value: biochemical composition of microalgae

Gross biochemical composition, in terms of total protein, lipid and carbohydrate content, and the presence of certain micronutrients, including highly unsaturated fatty acids (HUFA), amino acids and vitamins, determine the nutritional value of microalgae assuming appropriate ingestion and digestion (Robert and Trintignac, 1997). Biochemical composition of microalgae varies between species and according to culture conditions (Brown et al., 1997). However, a general pattern can be established as microalgae typically contain between 10-40% of their dry weight as protein, 10-30% as lipid and 5-30% as carbohydrate (e.g. Volkman and Brown, 2005).

Based on a multi-dimensional model developed to correlate larval growth with algal biochemical components, it has been suggested that some fatty acids were significant in determining microalgal nutritional value, with protein and carbohydrate playing a secondary 'modifying' role (Leonardos and Lucas, 2000).

Adequate levels of HUFA in the microalgae add nutritional value to the diet when this supplies essential HUFA according to specific bivalve larval requirements. Docosahexaenoic acid (22:6*n*-3, DHA), eicosapentaenoic acid (20:5*n*-3, EPA) and arachidonic acid (20:4*n*-6, ArA) are thought to be essential dietary nutrients for marine bivalves (Langdon and Waldock, 1981; Pernet and Tremblay, 2004) while the saturated fatty acids myristic acid (14:0) and palmitic acid (16:0) have been characterized as important energy sources for bivalves which also function as precursors for chain elongation and desaturation during synthesis of longer chain fatty acids including polyunsaturated fatty acids (PUFA) (Renaud et al., 2002). It is important to consider these fatty acid requirements when selecting microalgae as potential food sources for bivalves.

The relationship between HUFA contents of microalgae and their value as food for bivalve larvae has been examined in numerous studies (i.e. Roadhouse et al., 1983; Thompson et al., 1993; Mansour et al., 2005). It is generally accepted that bivalves have a limited capacity or total inability to synthesize HUFA, specifically EPA and DHA, because most bivalve species are unable to produce them from shorter chain precursors (Langdon and Waldock, 1981; Waldock and Holland, 1984; Delaunay et al., 1993).

These essential fatty acids must therefore be supplied in the diet. Microalgae differ in their lipid contents (Brown et al., 2001) and more specifically, PUFA contents (Leonardos and Lucas, 2000, Renaud et al., 2000). However, the relationship between EPA and DHA contents of the diet and the response of the larvae (growth and survival) seem to be species-specific. For example, growth of oyster, *Ostrea edulis*, larvae was found to be significantly related to dietary DHA contents with no similar correlation to EPA content (Jonsson et al., 1999). However, for *Crassostrea gigas* larvae, growth was found to be negatively correlated with EPA content (Thompson et al., 1996). So it is important to test the effectiveness of different microalgae as a source of these nutrients when used as food for bivalve larvae.

Research has shown that the biochemical components of microalgae can be modified according to cultures parameters (e.g. Thomas et al., 1984; Wikfors, 1986; Raven and Geider, 1988; Valenzuela-Espinoza, 2002), and much of this research has been conducted with temperate microalgae species. For example, increasing culture temperature (25 to 29ºC) may increase specific growth rate of microalgae cultures (Thompson and Guo, 1996; Sandnes et al., 2005); however, there is a concomitant modification in biochemical content. The most common change will be reflected in the protein content of microalgae as reported for *Skeletonema costatum* (Falkowski, 1977) and *Euglena gracilis* (Cook, 1963). Similarly, when testing eight temperate microalgae species, water temperature had to be reduced from 21ºC to 15ºC to obtain an increase in protein content with no apparent modification in lipid and carbohydrate levels (Thompson et al., 1993). In terms of fatty acid contents, it seems that there is a general tendency towards decreasing PUFA contents

with increasing culture temperature (Shifrin and Chisholm, 1980) and there is also a decrease in the degree of unsaturation of fatty acids in response to the elevation of culture temperature (25-30ºC) (Cohen et al., 1998). Understanding such changes in the biochemical (nutritional) composition of microalgae in response to culture conditions offers a means by which the nutritional value of the microalgae can be improved by optimizing culture conditions. This premise, however, relies on an understanding of the nutritional requirements of the organisms to which the microalgae will be fed.

1.5 Use of tropical microalgae species.

Tropical bivalve species (specifically pearl oysters) are generally cultured at a temperature range of between 25 to 30ºC. Early research into the artificial rearing of tropical pearl oyster larvae highlighted problems when temperate microalgae species were used as a food source for larvae cultured at tropical water temperatures (Minaur, 1969; Tanaka et al., 1970b). Although the greater suitability of tropical microalgae for rearing larvae of tropical pearl oysters was proposed by Minaur (1969), further study in this field has been limited. Increasing availability of tropical microalgae over recent years has, however, allowed some species to be assessed for their physical suitability as a food source for pearl oyster larvae (Doroudi et al., 2003) as well as their nutritional value (Southgate et al., 1998b). However, no prior study has determined the nutritional value of tropical microalgae for pearl oyster larvae on the basis of their nutrient compositions.

Description and determination of the biochemical profiles of tropical microalgae species has been investigated recently (Brown, 1991; Volkman et al., 1991; Brown and Jeffrey, 1992; Brown et al., 1997). From these profiles, 'new' or never previously used tropical microalgae species have been identified with potential as a food in tropical mariculture hatcheries (Volkman et al., 1991). Some of these have been assessed for their nutritional value for bivalves (e.g. Brown et al., 1998); however, there is generally limited information on the nutritional compositions of available tropical microalgae species and virtually no information on the nutritional value of these species for tropical bivalves.

Information on the nutritional value of tropical microalgae for pearl oyster larvae species is limited. *Pavlova salina,* TISO and *Chaetoceros muelleri* have been used successfully as food for black-lip pearl oyster (*P. margaritifera*) larvae (Southgate and Beer, 1997) but it is interesting to note that there was low survival during hatchery culture of *P. margaritifera*, i.e. survival from D-stage to spat generally ranges between 4.3 and 7.7% (Alagarswami et al., 1989; Southgate and Beer, 1997, Doroudi and Southgate, 2000). It may be possible to improve survival of *P. margaritifera* larvae during hatchery culture through selection and use of appropriate species of tropical microalgae which provide optimal nutrition.

1.6 Aims of this study.

This study investigated the use of tropical microalgae species as a food source for *P. margaritifera* larvae. The specific aims of this study were:

- to describe the proximate biochemical profile and fatty acid and amino acid contents of 'new' or not previously used species of tropical microalgae (Chapter 2);
- to determine the nutritional value of seven selected tropical microalgae species for *P. margaritifera* larvae (Chapter 3);
- to examine growth and survival of *P. margaritifera* larvae fed tropical microalgae as components of multi-species diets (Chapter 4); and
- to assess the effects of different light:dark cycles during culture on the proximate composition and fatty acid contents of tropical microalgae (Chapter 5).

No prior study has determined the nutritional value of tropical microalgae for pearl oyster larvae on the basis of their nutrient compositions. The results of this study will provide information related to the use of tropical microalgae species and their utilization as food for *P. margaritifera* larvae. The results will also assist in the development of more effective larval culture techniques for this species and facilitate the use of hatchery propagation as a source of oysters for the 'black pearl' industry.

Chapter 2

Biochemical compositions of tropical microalgae

2.1 Introduction

Microalgae culture is fundamentally important to commercial hatcheries rearing marine molluscs, since they are currently the only suitable food source (Müller-Feuga et al., 2003). Factors such as cell size and shape, rate of ingestion, digestibility and biochemical composition determine the nutritive quality of microalgae and their utility as food (Utting, 1986; Whyte et al., 1987; Doroudi et al., 2003; Martínez-Fernández et al., 2004). The species of microalgae investigated in this study were selected on the basis of suitable cell size, previously reported biochemical profiles (Brown et al., 1997) and nutritional studies with temperate species of the same genera (e.g. *Pavlova*, *Isochrysis* and *Chaetoceros*) which have shown superior nutritional value (e.g. Delaunay et al., 1993; Thompson et al., 1993; O'Connor and Heasman, 1997).

2.1.1 Tropical microalgae used during this study

a) Bacillariophyceae

Species in this group of microalgae, generally called diatoms, are characterised by being unicellular or colonial coccoid. Each cell is encased by a unique type of cell wall, which is siliceous and it simulates a box with an overlapping lid, this is termed the frustule (Van

den Hoek et al., 1995). The siliceous cell walls and lack of flagellae cause these microalgae to be negatively buoyant. In aquaculture, diatoms are used for their high nutritional value as food (Brown et al., 1997; O'Connor and Heasman, 1997; Lora-Vilchis et al., 2004b) and especially for their ability of accumulate lipid during culture (Opute, 1974). Because of their siliceous cell walls, the culture medium used for diatoms is supplemented with silica ($NaSiO_3$) unlike those used for culture of other classes of microalgae (Guillard, 1975). The most common diatoms used as food for mollusc larvae are *Chaetoceros* spp. and *Skeletonema* spp. Diatoms in particular seem to be important lipid sources not only for their high lipid and fatty acid contents but also because of their abundance of PUFA (Renaud and Parry, 1994b).

b) Haptophyceae (Prymnesiophyceae)

This group of microalgae also known as golden-brown flagellates, is broadly used in aquaculture; the coccoid flagellated cells of these organisms make them ideal food for any culture organism, since the action of flagellae provide them with motility and allow them to maintain their position within the water column. Almost all the planktonic haptophytes are small algae, belonging to the nanoplankton (2–20-µm in length) (Van den Hoek et al., 1995), a characteristic that also makes them a suitable choice as food for small mollusc larvae (Delaunay et al., 1993; Brown et al., 1998). The most common golden-brown flagellates used as food for mollusc larvae are *Isochrysis* spp. and *Pavlova* spp.

c) Prasinophyceae

Prasinophycea are free-living, flagellated green algae (Van den Hoek et al., 1995). The most common microalgae species used in aquaculture from this group belong to the genus *Tetraselmis*. Even though green flagellates are not frequently used as food for bivalve larvae, like diatoms and golden-brown flagellates, they do contain significant amounts of high unsaturated fatty acids (HUFA) specifically EPA and DHA (Brown and Jeffrey, 1992).

This chapter aims to determine the biochemical profiles of eight selected tropical microalgae species. These microalgae were assessed for their nutritional value for *P. margaritifera* larvae in Chapter 3 of this thesis. Detailed biochemical analyses of these species were undertaken in an effort to identify important nutritional components for *P. margaritifera* larvae and nutrients imparting high nutritional value to microalgae. Algae were batch cultured, since this type of culture is widely used for commercial cultivation of microalgae because of its ease of operation and simple culture system (Lee and Shen, 2004).

2.2 Materials and Methods

2.2.1 Microalgae culture

The microalgae used in this study were obtained from CSIRO Marine Laboratories in Hobart, Tasmania (Australia). Species were initially selected from the range of available

tropical microalgae on the basis of suitable cytomorphological characteristics such as cell size (within a range of 1.8 to 9-µm) and the high nutritional value associated with certain genera of temperate microalgae (e.g. Whyte et al., 1987; Brown et al., 1998). The microalgae used in this study included two species of diatom (Bacillariophyceae) *Chaetoceros muelleri* and *Chaetoceros* sp., three species of golden-brown flagellate (Haptophyceae) *Isochrysis* sp. (TISO), *Pavlova salina* and *Pavlova* sp., and two species of green flagellate (Prasinophyceae) *Micromonas pusilla* and Prasinophyte sp.

Microalgae were batch cultured in 0.5-µm filtered, UV irradiated and autoclaved seawater (34‰) enriched with f_2 medium (Guillard, 1975) (with the exception of *Pavlova* sp. where a double concentration of f_2 medium was used, since preliminary testing showed that this species grew better under such conditions. Cultures were maintained at 26 ± 1°C under a 12 h light: 12 h dark photoperiod regime at a light intensity of 200 µmol photons $m^{-1}s^{-1}$ provided by cool white fluorescent tubes. Microalgae cultures were aerated without supplemental CO_2. All microalgae were grown under the same conditions and harvested during the late logarithmic growth phase. Microalgae were grown in triplicate 20-L blue-tinted polycarbonate carboys containing 16-L of algal culture. Scale-up of microalgae cultures from starter cultures used during this study is shown in Fig. 2.1.

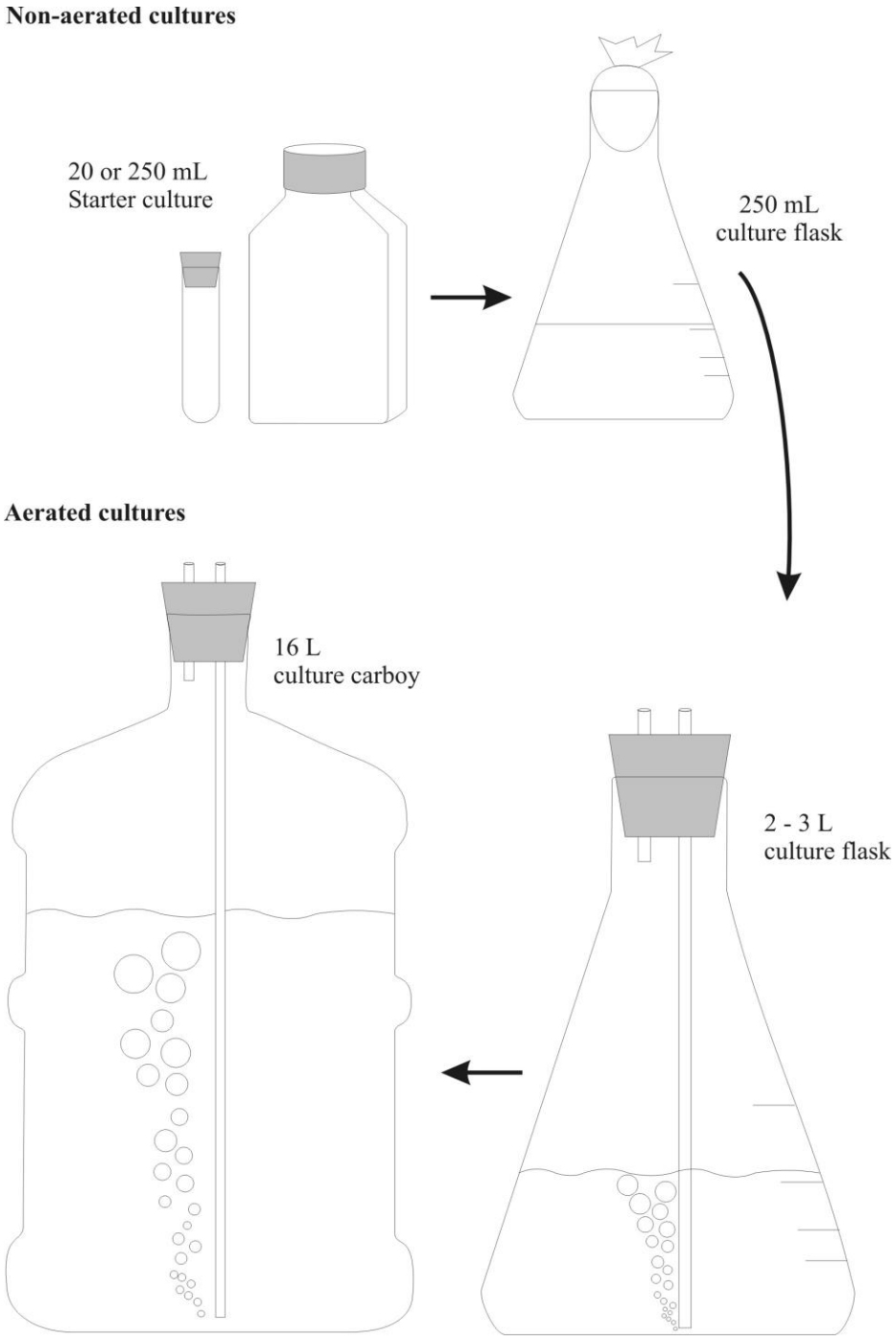


Fig. 2.1. Scale-up of microalgae cultures from starter cultures used during this study.

Table 2.1. Microalgae species used in this study, their growth rates (divisions/day), cell sizes and mean (± s.e.) dry-weight.

Microalgal species	Code[a]	Cell size (μm)	Dry weight (pg cell^{-1})	Divisions day^{-1} (k)	Strain origin
Bacillariophyceae					
Chaetoceros muelleri	CS-176	5 - 8	23.15 (2.50)	0.7	Hawaii, USA
Chaetoceros sp.	CS-256	8 x 3	49.47 (10.70)	0.3	North Queensland, Australia.
Skeletonema sp.	CS-252	8 x 5	26.38 (4.08)	0.3	North Queensland
Haptophyceae					
Isochrysis sp.	CS-177	3 x 5	68.89 (10.01)	0.6	French Polynesia
Pavlova salina	CS-49	4 x 8	25.20 (3.66)	0.4	Sargasso Sea
Pavlova sp.	CS-50	5 x 5	46.45 (9.58)	0.3	Sargasso Sea
Prasinophyceae					
Micromonas pusilla	CS-170	1.8 x1.8	3.64 (1.13)	0.4	N.W. Australia
Prasinophyta sp.	CS-126	3 x 3	8.72 (2.91)	0.4	Coral Sea, Australia

[a] CSIRO identification catalogue code

2.2.2 Cell counting

Aliquots (3-mL) from each carboy were collected for all species for cell counting and daily cell counts (six replicate counts per species) were undertaken using a Neubauer haemocytometer. Growth rate of each culture (Ke or μ = divisions per day) was calculated using the equation:

$$\text{Ke or } \mu = (\ln N_2 - \ln N_1)\,(t_2 - t_1)^{-1}$$

where: N_2 = number of cells mL^{-1} at time of harvest t_2 and N_1 = cells mL^{-1} at time t_1 (Guillard, 1973) from day 2 until harvesting day depending on the species (7 to 10 days). The Ke or μ values obtained were then converted to divisions per day following the equation:

$$k = Ke / 0.6931 \qquad \text{(Guillard, 1973).}$$

2.2.3 Harvesting and algal paste processing

The content of each carboy was centrifuged (3000 g, 30 min.) using a large volume centrifuge with 3-L capacity (4 x 750 mL vessels per cycle). The resulting microalgae concentrates were washed with 0.5 M of ammonium formate (200-mL) and centrifuged a second time (3000 g, 15 min). The resulting supernatants were discarded and the microalgae concentrates were placed in vials which were frozen in liquid nitrogen and stored at -80°C until analysis for protein, total lipid and fatty acid contents.

Microalgae dry-weight was obtained the day of harvesting. Triplicate aliquots of 15 to 30-mL, taken from the initial 100-mL removed from the carboys, were filtered through pre-weighed, precombusted (450ºC; 2 h), glass-fiber micro filters (Whatman GF/F, 25 mm). Isolated microalgae were then washed with 0.5 M ammonium formate (20-mL) and dried at 100 ºC for 16 h to volatilise the ammonium formate (Epifanio, 1979). Filters were then re-weighted to determine the dry weight of microalgae mL^{-1} (Brown et al., 1998).

2.2.4 Biochemical analysis of microalgae

Samples analysed for protein, total lipid and fatty acid content were freeze-dried prior to analysis. Protein contents were calculated from total Kjeldahl nitrogen (x 6.25) by the combustion method of Sweeny (1989) using an Elementar RapidN analyser. Lipids were extracted with chloroform-methanol (Folch et al., 1957) and total lipid content was determined gravimetrically. Further portions of the total lipid extract were taken to determine fatty acid compositions of the microalgae. Fatty acids within the lipid extracts were derivitised to fatty acid methyl esters (FAME) using 14% boron trifluoride-methanol (Van Wijngaarden, 1967). FAME were analysed on an Agilent Technologies 6890 gas chromatograph using helium as the carrier gas and a flame ionization detector. The column used was a DB23 fused silica column, 30 m x 0.25 mm, with a 0.25 µm coating (Agilent Technologies, USA). Column temperature was held at 140°C for 5 min and then elevated at a rate of 3°C min^{-1} to 210°C where it was held until all FAME of interest had been eluted. FAME were identified by comparing their retention times with

those of authentic standards (Sigma-Aldrich Co, USA), and were quantified using heptadecanoic acid as the internal standard.

Carbohydrate content was determined from triplicate aliquots of 60 to 100-mL of microalgae culture which were removed from the carboys, filtered through a glass-fibre micro-filter (Whatman GF/F, 25mm) and washed with 0.5 M ammonium formate. Samples were freeze dried and carbohydrate contents were determined by the phenol-sulphuric acid method of Dubois et al. (1956).

2.2.5 Amino acid determinations of microalgae

For amino acid (AA) determination, freeze-dried samples of the microalgae paste were hydrolysed in 6N HCl at 110°C for 18 h in sealed tubes blanketed with nitrogen. Amino acids were quantitated via reverse phase high-pressure liquid chromatography (HPLC) using a Waters Alliance 2690 system with Waters 474 variable wavelength detector (Moore, 1963) and pre-column derivatisation with 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Accq-Tag method, Waters Corporation) (Finlayson, 1964). Samples for cystine and methionine determination were oxidised prior to hydrolysis (as above) with performic acid at 0°C overnight. Cystine and methionine were separated by HPLC (as above) and detected using a Waters 474 fluorescence detector (Waters Corporation) (Moore, 1963). Tryptophan was determined following hydrolysis in 4.2 N NaOH at 110°C for 20 h in sealed tubes blanketed with nitrogen. Separation was

via reverse phase HPLC (as above) fitted with a UV/Vis. detector at 280 nm (Spackman et al., 1958; Helrich, 1990).

2.2.6 Data analyses

Data for proximate composition (total protein, lipid and carbohydrate) were tested for homoscedasticity using Levene's test. One-way ANOVA followed by a Tuckey HDS post hoc comparison was used to determine significant differences between biochemical contents in the different microalgae species tested.

2.3 Results

2.3.1 Proximate biochemical composition

The proximate compositions (total protein, lipid and carbohydrate) of the eight species of microalgae used in this study are shown in Table 2.2. Protein contents were very similar between species and ranged from 429.9 ± 8.4 mg g^{-1} in *Skeletonema* sp. to the highest levels in *M. pusilla* (568.4 ±1.7 mg g^{-1}) and *Pavlova* sp. (637.2 ± 0.7 mg g^{-1}). Total carbohydrate contents ranged from 12.6 ± 0.2 mg g^{-1} in CS-126 to 52.3 ± 2.3 mg g^{-1} in *Pav. salina*. There were no significant differences between the total carbohydrate contents of *Pavlova* sp. (31.3 ± 0.2 mg g^{-1}), TISO (33.3 ± 3.7 mg g^{-1}), *Chaetoceros muelleri* (37.4 ± 3.5 mg g^{-1}) and *Micromonas pusilla* (38.5 ± 0.7 mg g^{-1}). The golden-brown flagellates (*Pavlova* sp., TISO and *Pav. salina*) had the highest lipid contents

(271.6 ± 9.4, 299.9 ± 2.7 and 305.5 ± 17.2 m g g^{-1}, respectively) with similar lipid content shown by the green microalgae *M. pusilla* (241.6 ± 7.7 mg g^{-1}). CS-126 and *C. muelleri* had the lowest lipid contents of 101.4 ± 2.3 mg g^{-1} and 121.4 ± 7.4 mg g^{-1}, respectively.

Table 2.2. Proximate compositions (mg g^{-1} dry weight) of the eight microalgae species investigated in this study. Values are means (± s.e.) where n = 3 for diatoms species and TISO and n=2 for other species. Means in the same column with different superscripts are significantly different (P <0.05).

Species	Carbohydrate	Lipid	Protein
Chaetoceros muelleri	37.4 (± 3.5)^{c}	121.4 (± 7.4)^{ab}	455.6 (± 0.5)^{ab}
Chaetoceros sp.	13.1 (± 1.9)^{a}	193.9 (± 2.4)^{c}	430.0 (± 2.6)^{a}
Skeletonema sp.	21.1 (± 4.3)^{ab}	158.9 (± 6.2)^{bc}	429.9 (± 8.4)^{bc}
Isochrysis sp. (TISO)	33.3 (± 3.7)^{bc}	299.9 (± 2.7)^{e}	477.7 (± 1.5)^{abc}
Pavlova salina	52.3 (± 2.3)^{d}	305.5 (± 17.2)^{e}	529.1 (± 4.3)^{cd}
Pavlova sp.	31.3 (± 0.2)^{bc}	271.6 (± 9.4)^{de}	637.2 (± 0.7)^{e}
Micromonas pusilla	38.5 (± 0.7)^{c}	241.6 (± 7.7)^{d}	568.4 (± 1.7)^{d}
CS-126	12.6 (± 0.2)^{a}	101.4 (± 2.3)^{a}	437.8 (± 3.1)^{ab}

2.3.2 Fatty acids compositions

Fatty acid compositions of the eight species of microalgae used in this study are shown in Table 2.3. Saturated fatty acid (SFA) contents were generally higher in golden-brown flagellates (23.1-27 mg g^{-1}) than in diatoms (8.9–17.3 mg g^{-1}). The SFA contents of *M. pusilla* (17.6 mg g^{-1}) and Prasinophyta sp. (CS-126) (12.1 mg g^{-1}) were similar to that of the diatoms. Myristic acid (14:0) and palmitic acid (16:0) were the dominant SFA in all species of microalgae. Golden-brown flagellates had the highest levels of total SFA ranging from 27 mg g^{-1} in *Pavlova* sp. to 23.1 mg g^{-1} in TISO compared to the rest of the microalgae analysed. *M. pusilla* (17.6 mg g^{-1}) and *Chaetoceros* sp. (17.3 mg g^{-1}) had higher levels of SFA than *C. muelleri* (8.9 mg g^{-1}) and CS-126 (12.1 mg g^{-1}).

Monounsaturated fatty acid (MUFA) contents were generally higher in diatoms (12.2–15 mg g^{-1}) than in the golden-brown flagellates (8.8-9.4 mg g^{-1}), with the exception of T-ISO which showed the highest MUFA content (17.5 mg g^{-1}) of all species analysed. The MUFA contents of green flagellates (7.5 mg g^{-1} and 6.1 mg g^{-1} for *M. pusilla* and CS-126, respectively) were similar to those of the golden-brown flagellates *Pav. salina* and *Pavlova* sp. (9.4 mg g^{-1} and 8.8 mg g^{-1}, respectively). Predominant MUFA were 16:1*n*-7 (palmitoleic acid) and 18:1*n*-9 (oleic acid) in all microalgal groups. Diatoms had the highest levels of palmitoleic acid (10.5-12.8 mg g^{-1}) while Prasinophytes showed the lowest values (< 0.1-1.2 mg g^{-1}). Levels of oleic acid were very similar between the microalgae species (0.4-1.6 mg g^{-1}) with the exception of TISO which showed the highest content of this fatty acid (8.8 mg g^{-1}) (Table 2.3).

Polyunsaturated fatty acid (PUFA) contents showed a different pattern to that of MUFA, with golden-brown flagellates containing the highest levels of PUFA (57.5-72.1 mg g^{-1}) compared to diatoms (13.5–19.5 mg g^{-1}). The PUFA content of *M. pusilla* (36.7 mg g^{-1}) was similar to that of the golden-brown flagellates (58-72 mg g^{-1}), while the PUFA content of Prasinophyta sp. (CS-126) (19.3 mg g^{-1}) was more similar to the range found in diatoms (14-20 mg g^{-1}). TISO showed the highest content of 18:2*n*-6 (linoleic acid, 19.1 mg g^{-1}), while *M. pusilla* had the highest content of 18:3*n*-3 (α-linolenic acid, 9.5 mg g^{-1}) followed by TISO (8.8 mg g^{-1}). *Pav. salina* and *M. pusilla* were characterised by their relatively high levels of 18:4*n*-3 (stearidonic acid, 17.6 and 18.2 mg g^{-1}, respectively). EPA (20:5*n*-3) and DHA (22:6*n*-3) were present in all the microalgae species. *Pavlova* sp. had the highest EPA content of all microalgae (35.2 mg g^{-1}) analysed followed by *Pav. salina* (30.8 mg g^{-1}). The EPA contents of diatoms ranged from 10.4-15.4 mg g^{-1}, while the lowest EPA levels were shown by the Prasinophycea group and TISO (0.5-4.0 mg g^{-1}). Golden-brown flagellates showed the highest contents of DHA (8.7-12.1 mg g^{-1}) of all species analysed. Relatively low levels of arachidonic acid (ArA, 20:4*n*-6) were found in all microalgae species (<0.1-1.9 mg g^{-1}) (Table 2.3).

Table 2.3 Fatty acid compositions (mg g^{-1}) of the eight species of microalgae investigated in this study

	Microalgae species							
Fatty Acid	*C. muelleri*	*Chaetoceros* sp.	*Skeletonema* sp.	TISO	*Pav.salina*	*Pavlova* sp.	*M.pusilla*	*CS-126*
14:0	5.5(±0.4)	13.4(±0.3)	9.9(±0.3)	14.2(±0.2)	10.5(±0.4)	19.1(±0.5)	2.6(±0.3)	0.7(±0.1)
15:0	0.2(±0.0)	0.3(±0.0)	0.3(±0.0)	0.3(±0.0)	0.2(±0.0)	0.3(±0.1)	-	0.9(±0.3)
16:0	2.7(±0.1)	3.0(±0.1)	3.0(±0.1)	8.2(±0.2)	12.4(±0.2)	7.4(±0.4)	14.3(±0.6)	10.0(±0.3)
18:0	0.5(±0.0)	0.4(±0.0)	0.2(±0.0)	0.3(±0.1)	0.2(±0.1)	0.2(±0.1)	0.7(±0.1)	0.5(±0.1)
20:0	-	-	-	-	-	-	-	-
22:0	-	0.2(±0.0)	0.1(±0.1)	0.2(±0.1)	-	-	-	-
24:0	-	-	-	-	-	-	-	-
Sum SFA	8.9	17.3	13.5	23.1	23.3	27.0	17.6	12.1
14:1*n*-5	0.1(±0.4)	0.3(±0.1)	0.1(±0.0)	0.5(±0.0)	-	-	-	-
16:1*n*-7	10.5(±0.4)	12.8(±0.1)	11.7(±0.4)	6.3(±0.2)	4.1(±0.4)	7.3(±0.3)	1.8(±0.2)	-
18:1*n*-9	0.6(±0.1	1.6(±0.1)	0.6(±0.1)	8.8(±0.1)	0.5(±0.1)	0.4(±0.2)	0.7(±0.3)	1.1(±0.0)
18:1*n*-7	1.0(±0.2)	0.3(±0.1)	1.0(±0.1)	1.9(±0.1)	0.2(±0.2)	0.2(±0.3)	3.9(±0.2	0.5(±0.2)
Sum MUFA	12.2	15.0	13.4	17.5	4.9	8.0	6.4	1.6
18:2*n*-6	0.5(±0.1)	0.8(±0.1)	0.4(±0.1)	19.1(±0.3)	1.1(±0.1)	0.4(±0.1)	2.3(±0.2)	2.8(±0.5)
18:3*n*-6	0.4(±0.1)	0.4(±0.1)	0.3(±0.1)	2.4(±0.1)	0.7(±0.2)	0.1(±0.1)	1.0(±0.1)	1.5(±0.5)
18:3*n*-3	-	-	0.1(±0.0)	8.8(±0.3)	1.4(±0.2)	3.0(±0.2)	9.5(±0.8)	1.9(±0.2)
18:4*n*-3	0.3(±0.1)	0.5(±0.1)	1.8(±0.1)	11.9(±0.3)	17.6(±0.6)	15.4(±0.7)	18.2(±0.8)	7.8(±0.5)
20:2*n*-6	-	-	-	0.1(±0.1)	-	0.1(±0.1)	-	-
20:3*n*-6	-	-	-	-	-	-	-	-
20:3*n*-3	-	-	-	-	-	-	-	-
20:4*n*-6	1.4(±0.2)	1.9(±0.1)	0.2(±0.1)	0.6(±0.1)	0.9(±0.1)	1.5(±0.1)	-	0.7(±0.3)
20:4*n*-3	-	-	-	-	-	-	-	-

(-) denotes values less than 0.1.

Table 2.3 Continued

Fatty Acid	*C. muelleri*	*Chaetoceros* sp.	*Skeletonema* sp.	TISO	*Pav.salina*	*Pavlova* sp.	*M.pusilla*	*CS-126*
20:5*n*-3	10.4(±0.5)	15.4(±0.2)	11.6(±0.4)	0.7(±0.1)	30.8	35.2(±1.1)	0.5(±0.3)	4.0(±0.4)
22:2*n*-6	-	-	-	-	-	-	-	-
22:4*n*-6	-	-	-	-	-	-	-	-
22:5*n*-6	-	-	-	1.8(±0.1)	3.7(±0.2)	7.6(±0.4)	-	-
22:6*n*-3	0.5(±0.1)	0.5(±0.1)	1.2(±0.1)	12.1(±0.2)	10.6(±0.7)	8.7(±0.5)	5.3(±0.3)	0.3(±0.1)
Sum PUFA	13.5	19.5	15.6	57.5	66.8	72.1	36.7	19.3
Total FA	34.6	51.8	42.5	98.1	98.7	106.9	60.7	33.0
Sum EPA+DHA	0.5	0.5	12.8	12.8	41.4	43.9	5.8	4.3
Sum *n*-3	11.2	16.4	14.8	33.5	60.5	62.3	33.5	14.2
Sum *n*-6	2.3	3.1	0.8	23.9	6.3	9.7	3.3	5.1
n-3 / *n*-6	4.9	5.2	18.2	1.4	9.6	6.4	10.3	2.8

(-) denotes values less than 0.1.

2.3.3 Amino acid composition

Amino acid compositions of the seven species of microalgae used in this study are shown in Table 2.4. Amino acid contents were fairly similar between the different microalgae classes. Of the essential amino acids, leucine showed the highest relative concentration (7.9-10.4% of total amino acids) followed by arginine (6-9.3% of total amino acids) and threonine (5-8.2%). Non essential amino acid levels were characterized by relatively high levels of glutamic acid (8.3-12.9% of total amino acids), aspartic acid (6.2-9.4% of total amino acids) and glycine (6.3-7.6 %). All microalgae showed relatively low concentrations of cystine (1-2.9%), methionine (1.8-4 %) and histidine (1.6-2.3%).

Table 2.4. Amino acid profiles of the microalgae used in this study (% of total amino acids). Na = not analysed

Amino acid	*C.muelleri*	*Chaetoceros sp.*	*Skeletonema sp.*	T-iso	*Pav.salina*	*Pavlova sp.*	*M. pusilla*	CS-126
Essential								
Arginine	6.5	6.0	7.9	8.3	8.5	9.3	6.5	7.5
Histidine	1.6	1.8	1.5	2.3	1.9	2.3	1.8	1.7
Iso-Leucine	5.3	5.5	5.4	4.9	4.7	4.3	4.8	4.4
Leucine	8.5	7.9	9.5	10.0	10.4	9.7	9.4	8.4
Lysine	5.0	5.1	4.5	3.9	3.8	3.8	5.4	4.0
Methionine	1.8	2.1	2.4	4.0	3.2	3.1	na	2.5
Phenylalanine	5.8	5.9	6.9	7.1	7.3	8.1	6.4	6.0
Proline	4.6	5.6	4.9	5.4	5.1	5.2	5.9	5.9
Threonine	5.0	5.1	5.4	5.7	5.2	5.2	5.6	8.2
Trypthophan	6.2	3.2	2.9	3.6	2.8	3.1	na	na
Valine	5.5	5.5	5.9	5.8	6.1	6.0	6.5	6.9
Subtotal EAA	55.8	53.7	57.1	60.9	59.1	60.2	52.3	55.6
Non-essential								
Alanine	5.9	6.2	4.9	5.6	5.6	4.8	7.2	6.7
Asppartic acid	8.8	9.4	8.6	6.5	6.8	6.2	11.2	8.1
Cystine	1.6	2.5	1.0	1.5	1.7	2.5	na	2.9
Glutamic acid	11.7	11.9	10.2	8.4	9.3	8.3	12.9	10.7
Glycine	6.5	6.3	7.6	7.0	7.3	7.0	7.6	6.6
Serine	5.5	5.6	6.0	5.2	5.5	5.6	4.7	5.6
Tyrosine	4.1	4.4	4.6	4.9	4.6	5.3	4.1	3.8
Subtotal NEAA	44.2	46.3	42.9	39.1	40.9	39.8	47.7	44.4

2.4 Discussion

2.4.1 Proximate biochemical composition

The biochemical composition of microalgae varies between species and according to culture conditions (Lourenco et al., 2002; Renaud et al., 2002; Valenzuela-Espinoza et al., 2002). It is influenced by growth phase (Brown et al., 1993) and culture medium composition (Valenzuela-Espinoza et al., 2002) as well as abiotic factors such as irradiance (Thompson et al., 1993) and temperature (Renaud et al., 2002). Despite these influences, generalisations can be made regarding the gross biochemical composition of microalgae where the major organic component is protein (30-40% of total dry weight), followed by lipid (10-20% of total dry weight) and carbohydrate (5-15% of total dry weight) (Volkman and Brown, 2005). The culture conditions used for microalgae in this study favoured relatively high protein (43-63% of total dry weight) and lipid (10-30% of total dry weight) contents in the species used and relatively low levels of carbohydrate (1-5% of total dry weight). The total accountable organic matter, determined as the sum of the measured protein, carbohydrate and lipid components of microalgae, ranged from 55.2% of dry weight for CS-126 to 94.0% of dry weight for *Pavlova* sp. (CS-50). These values are within the ranges reported in similar studies where the proximate compositions of microalgae have been determined (e.g. Brown and Jeffery, 1992; Brown et al., 1998; Thinh et al., 1999).

2.4.2 Fatty acid composition

There were clear differences in fatty acid composition according to taxonomic groupings of microalgae. Golden-brown flagellates had generally higher levels of SFA, with myristic acid (14:0) and palmitic acid (16:0) being predominant. Polyunsaturated fatty acids (PUFA) were also present in relatively high levels in the golden-brown flagellates. DHA in particular showed the highest levels in TISO, *Pavlova* sp. and *Pav. salina* while EPA levels were highest in species of *Pavlova*. Diatoms were characterized by relatively high levels of MUFA composed predominantly of 16:1*n*-7. The PUFA contents of diatoms were low compared to golden-brown flagellates and, although containing higher levels of EPA than Prasinophyte species, contained around half the levels of EPA than the golden-brown flagellates. The two Prasinophytes assessed in this study showed differences in their SFA, MUFA and PUFA contents. *M. pusilla* had higher levels of SFA and considerably higher levels of PUFA compared to CS-126. Palmitic acid was the predominant SFA in this group while 18:3*n*-3 and 18:4*n*-3 were the major PUFA. These results are similar to those of previous studies reporting on the fatty acid compositions of various microalgae (Helm and Laing, 1987; Volkman et al., 1989; Dunstan et al., 1992; Brown et al., 1997; Müller-Feuga et al., 2003).

2.4.3 Amino acid composition

It has been suggested that essential amino acid composition determines differences in the nutritional value of microalgae species (Brown, 1991) and that nutritive quality of protein is determined by content, proportion and availability of its amino acids (James et al.,

1989). However, it is generally accepted that amino acid levels in microalgae are very similar irrespectively of algal class (Brown et al., 1997) with only minor differences found in the levels of specific amino acids in most microalgae species (Brown, 1991).

Amino acid values in this study are within the ranges reported in similar studies where the amino acid compositions of microalgae have been determined. For example, aspartic acid and glutamic acid are usually found in the highest concentration (7.1-12.9%) compared to the other amino acids in microalgae (Brown, 1991). Cystine, methionine, histidine and tryptophan are generally found within the range of 0.4 to 3.2% (Brown, 1991; Brown and Jeffrey, 1992; Volkman et al., 1993; Dunstan et al., 1994). However, levels of tryptophan found in this study (2.9-6.2%) were relatively high compared to those reported in previous studies (0.4-3.1%) (Brown, 1991; Brown and Jeffrey, 1992) ranged between of total amino acids.

2.4.4 Microalgae as food for bivalve

Assuming appropriate cell size allows ingestion and that ingested microalgae cells are readily digested, the nutritional value of microalgae for given species depends on the degree to which it satisfies the nutritional requirements of that species. Few studies have assessed the nutritional value of tropical microalgae for pearl oyster larvae and no prior study has attempted to relate the observed nutritional value of tropical microalgae to their nutrient compositions. The nutritional values of seven of the eight species of microalgae analysed in this chapter are determined for *Pinctada margaritifera* larvae in Chapter 3.

The detailed biochemical analyses undertaken in this chapter will allow identification of important nutritional components for *P. margaritifera* larvae and nutrients imparting high nutritional value to microalgae.

Chapter 3

The nutritional value of seven species of tropical microalgae for *Pinctada margaritifera* (L.) larvae

3.1 Introduction

Cultured pearls provide the basis for major mariculture industries in the Asia-Pacific region (Gervis and Sims, 1992; Fassler, 1997). The majority of cultured pearl production occurs in tropical regions using the silver- or gold-lip pearl oyster, *Pinctada maxima*, and the black-lip pearl oyster, *P. margaritifera*. Over recent years, hatchery production has become an increasingly important source of pearl oyster stock for the pearling industry (Gervis and Sims, 1992), and hatchery culture methods are well established for both *P. maxima* and *P. margaritifera* (Rose and Baker, 1994; Southgate and Beer, 1997). Early research into the artificial rearing of tropical pearl oyster larvae highlighted problems when temperate microalgae species were used as a food source for larvae cultured at tropical water temperatures (Minaur, 1969; Tanaka et al., 1970b). Although increasing numbers of tropical microalgae species have become available to aquaculture over recent years, no prior study has determined the nutritional value of tropical microalgae for pearl oyster larvae on the basis of their nutrient compositions.

Biochemical composition is a major factor in determining the nutritive quality of microalgae and their utility as food for bivalves. Much of the research in this field has

been conducted with juvenile or adult bivalves (e.g. Rodhouse et al., 1983, Taylor et al., 1997) and has focused on the highly unsaturated fatty acid (HUFA) profiles of microalgae as an indicator of nutritional quality (e.g. Albentosa et al., 1996). The link between the presence of HUFA in microalgae, specifically EPA and DHA, 22:6*n*-3, and high nutritional value is now well established (Knauer and Southgate, 1999; Volkman and Brown, 2005). Fewer studies in this field have been conducted with bivalve larvae and, while dietary HUFA have been shown to be similarly important (Chu and Webb, 1984; Marty et al., 1992; Pernet et al., 2004), other studies have reported a negative influence of PUFA and *n*-3 fatty acids on growth of bivalve larvae and positive correlations between the levels of saturated fatty acids and larval growth (Thompson et al., 1996; Leonardos and Lucas, 2000). Furthermore, Whyte et al. (1990) reported a high correlation between dietary carbohydrate level and the quality of scallop, *Crassodoma gigantea*, larvae, where carbohydrate was thought to spare other major nutrients for tissue synthesis.

While there is limited and somewhat conflicting information on the relationship between the nutrient composition of microalgae and their nutritional value for bivalve larvae, a number of recent studies have used biochemically-based models to simulate growth and survival of bivalve larvae under varying conditions (Powell et al., 2002, 2004; Hofmann et al., 2004). Models suggested that increasing survival of oyster, *Crassostrea gigas*, larvae was associated with low dietary protein and high dietary lipid levels (Powell et al., 2002, 2004) and that relatively high levels of carbohydrate are important for larvae in preparation for settlement (Powell et al., 2004). Furthermore, Hofmann et al. (2004)

suggested that the quality of microalgae, as a factor influencing growth and survival of *C. gigas* larvae, was best described by the ratio of dietary protein to the sum of lipid and carbohydrate. Knowledge of the biochemical compositions of microalgae, the presence of key nutrients, and models based on biochemical changes in larvae, provide a useful guide to the potential nutritional value of microalgae and larval nutrient requirements. However, variation in the results obtained in growth trials with bivalve larvae and differences in cytomorphological characteristics of microalgae, such as cell wall thickness and resulting digestibility, require elucidation of their true nutritional value in growth trials with the target species.

This Chapter assessed the nutritional value of seven selected species of tropical microalgae for larvae of *P. margaritifera*. The detailed biochemical analyses of these species outlined in Chapter 2 will be used to identify nutrients imparting high nutritional value to microalgae when fed to *P. margaritifera* larvae.

3.2 Materials and Methods

The tropical microalgae used in these experiments are listed in Table 2.1 (page 25) and they were cultured as outlined in Chapter 2 (section 2.2.1). The microalgae used in this study included two species of diatom (Bacillariophyceae), *Chaetoceros muelleri* and *Chaetoceros* sp., three species of golden-brown flagellates (Haptophyceae), *Isochrysis* sp. (TISO), *Pavlova salina* and *Pavlova* sp., and two species of green flagellate

(Prasinophyceae) *Micromonas pusilla* and Prasinophyte sp. Microalgae species were harvested in the late-log growth phase when used as food for the larvae.

3.2.1 Spawning induction and larval rearing

Pinctada margaritifera adults were obtained from culture stock held on a long-line at Orpheus Island, north Queensland, Australia (18º35´S, 146º29´E). Spawning induction, fertilization and larval rearing were conducted as described by Southgate and Beer (1997). Twenty-four hours after fertilization, D-stage veliger larvae were drained from 500-L incubation tanks and counted. Larvae were either used in Experiment 1 or cultured for 10 more days to be used in Experiment 2. Larvae used in Experiment 2 were stocked into 500-L tanks containing gently aerated, 34‰ salinity, 1-µm filtered seawater at a temperature of 28 (± 1) ºC. Initial larval stocking density was 3 mL^{-1}. Larval culture tanks were provided with a 1:1 mixture (on dry weight basis) of *Pav. salina* and TISO at a density of 5 to 10 cells per µL during larval development (Doroudi and Southgate, 2000); prior to the start of Experiment 2.

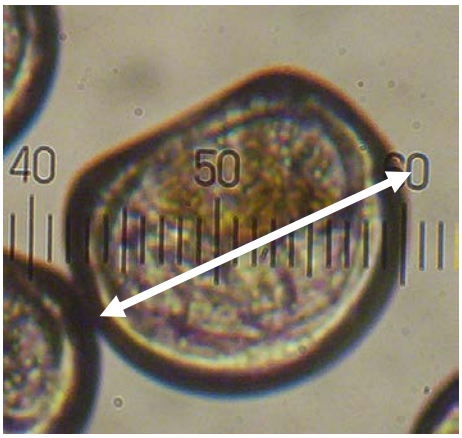
3.2.2 Experiment 1. Assessing tropical microalgae for D-stage larvae

One-day old *P. margaritifera* larvae with a mean (± s.e.) antero-posterior shell measurement (APM) of 81.3 ± 0.06 µm (n = 30) were placed in 12-L aquaria containing 10-L of gently-aerated 1-µm filtered seawater (FSW) at a density of 4.1 larvae mL^{-1}. Larvae were fed one of seven microalgae species (section 3.2) on a daily basis in three

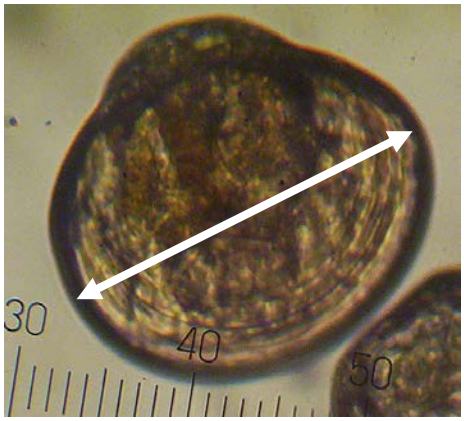
randomly allocated replicate aquaria. A 100% water exchange was conducted in each aquarium every second day.

Prior research in this laboratory determined optimal rations of microalgae for *P. margaritifera* larvae of different ages, using a standard diet composed of a 1:1 mixture of *Pav. salina* and TISO (Doroudi et al., 1999a; Doroudi and Southgate, 2000). Based on this research, the rations chosen for use throughout the present study were the equivalent of 5 cells μL^{-1} (of a 1:1 mixture of *Pav. salina* and TISO) for 2-8 day old larvae, 10 cells μL^{-1} for 9-14 day old larvae, and 15 cells μL^{-1} for older larvae. Using the mean dry weights of individual cells of *Pav. salina* and TISO shown in Table 2.1, the total dry weights of these rations were calculated as 0.235 $\mu g\ mL^{-1}$, 0.470 $\mu g\ mL^{-1}$ and 0.705 $\mu g\ mL^{-1}$, respectively. These dry weight values were used to calculate the ration of each microalgal species fed to larvae (i.e. the number of cells mL^{-1}) by dividing the required ration dry weight (appropriate for the age of larvae) by the known dry weight of individual cells of a particular species of microalgae (see Table 2.1). On this basis, each treatment received the same ration on a dry weight basis although microalgal cell density varied (Southgate et al., 1998b). The experiment was terminated after 10 days when larvae from each aquarium were collected on a 37 µm mesh sieve and washed into a 500-mL beaker. The beaker was topped up to 500-mL with FSW, then three 250-µL aliquots were taken for counting using a Sedwick-Rafter counting chamber. Survival estimation was the difference between the initial and final larvae density. Aliquots were removed from the beaker and preserved (Culliney et al., 1974) for shell length measurement. The

APM of 30 randomly selected larvae from each aquarium was measured using an optical microscope at the end of the experiment (Fig. 3.1).



D-stage veliger larvae



Umbone veliger larvae

Fig. 3.1. Antero-posterior shell measure (APM) or shell length of D-stages and umbone *P. margaritifera* larvae.

3.2.3 Experiment 2. Assessing tropical microalgae for umbo-stage larvae

This experiment was conducted under the same conditions as Experiment 1. Day-11 larvae with mean (± s.e.) APM of 122.9 ± 0.09 µm (n= 30) were distributed in 12-L plastic aquaria at a density of 2 larvae mL^{-1}. Larvae were fed with the same microalgae species used in Experiment 1. Ration (number of cells mL^{-1}) was calculated as detailed in s*ection 3.2.2* and was equivalent to a dry-weight ration of 0.470 µg mL^{-1} for the first 3 days and 0.705 µg mL^{-1} for the remainder of the 8 day experiment. The APM of 30 randomly selected larvae from each replicate was measured (Fig. 3.1) using an optical

microscope at the start and end of the experiment and survival was estimated as detailed above (section 3.2.2).

3.2.4 Data analyses

Homoscedasticity was tested using Levene's test. Shell length and survival of the larvae in Experiment 1 were analysed using one-way ANOVA followed by a Tukey HSD post-hoc comparison to determine significant differences between treatment means (*P* being set at 0.05). Shell length and survival of larvae in Experiment 2 were analysed using the Kruskal–Wallis test followed by the Mann-Whitney U test to determine significant differences between means and the level of significance was adjusted using a Bonferroni correction (Zar, 1999).

Correlations between the nutritional components of the seven species of microalgae determined in Chapter 2 (total carbohydrate, lipid, protein, as well as total saturated, monounsaturated and polyunsaturated fatty acids and specific fatty acids) and larval growth were determined for data generated during both experiments in an effort to identify important nutritional components for *P. margaritifera* larvae and nutrients imparting high nutritional value to microalgae. Pearson's correlation test was performed to analyse these relationships (Zar, 1999).

3.3 Results

3.3.1. Experiment 1. Assessing tropical microalgae for D-stage larvae

Increase in mean APM of larvae fed the different microalgae species over the 10 day period of Experiment 1 is shown in Fig. 3.2A. The golden-brown flagellates *Pav. salina* and *Pavlova* sp. supported significantly greater increases in larval APM (P <0.05) than any other species (15.9 ± 0.9 and 15.9 ± 0.8 µm, respectively). TISO (10.8 ± 0.9 µm), *M. pusilla* (9.0 ± 0.4 µm) and *C. muelleri* (7.8 ± 0.6 µm) supported significantly lower larval growth rates than both species of *Pavlova* (P < 0.05) but significantly greater increase in larval APM than Prasinophyta sp. (CS-126) (4.0 ± 0.6 µm) and *Chaetoceros* sp. (3.7 ± 0.6 µm). The mean increase in APM of larvae fed *Chaetoceros* sp. and CS-126 did not differ significantly from that of unfed larvae (3.8 ± 0.6 µm).

Survival of larvae in Experiment 1 is shown in Fig. 3.2B and ranged from 15.2% to 64.6%. Highest survival was shown by larvae fed *Pavlova* sp. (64.6%) but this did not differ significantly from that of larvae fed TISO (57.7%), *Pav. salina* (52.4%), *M. pusilla* (41.9%) or unfed larvae which showed high survival (60.6%).

Relationships between the levels of various nutritional components of microalgae used in this study and resulting larval growth (increase in APM) are shown in Table 3.1. Protein (r = 0.790, *P= 0.035*), lipid (r = 0.782, *P= 0.038*) and carbohydrate (r = 0.774, *P= 0.041*) contents of microalgae were found to be significantly correlated to larval growth (Table 3.1). Not surprisingly, there was also a significant positive correlation between

accountable organic matter (the sum of protein, lipid and carbohydrate) and larval growth (r = 0.835, *P= 0.019*). However, the relationships between the ratio of protein to the sum of lipid and carbohydrate (P:L+C) and larval growth (r= -0.616, *P= 0.141*) (Table 3.1) and larval survival (r= 0.070, P = 0.882) were non significant. The SFA content of microalgae was significantly correlated to larva growth (r = 0.820, *P = 0.*024) specifically the sum of myristic acid (14:0) and palmitic acid (16:0) (r = 0.791, *P = 0.034*). Larval growth was also strongly correlated with PUFA content of microalgae (r = 0.922, *P = 0.003*), specifically DHA contents (r = 0.834, *P = 0.020*). Relationships between larval growth and the levels of 14:0, 16:0, MUFA, ArA and EPA were not significant (Table 3.1).

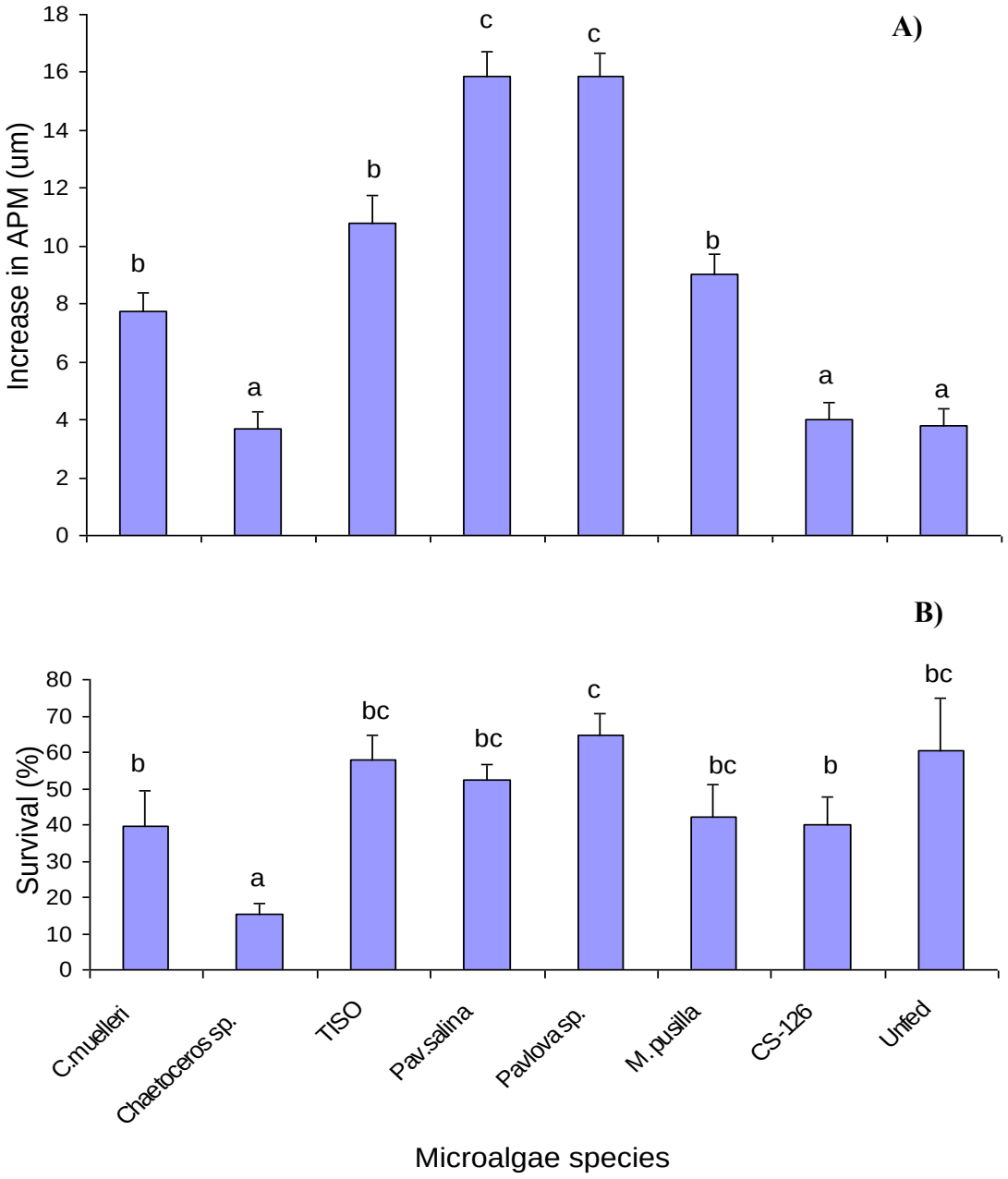


Fig. 3.2. Mean (± s.e. n= 30) increase in antero-posterior shell length (APM) (µm) (A) and survival (B) of *P. margaritifera* larvae fed seven species of microalgae during Experiment 1 (day 2 to day 11 after fertilization). Means with the same superscript are not significantly different (P >0.05).

Table 3.1. Pearson's correlations between increase in antero-posterior shell length (APM) of larvae during Experiment 1 and nutrient components of microalgae investigated in this study. * = Significant correlation; NS = non significant correlation.

Component	Correlation coefficient	Probability	
Accountable Organic Matter	0.835	0.019	*
Carbohydrate	0.774	0.041	*
Lipid	0.782	0.038	*
Protein	0.790	0.035	*
Protein: Lipid + Carbohydrate ratio			
Growth	-0.616	0.141	NS
Survival	0.070	0.882	NS
SFA	0.820	0.024	*
MUFA	- 0.115	0.806	NS
PUFA	0.922	0.003	**
ArA (20:4*n*-6)	-0.096	0.838	NS
DHA (22:6*n*-3)	0.834	0.020	*
EPA (20:5*n*-3)	0.670	0.100	NS
EPA + DHA	0.836	0.019	*
Myristic acid (14:0)	0.546	0.205	NS
Palmitic acid (16:0)	0.367	0.418	NS
14:0 + 16:0	0.791	0.034	*

*Correlation is significant at the 0.05 level (2-tailed)

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

3.3.2 Experiment 2. Assessing tropical microalgae for umbo-stage larvae.

Increases in mean APM of larvae during Experiment 2 are shown in Fig. 3.3A. Larvae fed *Pavlova* sp. and *Pav. salina* showed the greatest mean increase in APM (20.8 ± 1.8 and 20.4 ± 2.0 µm, respectively) but these were not significantly different to those of larvae fed TISO (11.0 ± 1.6 µm) and *C. muelleri* (13.8 ± 0.6 µm)(P >0.05). The APM increase of larvae fed *M. pusilla* (6.5 ± 1.5 µm), *Chaetoceros* sp. (1.5 ±1.2 µm) and Prasinophyta sp. (CS-126) (1.5 ± 1.1 µm) did not differ significantly from that of unfed (3.1 ± 1.2 µm) larvae. Larval survival during Experiment 2 is shown in Fig. 3.3B and ranged between 48.3% (unfed) and 68.9% (*Pavlova* sp.); however, there were no significant differences between treatments (P >0.05).

The same flagellate species shown to have good nutritional value for *P. margaritifera* larvae in Experiment 1 (*Pav. salina* and *Pavlova* sp., TISO and *M. pusilla*), plus the diatom *C. muelleri*, were the best performing microalgae for older *P. margaritifera* larvae in Experiment 2. Larval growth in Experiment 2 was significantly correlated with carbohydrate contents of microalgae (r = 0.769, *P = 0.043*); however, relationships between larval growth and the levels of lipid, protein, SFA, 14:0, 16:0, MUFA, ArA, DHA and EPA in microalgae were not significant. The relationship between larval growth and accountable organic matter was also non significant. The relationships between P:L+C and larval growth (r= 0.464, *P= 0.295*) and survival (r = -0.185, *P = 0.692*) were again non significant. Larval growth in Experiment 2 was significantly correlated with PUFA content (r =0.755, *P = 0.050*) and with the sum of EPA + DHA in microalgae (r = 0.829, *P = 0.21*) (Table 3.2).

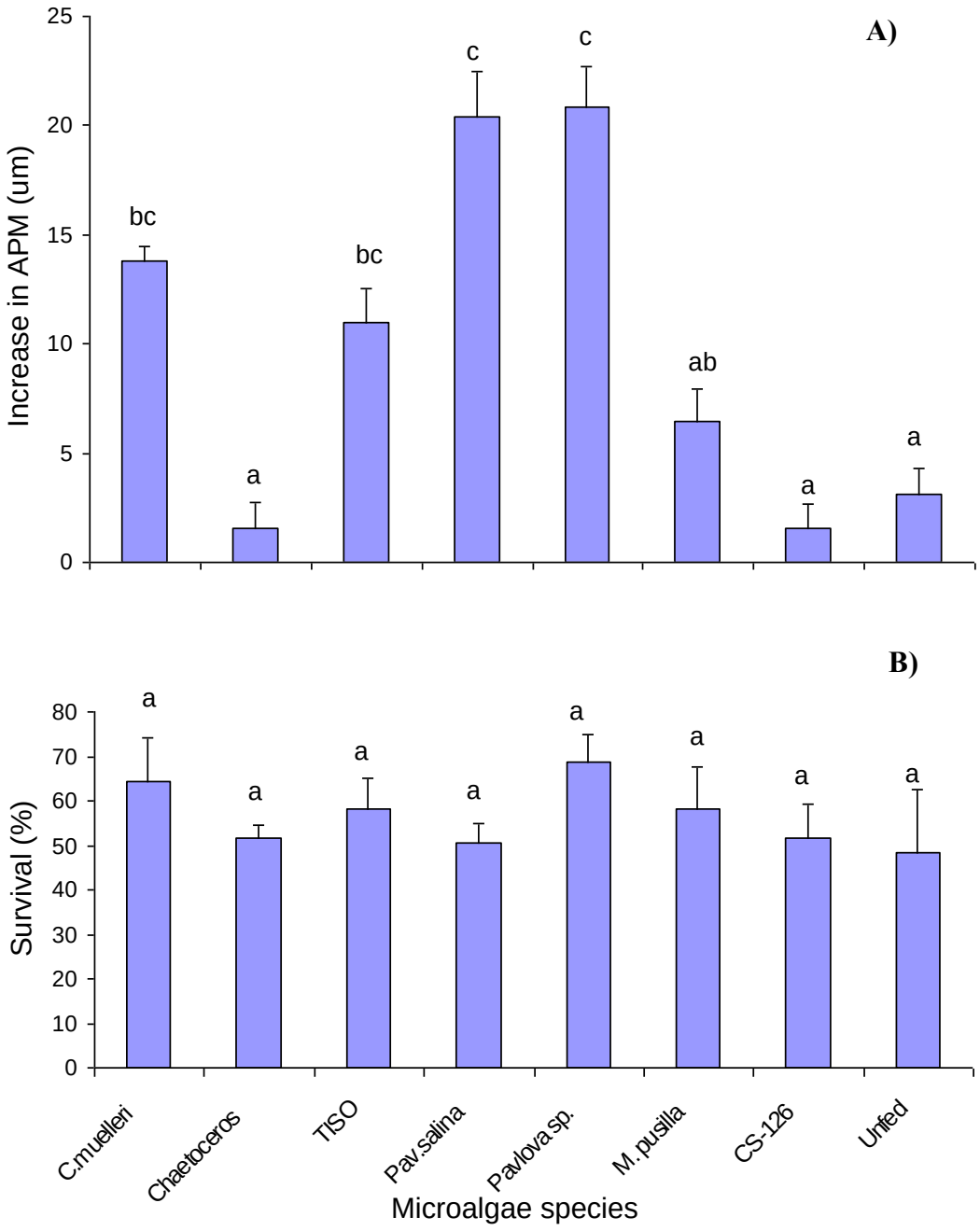


Fig. 3.3 Mean (± s.e. n= 30) increase in antero-posterior shell length (APM) (µm) (A) and survival a (B) of *P. margaritifera* larvae fed seven species of microalgae during Experiment 2 (day 11 to day 19 after fertilization). Means with the same superscript are not significantly different (P >0.05).

Table 3.2. Pearson's correlations between increase in antero-posterior shell length (APM) of larvae during Experiment 2 and nutrient components of microalgae investigated in this study. * = Significant correlation; NS = non significant correlation.

Component	**Correlation coefficient**	**Probability**	
Accountable Organic Matter	0.717	0.070	NS
Carbohydrate	0.769	0.043	*
Lipid	0.572	0.179	NS
Protein	0.656	0.109	NS
Protein: Lipid + Carbohydrate ratio			
Growth	-0.464	0.295	NS
Survival	-0.185	0.692	NS
SFA	0.622	0.139	NS
MUFA	-0.051	0.914	NS
PUFA	0.755	0.050	*
ArA (20:4*n*-6)	0.118	0.801	NS
DHA (22:6*n*-3)	0.655	0.110	NS
EPA (20:5*n*-3)	0.726	0.065	NS
EPA + DHA	0.829	0.021	*
Myristic acid (14:0)	0.521	0.231	NS
Palmitic acid (16:0)	0.098	0.835	NS
14:0 + 16:0	0.588	0.165	NS

*Correlation is significant at the 0.05 level (2-tailed)

3.4 Discussion

When the seven species of tropical microalgae used in this study were assessed as unialgal diets for *P. margaritifera* larvae, a similar pattern of growth was evident when fed to both D-stage larvae in Experiment 1 and umbone larvae in Experiment 2. The two species of microalgae supporting the best larval growth in both experiments were *Pav. salina* (CS-49) and *Pavlova* sp. (CS-50). Microalgae from the Prymnesiophytes which includes *Pavlova* spp. and *Isochrysis* spp., have been shown to be preferentially ingested by young pearl oyster larvae because their size and shape facilitate ingestion (Doroudi et al., 2003; Martínez-Fernández et al., 2004). Similarly in both experiments, *Chaetoceros* sp. (CS-256) and CS-126 supported the poorest larval growth and the mean APM of larvae receiving these species did not differ significantly from that of unfed larvae. Cytomorphological characteristics of microalgae are a major influence on their suitability as a food source for bivalve larvae. For example, large spiny appendages (Bacillariophyta) and 'cell wall' thickness (chlorophytes), respectively, may hamper ingestion and digestion by small pearl oyster larvae (Rose and Baker, 1994; Doroudi et al., 2003; Martínez-Fernández et al., 2004).

Young bivalve larvae may also lack an appropriate suite of digestive enzymes necessary to efficiently digest microalgae (Robert and Trintignac, 1997). The cell size of all species of microalgae used as food for *P. margaritifera* larvae in this study ranged between 1.8–8

μm. This size range is within that generally accepted as appropriated for mollusc larvae (Webb and Chu, 1982; Knauer and Southgate, 1999).

On the basis of larval growth in Experiment 1, the microalgae can be divided into three groups: (1) larvae fed *Pav. salina* and *Pavlova* sp., showed significantly greater growth than those fed other microalgae; (2) those fed *Isochrysis* sp., *C. muelleri* and *M. pusilla* showed significantly greater growth than unfed larvae; and (3) larvae fed *Chaetoceros* sp. and CS-126 did not grow at a rate greater than unfed larvae. For umbo-stage larvae, the same microalgae supported similar growth to those in Experiment 1 with those fed *Pav. salina* and *Pavlova* sp. again showing the greatest growth while those fed *Chaetoceros* sp. and CS-126 again grew at a rate similar to unfed larvae.

Available literature does not suggest a strong correlation between proximate composition of microalgae and their nutritional value (e.g. Volkman and Brown, 2005). Protein is the largest organic component of microalgae and, while high dietary protein has been associated with good growth of juvenile bivalves (Knuckey et al., 2002), this relationship is less clear for bivalve larvae (Webb and Chu, 1982). The protein content of the tropical microalgae used in this study ranged from 43-63% and there was a significant correlation between dietary protein content and larval growth in Experiment 1. Biochemically-based models suggest that high levels of dietary protein may improve growth rates of oyster, *Crassostrea gigas* larvae but, as a consequence, reduce their capacity to accumulate lipid reserves to the necessary levels for successful metamorphosis (Powell et al., 2002). Conversely, low protein diets reduce larval growth rates but allow greater storage of lipid,

resulting in increased metamorphic success (Powell et al., 2002). A number of studies have reported the importance of algal protein content, in association with other important algal nutrients, as a factor influencing growth and survival of bivalve larvae. Leonardos and Lucas (2000) used multidimensional models to correlate algal biochemical composition with growth of *Mytilus edulis* larvae. Protein content had an important influence on the nutritional value of microalgae in association with fatty acids and carbohydrate, and had either a primary role in determining nutritional value or a secondary 'modifying' role depending on the composition of the microalgae (Leonardos and Lucas, 2000). Similarly, Hofmann et al. (2004) reported that the quality of microalgae, as a factor influencing growth and survival of *C. gigas* larvae, was best described by the ratio of dietary protein to the sum of lipid and carbohydrate (P:L+C). The results of this study show that the growth of early *P. margaritifera* larvae was positively and significantly correlated with dietary levels of protein, lipid and carbohydrate but the growth of older larvae was significantly correlated with carbohydrate content only. Furthermore, we did not find any significant correlation in either experiment between P:L+C and growth or survival of *P. margaritifera* larvae.

High levels of microalgal carbohydrate have been associated with high growth rates of scallop, *Crassodoma gigantea*, larvae provided that dietary fatty acid requirements are satisfied (Whyte et al., 1990). Similarly in this study, there was a significant correlation between the carbohydrate content of microalgae and growth of both early and umbo-stage *Pinctada margaritifera* larvae. It is interesting to note that the five species of microalgae supporting significantly greater growth than unfed larvae in Experiment 1 also had the

highest carbohydrate contents ranging from 31.3-52.3 mg g^{-1} dry weight. Of these five species, *Pav. salina* and *Pavlova* sp. supported significantly greater growth than the others, and contained more than three times the sum of EPA and DHA than TISO which supported the third highest growth increment. Furthermore, the two species with the lowest carbohydrate contents, *Chaetoceros* sp. (13.1 ± 1.9 mg g^{-1} dry weight), and CS-126 (12.6 ± 0.2 mg g^{-1} dry weight) supported larval growth increments that did not differ from that of unfed larvae.

The total lipid content of microalgae used in this study was also significantly correlated with larval growth in Experiment 1. Of the species investigated in this study, the golden-brown flagellates (*Pav. salina*, *Pavlova* sp. and TISO) and the prasinophyte, *M. pusilla*, had the highest lipid contents (Chapter 2). These species supported four of the five highest growth increments of *P. margaritifera* larvae in Experiment 1. Lipid is a primary energy source for the early life stages of bivalves (Gallager et al., 1986; Laing and Millican, 1986) including *P. margaritifera* larvae (Strugnell and Southgate, 2003). On this basis, the total lipid content of dietary microalgae is likely to be an important factor in determining nutritional value of dietary microalgae and in influencing larval growth and survival. This is reflected in the significant correlation between growth of *P. margaritifera* larvae and the lipid content of microalgae found in Experiment 1. Lipid is accumulated in larval tissues during development of *P. margaritifera* where it is thought to be an important energy source during metamorphosis (Strugnell and Southgate, 2003). Simulations of the growth and development of *C. gigas* larvae based on biochemical

modelling indicate that diets with a high lipid content increase larval success because more lipid can be stored for metamorphosis (Powell et al., 2002).

As well as total lipid content, the results show that the fatty acid compositions of dietary lipid are a major influence on the growth rates of *P. margaritifera* larvae. There were clear differences in fatty acid composition according to taxonomic groupings of microalgae (Chapter 2, section 2.3.2, Table 2.3). This study showed a significant correlation between the growth of D-stage *P. margaritifera* larvae and the SFA contents (mg g^{-1} dry weight) of microalgae with the two microalgae supporting the greatest larval growth (*Pav. salina* and *Pavlova* sp.) having the highest SFA contents. In a similar study, Thompson et al. (1993) reported a correlation between the growth of *C. gigas* larvae and the SFA content of microalgae (as % of total fatty acids). Similar positive correlation between 16:0 and SFA contents of dietary microalgae and growth of mussel, *M. edulis*, larvae fed *Pav. lutheri* and *Rhinomonas reticulata* were reported by Leonardos and Lucas (2000). Species of microalgae with high levels of SFA are thought to be more nutritious for bivalve larvae because energy is released more efficiently from saturated fats than from unsaturated fats (Thompson et al., 1993). As well as providing energy, SFA also function as precursors for chain elongation and desaturation during synthesis of longer chain fatty acids including PUFA (Renaud et al., 2002). However, despite a significant correlation between SFA content of microalgae and the growth of D-stage *P. margaritifera* larvae, a similar relationship was not evident for older larvae in Experiment 2. While *Pav. salina* and *Pavlova* sp. again supported the highest larval growth in

Experiment 2, the microalgae with the lowest SFA content (*C. muelleri*) supported the third highest larval growth.

A similar pattern to that for SFA was also observed for PUFA with the two better performing species of microalgae in both experiments (*Pav. salina* and *Pavlova* sp.) having the highest PUFA contents. Significant positive correlations between PUFA content of microalgae and larval growth were seen in both experiments. The importance of EPA and DHA within the PUFA component is indicated by similar positive correlations between larval growth and the EPA+DHA content of microalgae in both experiments, and with DHA alone in Experiment 1. The nutritional value of microalgae for bivalves has been strongly associated with PUFA content (Marty et al., 1992) and particularly with levels of EPA and DHA (Enright et al., 1986; Delaunay et al., 1993), which are essential fatty acids for bivalve molluscs (Langdon and Waldock, 1981; Knauer and Southgate, 1999). However, Thompson et al. (1996) reported that the growth rate of *C. gigas* larvae was negatively correlated with dietary PUFA, particularly EPA, which had a strong negative effect on larval growth rate. Similar negative correlations between the growth of *M. edulis* larvae and both the PUFA and *n*-3 fatty acid content of *Pav. lutheri* and the 18:3*n*-3 and *n*-3 content of *Rhinomonas reticulata* were reported by Leonardos and Lucas (2000). Arachidonic acid (ArA) has also been identified as an essential dietary component for fish larvae (Emata et al., 2003) but little is known about the influence of this fatty acid on growth and survival of bivalve larvae. A recent study with larvae of the scallop, *Placopetcen magallanicus*, however, showed that a diet deficient in ArA (0.3-0.8 % of total fatty acid) resulted in poor growth and survival and

restricted fatty acid incorporation by the larvae (Pernet and Tremblay, 2004). In this study however, no significant relationship between ArA content of microalgae and growth of *P. margaritifera* larvae was evident.

This study is the first comprehensive assessment of the nutritional value of tropical microalgae species for pearl oyster larvae. The results show *Pavlova* sp. and *Pav. salina* to be the most nutritious of seven microalgae fed to both D-stage and umbone larvae of *P. margaritifera*. Another golden-brown flagellate, TISO, was also shown to be of high nutritional value. Our results support those of Southgate et al. (1998b) who showed *Pav. salina* and TISO to be of high nutritional value for *P. margaritifera* larvae; however, unlike the present study, Southgate et al. (1998b) reported TISO to have superior nutritional value to *Pav. salina* for both D-stage and umbo-stage larvae. This inconsistency may reflect differences in the conditions used in the two studies or differences in the physiological or biochemical composition of either larvae or microalgae used in the studies. The high nutritional value of *Pavlova* spp. for *P. margaritifera* larvae shown in this study has been reported in similar studies with the larvae of other bivalve molluscs (Walne, 1963; O'Connor and Heasman, 1997; Leonardos and Lucas, 2000). In contrast, however, a recent study which assessed the nutritional value of six species of Pavlovophyceae for *C. gigas* and *Pecten maximus* larvae, reported that all species (including *Pav. salina*) had poor food value for *C. gigas* larvae (Ponis et al., 2006). They concluded that members of the class Pavlovophyceae are unsuitable for *C. gigas* larvae (Ponis et al., 2006).

TISO and *Pav. salina* have been commonly used in combination as a food source for *P. margaritifera* larvae as they have high nutritional value and are readily ingested (Southgate and Beer, 1997; Southgate et al., 1998b; Doroudi et al., 2003). The results of this study validate the use of these microalgae as a food for *P. margaritifera* larvae. This study has, for the first time, allowed correlation between microalgae composition and growth of pearl oyster larvae. The results help identify nutrients imparting high nutritional value to microalgae and allow some interpretation of the nutritional requirements of *P. margaritifera* larvae. Bivalve larvae are generally fed a mixture of microalgae species to provide a better nutrient balance (Southgate, 2003). Identification of tropical microalgae species of high nutritional value for *P. margaritifera* larvae, provides a basis for investigating the use of these microalgae in dietary combinations with other species of microalgae. Use of combinations of tropical microalgae as food source for *P. margaritifera* is the basis for experiments in Chapter 4.

Chapter 4

Nutritional value of combinations of tropical microalgae for *Pinctada margaritifera* (L.) larvae

4.1 Introduction

A number of species of tropical microalgae have been assessed for their physical suitability as a food source for pearl oyster larvae (Doroudi et al., 2003) as well as their nutritional value (Taylor et al., 1997; Southgate et al., 1998b). Furthermore, a number of studies have reported on larval culture of *P. margaritifera* (Southgate and Beer, 1997; Doroudi et al., 1999a, b; Doroudi et al., 2002). Despite this research, there is not yet a 'standard' combination of microalgae which has broad acceptance as a diet for *P. margaritifera*. Pearl oyster larvae are usually supplied with golden-brown flagellates throughout larval development and with at least one diatom as they reach the umbo stage and approach metamorphosis (Southgate and Beer, 1997; Southgate et al., 1998b; Doroudi et al., 2003). While it is generally accepted that a micro-algal diet composed of more than one species provides a better nutritional 'balance' for bivalves (Webb & Chu, 1982; Southgate, 2003), there is a paucity of information on the nutritional value of binary and ternary microalgal diets for pearl oyster larvae.

The nutritional values of seven species of tropical microalgae for both D-stage and umbone larvae of *P. margaritifera* were determined in Chapter 3. Growth of D-stage larvae showed significant positive correlation with a number of biochemical components of microalgae (total protein, total lipid, total carbohydrate, total saturated fatty acids, total PUFA, DHA, EPA+DHA and 14:0+16:0, while growth of umbone larvae was positively correlated with total carbohydrate, PUFA and DHA+EPA contents.

This Chapter describes the growth rate and survival of *P. margaritifera* larvae fed binary and ternary combinations of the tropical microalgae species shown to be of high nutritional value in Chapter 3. The results have major implications for the optimisation of hatchery culture protocols for *P. margaritifera*.

4.2. Material and Methods

Microalgae culture (Chapter 2, section 2.2.1) and spawning induction and larval rearing (Chapter 3, section 3.2.1) were conducted as previously described. The microalgae species used in this study were *Pavlova salina*, *Pavlova* sp., *Isochrysis* sp. (clone TISO), *Chaetoceros muelleri*, *Chaetoceros* sp., *Skeletonema* sp. and the Prasinophyte, *Micromonas pusilla*. The combined eggs from five *P. margaritifera* females were fertilised using a mixture of spermatozoa from seven males. Eggs were incubated at a density of 30-mL^{-1} in gentle aerated 500-L tanks (Southgate and Beer, 1997) and 24 h after fertilization, D-stage larvae were drained onto a 37-µm sieve and counted. Larvae were then split into groups and either used in Experiment 1 or reared for 14 more days to

be used in Experiment 2. Larvae used in Experiment 2 were kept in 500-L tanks at 28˚C at an initial density of 2 larvae mL^{-1} with total water exchange every second day. Larvae were fed with the standard diet use in our laboratory for *P. margaritifera* larvae (0.470 µg mL^{-1}) (Doroudi and Southgate, 2000).

4.2.1 Experiment 1. Nutritional value of tropical microalgae combinations for D-stage larvae.

Three day-old larvae with a mean (± s.e.) antero-posterior measurement (APM) of 82.2 ± 0.6-µm were placed into aquaria containing 10-L of gentle aerated 1-µm filtered and UV irradiated seawater (FSW) at a density of 4.3 larvae mL^{-1}. Larvae were fed the flagellates *Pavlova salina*, *Pavlova* sp, T-ISO and *Micromonas pusilla* in all possible binary and ternary combinations. *Pavlova* sp. was also fed as single species diet as this species was shown to have superior nutritional value for D-stage *P. margaritifera* larvae in Chapter 3. Three replicate aquaria were randomly allocated to each diet combination which was supplied on a daily basis. A 100% water exchange was conducted for each aquarium every second day.

All dietary microalgae combinations were fed on an equal dry weight basis (Table 4.1) as detailed in *section 3.2.2*. Food ration for binary and ternary combinations was formulated considering the contribution of each individual species by dry-weight in a proportion of 1:1 (binary) and 1:1:1 (ternary). A ration equivalent to a dry weight of 0.235 µg mL^{-1}

was fed to 2-8 day old larvae and this increased to 0.470 μg mL^{-1} for the remainder of the experiment.

Table 4.1. Dry weight, major biochemical components (% of dry weight) and fatty acid (% of total fatty acids) composition of the seven tropical microalgae used as food for *P. margaritifera* larvae during this study. Dry weight (pg cell^{-1}); SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid (22:6*n*-3); EPA, eicosapentanoic acid (20:5*n*-3). Data from Chapter 2.

Microalgae	Dry weight	Protein	Lipid	Carbohydrate	SFA	PUFA	DHA	EPA+DHA
Chaetoceros muelleri	23.2	45.5	12.1	3.7	19.6	29.7	1.0	24.0
Chaetoceros sp.	49.8	43.0	19.3	2.1	24.8	28.1	0.8	22.9
Skeletonema sp.	26.4	49.3	15.9	1.3	21.8	25.1	2.0	20.7
TISO	68.8	47.7	29.9	3.3	23.0	57.8	12.1	12.8
Pavlova salina	25.2	52.9	30.5	5.2	28.0	69.6	11.1	43.2
Pavlova sp.	46.4	63.7	27.2	3.1	24.7	65.8	7.9	40.4
Micromonas pusilla	3.6	58.6	29.9	3.8	23.0	48.0	7.0	7.7

The experiment was terminated after 10 days when larvae from each aquarium were collected on a 37-µm mesh sieve and washed into a 500-mL beaker. Aliquots were removed from the beaker and preserved in larval fixative (Culliney et al., 1974) for shell length measurement and counts to estimate survival (Chapter 3). The APM of 30 randomly selected larvae from each aquarium was measured using an optical microscope at the end of the experiment. Larval growth was evaluated as the increase in mean APM (shell length).

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4.2.2 Experiment 2. Nutritional value of tropical microalgae combinations for umbo-stage larvae

This experiment was conducted under the same conditions as Experiment 1 (section 4.2.1). Fourteen day old umbo-stage larvae with mean (± s.e.) APM of 138.6 ± 2.7 µm (n= 30) were distributed in aquaria at a density of 2 larvae mL^{-1}. Larvae were fed with the best binary algae combination of flagellates from Experiment 1 (*Pavlova* sp./ *M. pusilla*) or *Pavlova* sp. in combination with one of three species of diatom (either *C. muelleri*, *Chaetoceros* sp. or *Skeletonema* sp.). In addition, the best two ternary diets (flagellates only) from Experiment 1 (*Pav. salina*/*Pavlova* sp./TISO and T-ISO/*M. pusilla*/*Pavlova* sp.) were also used in this experiment.

All dietary microalgae combinations were fed on an equal dry weight basis (Table 4.1) as detailed in *sections 3.2.2* and *4.2.1.* A ration equivalent to a dry weight of 0.470 µg mL^{-1}

was fed to larvae for the first 3 days and this increased to 0.705 µg mL^{-1} for the remainder of the experiment. The experiment was terminated after 13 days. The APM of 30 randomly selected larvae was measured using an optical microscope at the start and end of the experiment and larval survival was estimated as detailed for Experiment 1. Increase in mean APM of larvae from all treatments was calculated as described for Experiment 1 (section 4.2.1).

4.2.3 Data analyses

Homoscedasticity was tested using Levene's test to determine whether to use parametric or not parametric analysis for larval and survival data obtained. Larval shell length was analysed using the Kruskal–Wallis test (P = 0.001) followed by the Mann-Witney U test to determine significant differences between means and the level of significance was adjusted using a Bonferroni correction (P = 0.05). Larval survival was analysed using one-way ANOVA and significant differences between group means (P = 0.05) were determine using the Tukey HSD test (Zar, 1999).

4.3. Results

4.3.1 Experiment 1. Nutritional value of tropical microalgae combinations for D-stage larvae.

Growth rates of D-stage *P. margaritifera* larvae fed combinations of tropical microalgae over the 10 days duration of Experiment 1 are shown in Fig. 4.1. As might be expected

all diets supported superior growth rates than that of unfed larvae. The ternary combination of *Pavlova* sp./*Pav. salina*/TISO supported the highest growth rate of larvae with the binary combination of *Pavlova* sp./*M. pusilla* supporting the second highest growth rate. It is interesting to note that the growth rate of larvae fed *Pavlova* sp. only did not differ significantly from that obtained with the best ternary (*Pavlova* sp./*Pav. salina/M. pusilla*) or binary (*Pavlova* sp./*M. pusilla*) combinations. Indeed, there was no apparent nutritional gain by larvae fed *Pavlova* sp. together with any other species in either binary or ternary combinations when compared to those fed *Pavlova* sp. alone. The binary combination of *Pavlova* sp./*Pav. salina* resulted in significantly reduced larval growth rates when compared to that of larvae fed *Pavlova* sp. alone. Survival of larvae during Experiment 1 (Fig. 4.1) ranged from 88.1% (unfed larvae) to 98.9% (*Pavlova* sp./*M. pusilla*) but did not differ significantly between treatments ($P > 0.05$).

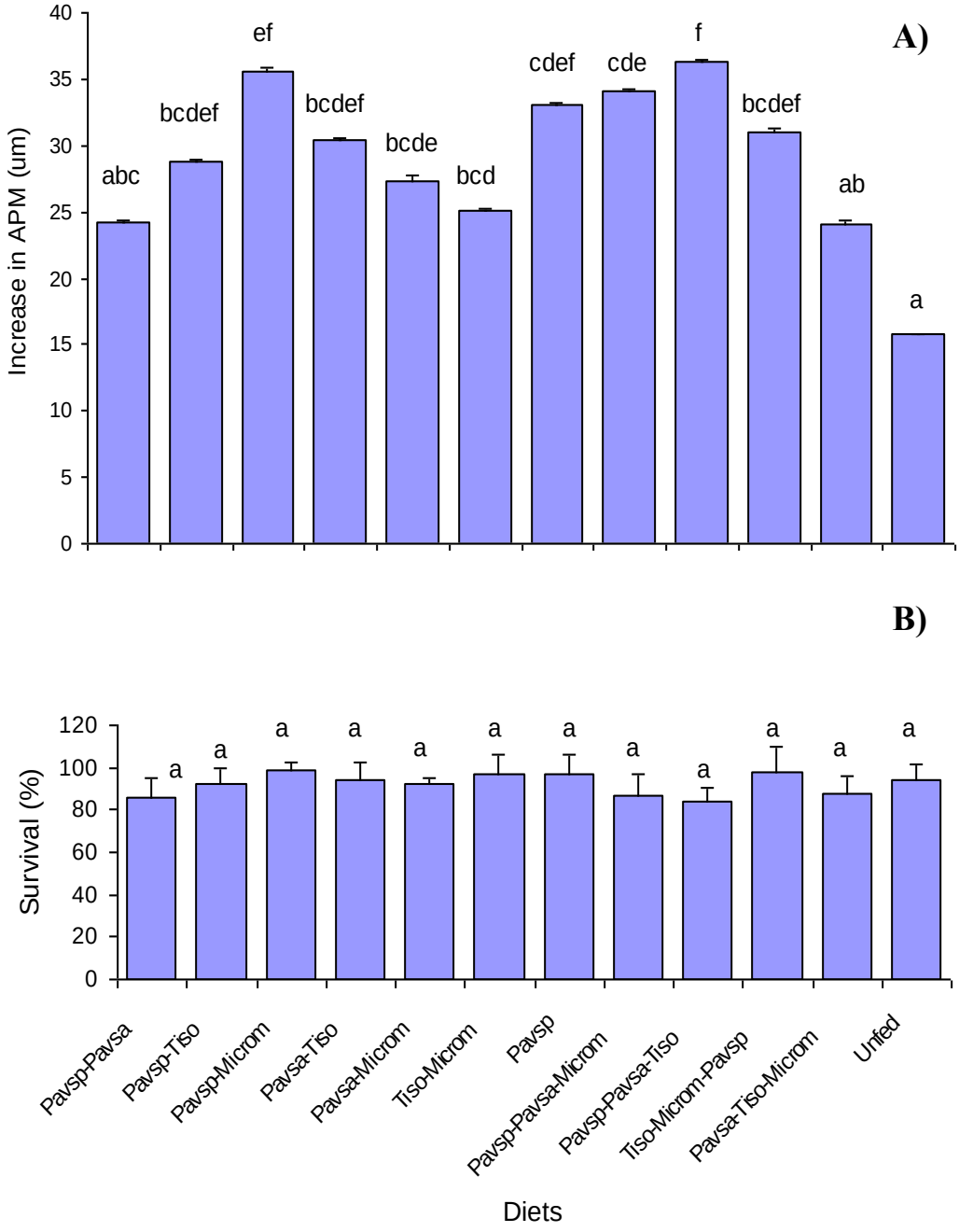


Fig.4.1. Mean (± s.e. n= 30) increase in antero-posterior shell length (APM) (µm) (A) and survival (B) of *P. margaritifera* larvae fed eleven microalgal diets during Experiment 1. Means with the same superscripts are not significantly different (P > 0.05). Abbreviations: Pavsp - *Pavlova* sp; Pavsa - *Pav .salina*; Microm - *M. pusilla*

4.3.2 Experiment 2. Nutritional value of tropical microalgae combinations for umbo-stage larvae

The growth rates of umbo-stage *P. margaritifera* larvae fed the best binary flagellate combination from Experiment 1 (section 4.3.1) plus a diatom are shown in Fig.4.2. Larvae fed combinations of microalgae which included a diatom showed a noticeable increase in growth rate compared to those fed diets lacking a diatom. For example, larvae fed the best binary combination from Experiment 1 (*Pavlova* sp./*M. pusilla*) showed significantly lower growth rates than larvae fed this diet in combination with either *C. muelleri* (*Pavlova* sp./*M. pusilla/C. muelleri*) or *Skeletonema* sp. (*Pavlova* sp./*M. pusilla/Skeletonema* sp.). The ternary combination of *Pavlova* sp./*M. pusilla/Skeletonema* sp. supported the highest growth rate of larvae in Experiment 2 followed by *Pavlova* sp./*M. pusilla*/*C. muelleri*. *Pavlova* sp. alone, which supported the second best growth rate of D-stage larvae in Experiment 1, was not so good for umbo-stage larvae in Experiment 2 where two ternary combinations (*Pavlova* sp./*M. pusilla*/*C. muelleri* and *Pavlova* sp./*M. pusilla/Skeletonema* sp.) and a binary combination (*Pavlova* sp./*C. muelleri*) supported significantly greater larval growth rates. Umbo-stage larvae fed diets composed of three flagellates (*Pavlova* sp./*Pav. salina*/ TISO and *Pavlova* sp./*M. pusilla*/TISO), which supported good growth rates of D-stage larvae in Experiment 1, showed relatively low growth rates. However, replacing T-ISO from the latter combination with either *C. muelleri* or *Skeletonema* sp., brought about a significant increase in larval growth rate (Fig. 4.2).

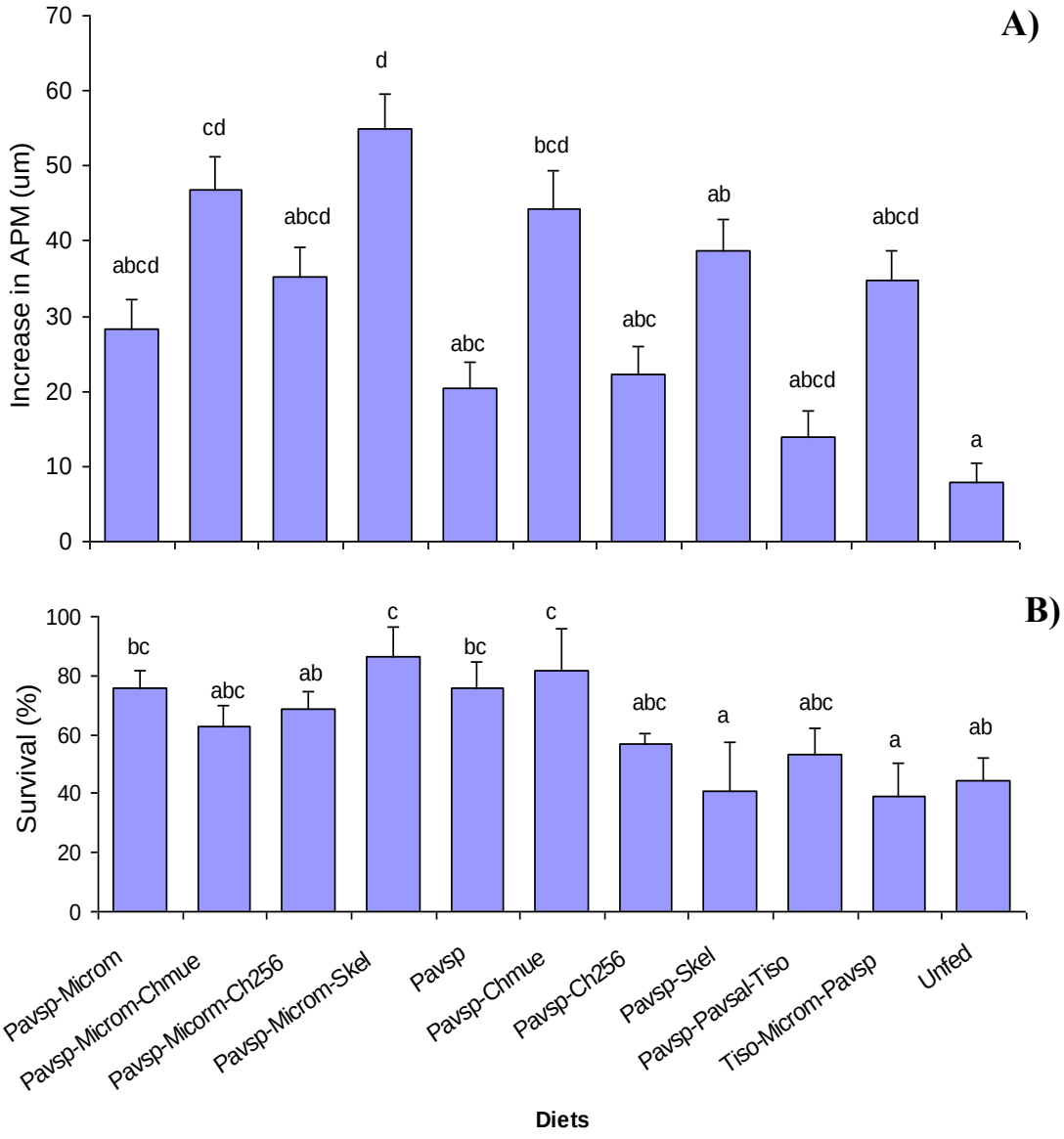


Fig. 4.2 Mean (± s.e. n= 30) increase in antero-posterior shell length (APM) (µm) (A) and survival (B) of Umbo-stage *P. margaritifera* larvae fed ten microalgal diets during Experiment 2. Means with the same superscripts are not significantly different (P > 0.05). Abbreviations: Pavsp- *Pavlova* sp; Pavsa- *Pav .salina*; Microm- *Micromonas pusilla*; Chmue- *Chaetoceros muelleri*; Ch256 - *Chaetoceros* sp; Skel- *Skeletonema* sp.

The growth rate of larvae fed the binary combination of *Pavlova* sp./*C. muelleri* did not differ significantly from that of larvae fed the ternary combinations of *Pavlova* sp./*M. pusilla*/*C. muelleri* and *Pavlova* sp./*M. pusilla/ Skeletonema* sp. Highest survival of larvae in Experiment 2 was recorded for those fed *Pavlova* sp./*M. pusilla*/*Skeletonema* sp. (86.5 %) and *Pavlova* sp./*C. muelleri* (81.7%) (Fig. 4.2).

4.4. Discussion

Microalgae culture is still the only reliable source of nutrients for bivalve larvae despite attempts to develop alternatives (Knauer and Southgate, 1999). This is particularly true for larval culture of pearl oysters where the potential of alternatives to live microalgae has received little research attention (e.g. Southgate et al., 1998b). Nutritional research with pearl oysters has focuses on the potential of species of tropical microalgae, many of which have only become available for aquaculture relatively recently. In Chapter 3 the nutritional value of seven species of tropical microalgae for *P. margaritifera* larvae was determined when fed as single species. These follow-up experiments assessed the nutritional value of combinations of microalgae shown to be of high nutritional value to *P. margaritifera* larvae in Chapter 3. These results report for the first time on the relative nutritional value of binary and ternary combinations of tropical micro-algae for *P. margaritifera* larvae.

The greatest growth rate of D-stage *P. margaritifera* larvae was recorded for those fed the ternary combination of *Pavlova* sp./*Pav. salina*/ TISO. However, this growth rate was not significantly greater than that of larvae fed the binary combination of *Pav. salina*/TISO which has been used as a 'standard' diet for hatchery culture of *P. margaritifera* in this laboratory (Southgate and Beer, 1997). Furthermore, the growth rate of *P. margaritifera* larvae fed *Pavlova* sp. alone was the fourth highest of the eleven algal combinations tested and was not significantly lower than that of larvae fed *Pavlova* sp./*Pav. salina*/ TISO. Previous experiments in Chapter 3 showed that species of *Pavlova* were of high nutritional value for *P. margaritifera* larvae and that *Pavlova* sp. in particular was an excellent nutrient source. Our results confirm the high nutritional value of *Pavlova* sp. for D-stage *P. margaritifera* larvae and indicate that it is not necessary to provide D-stage *P. margaritifera* larvae with more than one species of microalgae to obtain good growth rates. Indeed, *Pavlova* sp. supported a greater (although not significantly so) growth rate than the binary diet of *Pav. salina*/TISO used routinely for *P. margatifera* larvae in this laboratory (Southgate and Beer, 1997). Since larval survival did not show any significant differences between treatments, there are clear practical benefits in supplying only a single species of microalgae to *P. margaritifera* larvae during the first 10 days of hatchery culture. The high survival of unfed larvae during Experiment 1 is notable and confirms results from similar studies with the larvae of this species (e.g. Southgate et al., 1998a; Doroudi and Southgate, 2000, Doroudi et al., 2002). These studies showed that pearl oyster larvae have the ability to survive usually to around day 10 where they presumably draw on endogenous reserves (Strugnell and Southgate,

2003); however, as seen during this experiment, they are unable to develop beyond D-stage to the umbo stage.

The results of Experiment 2 showed that the addition of a diatom to microalgae diets composed of flagellates resulted in increased growth rates and survival of umbo-stage *P. margaritifera* larvae when compared to combinations without diatoms. It is notable that the ternary combination of *Pavlova* sp./*Pav. salina*/TISO which supported the highest growth rate of D-stage larvae in Experiment 1, was not the best diet when used for older umbo-stage larvae in Experiment 2. Two ternary (*Pavlova* sp./*M. pusilla*/*C. muelleri* and *Pavlova* sp./*M. pusilla*/*Skeletonema* sp.) and one binary (*Pavlova* sp. /*C. muelleri*) combination, all containing a diatom, supported significantly greater larval growth rates than the ternary flagellate combination of *Pavlova* sp./*Pav. salina*/TISO.

The results of Chapter 3 showed that the growth of umbo-stage *P. margaritifera* was positively correlated with a number of biochemical components of dietary microalgae (as determined in Chapter 2) including carbohydrate, PUFA and EPA+DHA content. However, the diatoms used in this study did not have consistently higher levels of any of these components compared to flagellates and, in fact, contained considerably less PUFA than the flagellates (Table 4.1). On the basis of nutrient composition of the microalgae used in this study, therefore, it is difficult to speculate about the reasons that the addition of diatoms improved larval growth rates in Experiment 2. Furthermore, there were apparent differences in the nutritional value of the three diatoms assessed in this study. For example, addition of either *C. muelleri* or *Skeletonema* sp. to the binary diet of

Pavlova sp./*M. pusilla* resulted in a significant increase in the growth rate of umbo-stage *P. margaritifera* larvae; this did not occur when *Chaetoceros* sp. was added to the binary diet. This indicates that *Chaetoceros* sp. has a lower nutritional value for umbo-stage *P. margaritifera* larvae than *C. muelleri* and *Skeletonema* sp.; however, again this difference is difficult to attribute to differences in the nutrient composition of *Chaetoceros* sp. compared to the other two diatoms (Table 4.1). Previous experiments (Chapter 3) showed that *Chaetoceros* sp. was of low nutritional value for both D-stage and umbo-stage *P. margaritifera* larvae and did not support significantly greater growth rates than were recorded for unfed larvae. It is interesting, therefore that the addition of *Chaetoceros* sp. to the binary diet of *Pavlova* sp./*M. pusilla* in Experiment 2 did not result in reduced larval growth rate.

These experiments have shown that diatoms may be of high nutritional value for umbo-stage *P. margaritifera* larvae and may be an important component of binary and ternary diets for the larvae of this species. Similar findings were reported for larvae of the scallop, *Placopecten magallanicus,* which sustained higher growth rates and survival when fed *C. muelleri*/*Isochrysis* sp. compared to those fed *Isochrysis* sp./*Pav. lutheri* (Pernet and Tremblay, 2003). Similarly, larvae of the doughboy scallop, *Mimachlamys asperrima*, showed significantly improved growth rates when fed a diet where *C. calcitrans* was added at a level of 10% (O'Connor and Heasman, 1997). In contrast, inclusion of a diatom in a binary diet (*I. galbana*/*C. calcitrans*) for oyster, *Ostrea edulis*, larvae resulted in lower growth rates and reduced settlement compare to larvae fed flagellates (*I. galbana*/*Pav. lutheri*) alone (Jonsson et al., 1999). It is generally accepted

that bivalve juveniles show better growth rates when a diatom is added to the diet (Enright et al., 1986; O'Connor et al., 1997; Lora-Vilchis et al., 2004a, b). Similar results were found in this study where the growth of umbo-stage *P. margaritifera* larvae was improved when a diatom was included in the diet. However, as stated above, it is difficult to determine the reasons for the improved growth rates of *P. margaritifera* larvae on the basis of chemical composition alone.

Chapter 5

Growth kinetics and proximate and fatty acid compositions of five tropical microalgae species grown under different light: dark cycles

5.1. Introduction

Recent years have seen improved availability of species of tropical microalgae in response to the growth of tropical mariculture and the resulting need for nutritious microalgae that are tolerant to tropical culture conditions. Tropical microalgae used for aquaculture must provide a good source of energy for larval stages and show high nutritional quality (Chapter 3 and 4). Microalgae are susceptible to physiological manipulation, and changes in the growth rates and nutritional composition of microalgae in response to changes in culture variables such as nutrient composition and concentration, light (photoperiod and intensity) and temperature, have been studied extensively (Zhu et al., 1997; Price et al., 1998; Tzovenis et al., 2003 a,b; Carvalho and Malaca, 2003). Proximate biochemical composition of cultured microalgae varies according to the growth phase (age) of the culture and the level and type of nutrients available to it (e.g.Valenzuela-Espinoza et al., 2002). Furthermore, variable responses were found when testing continuous and discontinuous light regimes in some temperate microalgae where some eight of the eleven microalgae tested grew faster under

continuous light (Price et al., 1998). In contrast, other species such as TISO and *Chrysochromulina* sp., grew significantly faster when cultured under discontinuous light (Price et al., 1998).

An appropriate food source for cultured animals must satisfy their nutritional requirements and, in the case of bivalve larvae, cultured microalgae are the exclusive source of such nutrients. Given the high costs associated with microalgae culture in bivalve hatcheries, which have been estimated to make up 30% of hatchery operating costs (Coutteau and Sorgeloos, 1993), it is important that growth conditions are optimised with regard to maximising the productivity of microalgae cultures as well as their nutrient composition. Testing the response of microalgae species to variations in culture conditions is therefore an important step that may result in improvements to both microalgae productivity and their nutritional value.

The results of Chapters 3 and 4 showed that many species of tropical microalgae have high nutritional value for *P. margaritifera* larvae; however, there is no current information relating to the optimal culture conditions for these species or the effects of varying culture conditions on their biochemical (nutritional) compositions. The results of Chapter 3 helped identified nutrients contained in microalgae that impart high nutritional value when fed to *P. margaritifera* larvae. On this basis, it is reasonable to assume that if the levels of these nutrients within microalgae can be increased through manipulation of their culture conditions, then optimal culture conditions can be identified for microalgae

which not only maximize their growth rates (i.e. culture productivity) but also optimize nutrient composition for *P. margaritifera* larvae.

The experiments in this Chapter were therefore performed to determine the response of batch cultured tropical microalgae to different light:dark photoperiod regimes in terms of their growth rate, proximate biochemical composition and fatty acid content.

5.2. Material and Methods

5.2.1 Microalgae cultures

Microalgae used in these experiments were the diatom *Chaetoceros muelleri* (CS-176), three species of golden-brown flagellates *Isochrysis* sp. (TISO) (CS-177), *Pavlova salina* (CS-49) and *Pavlova* sp.(CS-50), and a green flagellate, *Micromonas pusilla* (CS-170). Microalgae were culture as described in Chapter 2 (section 2.2.1). Microalgae was allowed to adapt to the new light regimes in 250-mL flasks (see Fig. 2.1) for 10 days using three different light:dark (L:D) regimes: 12hL:12hD, 18hL:6hD and 24hL:0hD at a light intensity of 50 to 80 µmol photons m^{-1} s^{-1} provided by cool white fluorescent tubes. Microalgae were then sub-cultured into 3-L flasks and then into the experimental units (20-L plastic carboys) which contained 16-L of culture medium (Fig. 2.1). Duplicate or triplicate batch cultures were established depending upon species. Microalgae cultures were aerated without supplemental CO_2. Microalgae were harvested at two points during the entire life of the culture (early and late stationary phase). Early stationary phase was

selected as Day 10 in all the microalgae species with the exception of TISO that was harvested at day seven. Late stationary phase samples were taken on day 15 for all microalgae species with exception of *Pavlova* sp. which was harvested at day 20.The cell density of each microalgae culture was counted every day (four replicate counts per species) using a Neubauer haemocytometer to determine growth patterns. Growth rates of each culture (μ = divisions per day) were calculated using the standard equation:

$$k = \log_2 (N_2 / N_1) (t_2 - t_1)^{-1}$$

where: N_2 = number of cells mL^{-1} at time of harvest t_2 and N_1 = cells mL^{-1} at time t_1. This formula generates division per day directly (unlike the formula used in Chapter 2 which required use of the inverse constant (k/0.6931) to do so). The doubling time (T_2) required for cell number (N) to reach two times N (also known as 'generation time') was calculated using the equation:

$$T_2 = 1/k$$

Algae productivity, considered as the production obtained per algae species related to the culture of the volume under the different light regimes was calculated using the equation:

$$P = (C-C_0)\ V\ (t-t_0)^{-1}$$

where: C = final cell density at time t, C_0 = initial cell density at t_0, $t-t_0$ = period of time and V = culture volume (Guillard, 1973).

5.2.2 Chlorophyll 'a' and proximate biochemical analyses

Four litre aliquots were removed from replicate culture units during early stationary growth phase (day 7 for TISO or day 10 for the rest of the species) and the late stationary phase (day 15 with exception of *Pavlova* sp. that was harvested at day 20). Samples were centrifuged (3000 g, 30 min.) and resulting microalgae concentrates were washed with iso-osmotic ammonium formate (3.2 % w/v) to remove salt from the samples (Whyte et al., 1987) and centrifuged a second time (3000 g, 15 min). The resulting supernatants were discarded and the microalgae concentrates were placed in separated, pre-weighed micro-tubes, frozen in liquid nitrogen and stored at –80 °C until analysed for chlorophyll 'a', total lipid, carbohydrate, protein and fatty acid contents.

Triplicate samples of the frozen microalgae concentrates for each microalgae species were processed for their chlorophyll 'a' contents. Chlorophyll was extracted from the microalgae with 90% aqueous acetone. The concentration of the pigment was determined by measuring the light absorption of the extract (ASTM Standard Practices for Measurement of Chlorophyll Content of Algae in Surface Waters. D3731; re-approved 1998). Microalgae concentrates were homogenized for 3 min at 500 rpm in 5-mL of 90% aqueous acetone. The homogenate was washed into a vial with a small amount of acetone and the volume of extract was brought to 10-mL by addition of 90% aqueous acetone. After mixing, the extract was placed in the dark at 4ºC to steep. The homogenate was then mixed by inverting the tube several times, and the extract was clarified by centrifugation (20 min. at 1000 g). After clarification, the extract was decanted directly

into a cuvette and the chlorophyll content of the sample was quantified spectrophotometrically (750, 664, 647 and 630 nm). The concentration of chlorophyll 'a' was calculated using the appropriate chlorophyll equations of Jeffrey and Humphrey (1975):

$$\text{Chl 'a', mg } L^{1} = 11.85\ (OD664) - 1.54\ (OD647) - 0.080\ (OD630)$$

Samples for proximate biochemical composition were freeze-dried and were homogenized with 1-mL of distilled water using an IKA UltraTurrax T25 homogeniser. A subsample of 300-µL of the homogenate was transferred to a 5-mL polypropylene centrifuge tube for determination of lipid content. Lipids were extracted in 1:2 v/v chloroform:methanol (Bligh & Dyer, 1959) followed by a second extraction in 2:1 v/v chloroform:methanol and purification by the addition of 0.7 % w/v NaCl solution (Folch et al., 1957). Aliquots of the lipid-containing organic phase were oven dried at 60 ºC until all chloroform evaporated. Concentrated sulphuric acid was added to the lipid residue and heated in a boiling water bath for 10 minutes. Lipid quantification followed the sulphophosphovanillin method of Barnes & Blackstock (1973) using cholesterol (Ajax Lab Chem 1729-100G) as the standard.

A further sub-sample (500-µL) of homogenate was used to determine carbohydrate and protein contents and was extracted overnight with 5% w/v trichloroacetic acid. After centrifugation, the carbohydrate-containing supernatant was removed to be quantified following the phenol-sulphuric acid method (Dubois et al., 1956) using glucose (AnalaR 10117) as the standard. The precipitate, used for protein analysis, was dissolved in 0.1 N

NaOH and heated at 60ºC for 1 h (modified from Holland & Gabbott, 1971). Protein determination followed the method of Bradford (1976) using Bio-Rad® protein assay dye reagent concentrate and bovine serum albumin (Sigma A-7888) as the standard. For detailed procedures of biochemical analyses see Appendix 1.

5.2.3 Fatty acid analysis

Fatty acid analyses were conducted as described by Isik et al. (1999). Briefly, samples were unfrozen and placed into Teflon capped Pyrex test tubes. Samples weights varied from 29.2-267.4 mg. Aliquots of 3-mL of methanol:benzene: 2,2-dimethoxypropane: H_2SO_4 (37:20:5:2 v/v) plus 2-mL of the internal standard (heptadecaenoic acid) were added to each tube. Test tubes were sealed with nitrogen and placed in a water bath at 80ºC for 2 h, with frequent shaking. Following centrifugation, the upper layer was aspirated and stored frozen for GC-MS analysis. The quantitative determination of the resulting fatty acid methyl esters (FAME) was undertaken using a mass spec-scan 50-300 and individual peaks were quantified using a single quantification ion. The column used was a Varian Factor Four column (CP8944) (30m x 0.25 mm) with a 0.25 µm coating. Column temperature was programmed to start at 50ºC and was held for 5 min. before being raised to 240ºC, at a rate of 2ºC min^{-1} where it was held for 20 min. FAME were identified by comparing their retention times with those of authentic standards (Sigma-Aldrich Co, USA), and they were quantified using heptadecaenoic acid as the internal standard.

5.2.4 Data analyses

Significant differences between the growth rates of the different microalgae species grown under the three different light regimes (12L:12D, 18L:6D and 24L:0D)were detected by a repeated measures analysis of variance (ANOVA) with light regime and time (early (t1) and late stationary phase (t2) as fixed factors.. Microalgae biochemical composition (protein, lipid and carbohydrate contents) was determined in triplicate taken from each of the two replicate samples for each species per light regime and day of harvesting (early and late stationary phase). The triplicate measures were averaged for each replicate. Fatty acid contents of microalgae grown under different light regimes were determined from two samples for each species per light regime and growth phase. Both analyses (gross biochemical and fatty acid content) were assessed by a repeated measures ANOVA with light regime and growth phase as fixed factors. Bonferroni adjustment was applied to each species and the probabilities less than 0.007 were considered significant.

Due to missing protein data for some of the replicates for *Isochrysis sp.* at t1 and *Pavlova salina* at t2, only a one-way ANOVA could be conducted on t2 and t1 measurements, respectively. There was also missing protein data from *Micromonas pusilla* light regime 24:0, therefore repeated measures ANOVA was only performed on light regimes 18:6 and 12:12. Moreover, no sufficient data was available for MUFA measurements for *Micromonas pusilla* and therefore this data is not presented here. Finally, data was missing from some of the replicates for SFA, PUFA and MUFA measurements for *Chaetoceros muelleri* for time regime 12:12 and therefore a repeated measures ANOVA was only conducted on the measurements for time regimes 24:0 and 18:6.

5.3 Results

5.3.1 Algae response to the three different light regimes

Microalgae were sampled at two different times during the life of the batch cultures. Cell density at each sampling time is shown in the Tables relating to each microalga (Tables 5.1, 5.3, 5.5, 5.7 and 5.9). The specific response to varying culture conditions for each microalgae species are described separately, however, general patterns can be observed. Growth rates of microalgae varied from 0.3 to 0.8 divisions per day with no apparent difference between species and light regime. Cell density doubled after the first sampling day for TISO and *Pavlova* sp. independently of light regime. On the other hand, *P. salina, M. pusilla* and *C. muelleri* slightly increased their density from day 10 to day 15 of growth. Furthermore, the different light regimes seemed not to influence productivity for most of the microalgae species, with the exception of *M. pusilla*. Chlorophyll 'a' contents increased from early to late stationary phase in most of the microalgae with the exception of *Pavlova* sp. where the opposite pattern was observed. However, no significant differences in chlorophyll 'a' contents were found ($P > 0.05$) between light regimes for each particular microalgae species. The specific reactions of each species of microalgae to varying culture conditions are described below.

a. ***Isochrysis* sp. (TISO)**

Growth patterns of TISO subject to the three different light regimes showed no significant difference (Fig. 5.1, Fig. A1a) The response of microalgae to the different

light regimes in terms of their proximate biochemical compositions is shown in Table 5.1 and Appendix 2. No significant differences were found in any biochemical component between different light regimes for proteins and carbohudrates. However, there was a significant difference in lipids for different light regimes with light regime 18:6 giving the height lipid concentration (Fig. A1c, Table A1). The fatty acid contents of TISO, in terms of total SFA, MUFA and PUFA, did not show significant differences when cultured under the three different light regimes or harvesting days. The pattern can be described as PUFA > SFA > MUFA (Table 5.2, Table A1, Fig. A1 e,f,g).

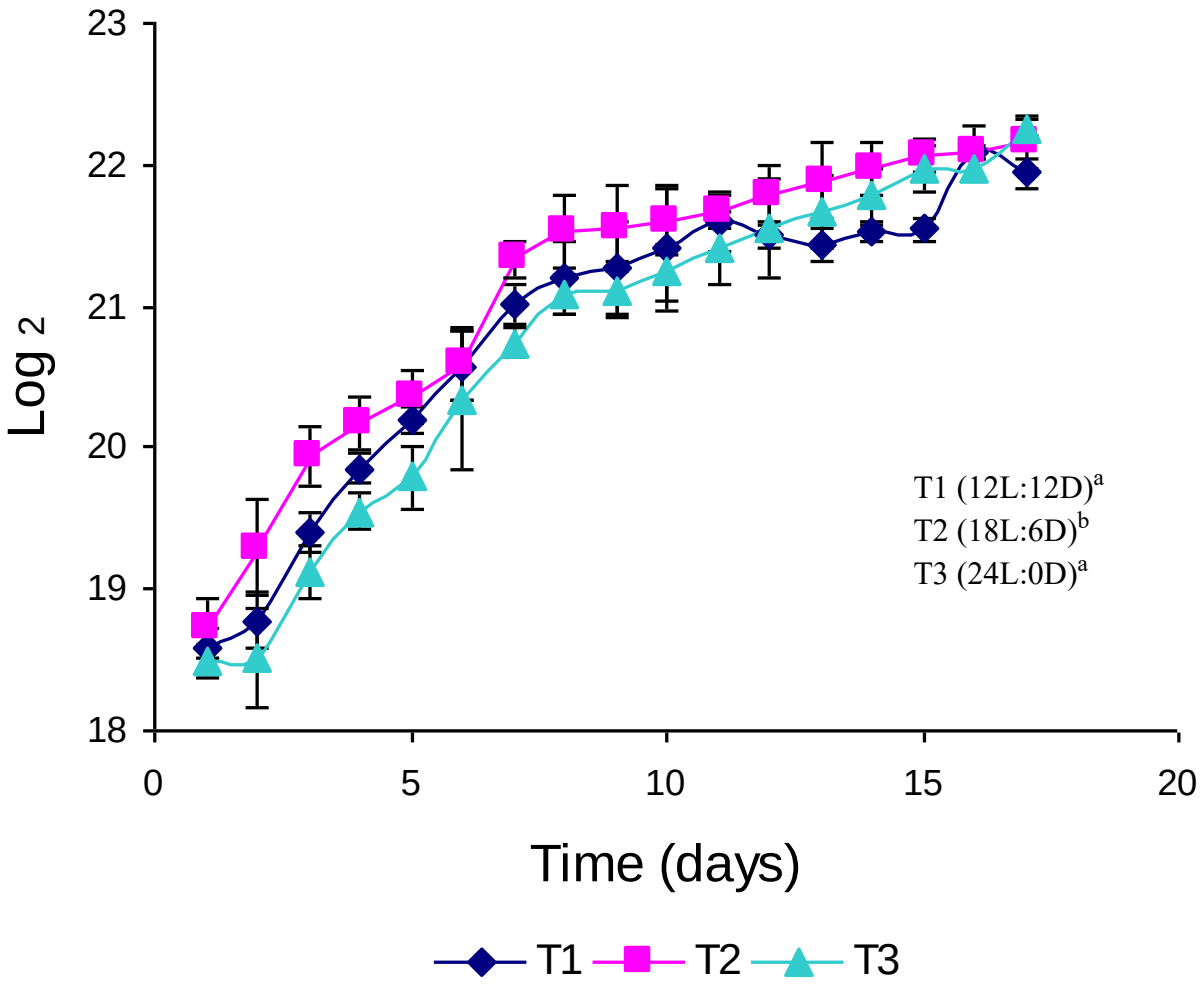


Fig. 5.1 Mean (n= 30 ± s.e.) growth patterns (log_2 of cell density mL^{-1}) of *Isochrysis* sp. (TISO) cultured using three different light regimes: T1 = 12L:12D; T2 = 18L:6D; and T3 = 24L:0D. The same superscript indicate no significantly difference between treatments (P >0.05).

Table 5.1 Response of TISO to different light regimes in relation to the following parameters: growth rates (k, divisions day^{-1}), generation time (T_2), productivity in 16-L culture (P, grams, mean ± s.e.), chlorophyll 'a' contents (µg/mg, mean ± s.e.) and proximate composition (mean ± s.e. % of dry weight).

TISO					
Light regime	**Day 5 (cells mL^{-1})**	**Day 7 (cells mL^{-1})**	**Day 15 (cells mL^{-1})**	**k**	
12L:12D	1.5 $x10^6$	2.4 $x10^6$	4.4 $x10^6$	0.40±0.1	Day1-5
18L:6D	1.5 $x10^6$	3.0 $x10^6$	4.4 $x10^6$	0.46±0.1	Day1-5
24L:0D	2.0 $x10^6$	2.2 $x10^6$	4.1 $x10^6$	0.64±0.1	Day1-5

	P		**Chlorophyll 'a'**	
		T_2	**Day 7**	**Day 15**
12L:12D	0.2103 ± 0.01	2.5±0.1	21.4 ± 4.0	34.7 ± 2.2
18L:6D	0.3110 ± 0.02	2.2±0.2	14.0 ± 1.0 [a]	30.0 ± 1.7
24L:0D	0.2657 ± 0.03	1.6±0.2	7.4 ± 6.7 [a]	15.5 ± 1.7

	Day 7			**Day 15**		
	Protein	**Lipid**	**Carbohydrate**	**Protein**	**Lipid**	**Carbohydrate**
12L:12D	50.4 (2.4	46.1 (2.6)	8.4 (1.2)	49.8 (0.9)	25.9 (4.0)	10.9 (0.4)
18L:6D	58.7 (2.1)	43.9 (1.8)	6.7 (0.4)	52.9 (1.6)	46.8 (0.8)	6.7 (1.2)
24L:0D	25.5 (1.9)	11.5 (0.5)	15.9 (3.0)	36.1 (1.2)	26.4 (1.4)	12.2 (3.6)

Table 5.2. Fatty acid composition (mean ± s.e. % of total fatty acids) of TISO grown under different light regimes and at different harvesting days. The table shows myristic acid (14:0) and palmitic acid (16:0) contents, the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total *n-3* and *n-6* fatty acids and *n-3/n-6* ratios. It also shows the degree of unsaturation (PUFA+MUFA/SFA) and the index (PUFA/SFA+MUFA).

	Day 7				**Day 15**		
	12L:12D	**18L:6D**	**24L:0D**		**12L:12D**	**18L:6D**	**24L:0D**
14:0	15.7 ±1.8	12.9 ±2.2	13.7 ±2.0		13.4 ±0.1	14.0 ±2.4	9.6 ±0.5
16:0	6.9 ±0.3	6.0 ±0.5	11.4 ±1.6		9.2 ±0.5	7.36 ±0.9	13.4 ±6.4
Total SFA	22.5 ±2.0	18.9 ±2.4	25.3 ±3.6		23.3 ±0.6	21.8 ±1.0	23.0 ±5.9
Total MUFA	11.6 ±2.6	8.0 ±2.5	5.6 ±1.1		16.9 ±0.0	10.5 ±4.4	9.9 ±0.1
Total PUFA	62.6 ±4.0	73.1 ±0.1	69.1 ±10.7		59.7 ±0.6	67.7 ±3.4	67.1 ±5.9
n-3	40.6	54.6	63.7		47.4	52.1	51.0
n-6	23.4	18.8	6.1		12.3	15.3	16.8
n-3/n-6	1.7	2.9	10.5		3.9	3.4	3.0
PUFA+MUFA/SFA	3.4	4.3	2.9		3.3	3.6	3.5
PUFA/SFA+MUFA	2.0	2.7	2.2		1.5	2.1	2.1

b. ***Pavlova salina***

The growth patterns for *P. salina* cultured using the three different light regimes showed that *P. salina* grew better under continuous light with a significant difference for light regimes being detected with highest growth at t2 for continuous light (Fig. 5.2, Fig. A2a, Table A2). The carbohydrate content of *P. salina* remained fairly constant independently of day of harvesting with no significant difference among light regimes (Table 5.3, Fig. A2d, Table A2). Lipid contents differed between days of harvesting and different light regimes, where the highest lipid content in *P. salina* in day 10 was recorded under the 18L:6D light regime (39 ± 1.2%) but this was not significantly greater than that obtained when algae was cultured at the 12L:12D light regime (38 ± 1.8 %). On the other hand, on day 15, relatively higher lipid contents were recorded in algae cultured under 18L:6D light regime (55.6 ± 1.2%) compared to those obtained in algae cultured under the 12L:12D light regime (48.6 ± 0.9% of total dry weight) and continuous light (32.4 ± 2.4% of total dry weight) (Table 5.3, Fig. A2c, Table A2). Protein values obtained during the culture of *Pav. salina* under the three different light regimes showed no significant differences at t2(Table 5.3, Fig. A2b, Table A2). Fatty acid contents in *P. salina* showed no significant differences when cultured under the three different light. The pattern of fatty acid content can be described as PUFA > SFA > MUFA (Table 5.4, Fig. A2 e,f,g, Table A2).

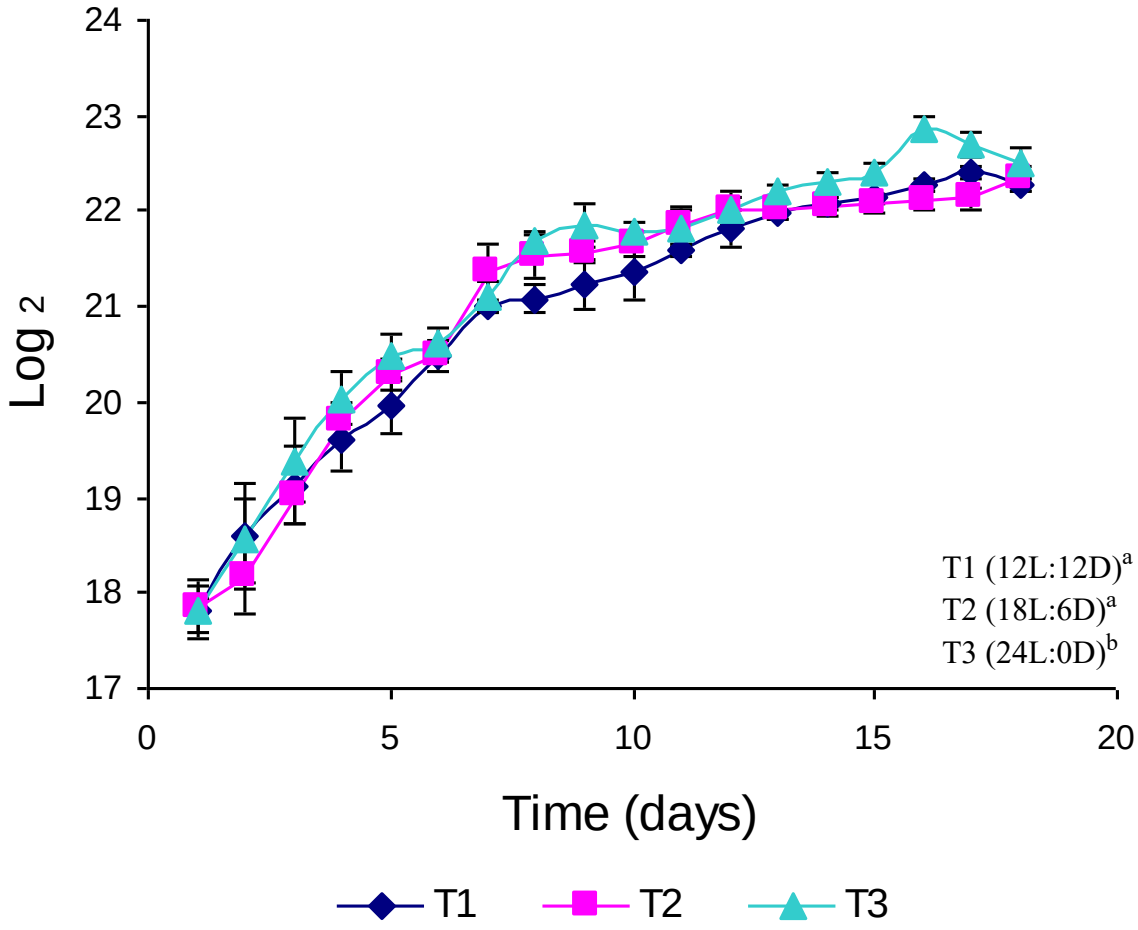


Fig. 5.2 Mean (n= 30 ± s.e.) growth patterns (log_2 of cell density mL^{-1}) of *Pavlova salina* cultured using three light regimes: T1 = 12L:12D; T2 = 18L:6D; and T3 = 24L:0D. The same superscript indicate no significantly difference between treatments (P >0.05).

Table 5.3 Response of *Pavlova salina* to different light regimes in relation to the following parameters: growth rates (k, divisions day^{-1}), generation time (T_2), productivity in 16-L culture (P, grams, mean ± s.e.), chlorophyll 'a' contents (µg/mg, mean ± s.e.) and proximate composition (mean ± s.e. % of dry weight).

Pavlova salina					
Light regime	**Day 5 (cells mL^{-1})**	**Day 10 (cells mL^{-1})**	**Day 15 (cells mL^{-1})**	**k**	
12L:12D	1.4 x10^6	3.1 x10^6	5.1 x10^6	0.47±0.1	Day 1-5
18L:6D	1.5 x10^6	3.7 x10^6	4.5 x10^6	0.57±0.1	Day 1-5
24L:0D	1.6 x10^6	3.7 x10^6	5.6 x10^6	0.52±0.5	Day 1-5

	P		**Chlorophyll 'a'**	
		T_2	**Day 10**	**Day 15**
12L:12D	0.2159 ± 0.00	2.1±0.4	16.6 ± 1.0	27.0 ± 1.5
18L:6D	0.1216 ± 0.00	1.7±0.2	18.8 ± 1.4	26.9 ± 3.2
24L:0D	0.1514 ± 0.01	1.9±0.3	17.4 ± 1.3	19.4 ± 2.4

	Day 10			**Day 15**		
	Protein	**Lipid**	**Carbohydrate**	**Protein**	**Lipid**	**Carbohydrate**
12L:12D	34.7 (0.7)	38.0 (1.8)	5.4 (0.3)	44.5 (3.2)	48.6 (0.9)	5.0 (0.1)
18L:6D	25.3 (6.3)	39.0 (1.2)	5.7 (0.1)	42.5 (6.1)	55.6 (1.2)	4.7 (0.3)
24L:0D	44.2 (1.1)	29.0 (1.3)	4.7 (1.4)	58.0 (2.2)	32.4 (2.4)	7.3 (0.4)

Table 5.4. Fatty acid composition (mean ± s.e. % of total fatty acids) of *Pavlova salina* grown under different light regimes and at different harvesting days. The table shows myristic acid (14:0) and palmitic acid (16:0) contents, the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total *n-3* and *n-6* fatty acids and *n-3/n-6* ratios. It also shows the degree of unsaturation (PUFA+MUFA/SFA) and the index (PUFA/SFA+MUFA).

	Day 10				**Day 15**		
	12L:12D	**18L:6D**	**24L:0D**		**12L:12D**	**18L:6D**	**24L:0D**
14:0	9.1 ±0.3	8.9 ±0.2	9.7 ±0.4		9.3 ±0.2	12.0 ±1.0	11.2 ±0.0
16:0	12.8 ±0.0	10.2 ±0.2	10.1 ±0.4		9.7 ±0.6	10.5 ±1.7	10.6 ±0.4
Total SFA	22.0 ±0.3	19.1 ±0.0	19.8 ±0.8		19.4 ±0.4	23.0 ±2.2	22.5 ±0.3
Total MUFA	2.0 ±0.1	2.4 ±0.8	18.7 ±7.1		3.2 ±1.6	4.5 ±0.0	4.3 ±2.7
Total PUFA	76.0 ±0.2	78.5 ±0.8	61.5 ±6.4		77.4 ±1.2	72.4± 0.7	73.3 ±3.0
n-3	73.4	73.0	46.6		72.3	65.9	62.5
n-6	3.7	5.7	9.2		4.4	6.5	10.1
n-3/n-6	20.1	12.9	5.1		16.3	10.1	6.2
PUFA+MUFA/SFA	3.6	4.2	4.1		4.1	3.3	3.5
PUFA/SFA+MUFA	3.2	3.7	1.6		3.4	2.6	2.7

c. ***Pavlova* sp.**

Pavlova sp. showed the best growth when cultured using the 18L:6D light regime but this growth rate was not significantly different to that of algae grown under the other two light regimes (Fig.5.3, Table A3, Fig. A3a). There was also no significant difference detected in carbohydrate or protein content of *Pavlova sp.* betwee different light regimes or sampling times (Table 5.5, Fig. A5b,c, Table A3). On both harvesting days the highest carbohydrate values were recorded when this species was cultured using the 12L:12D light regime (day 10, 6.0 ± 0.4%; day 20, 6.3 ± 0.2%) but these values did not differ significantly from those of algae cultured under other light regimes. Lipid contents of *Pavlova* sp. were significantly different on day 10 where algae cultured under continuous light showed the highest lipid contents (48 ± 0.9%). However, on day 20, lipid contents of the microalgae cultured using the three different light regimes were not significantly different (Table A3, Fig. A3c); lipid values varied from (33 ± 1.1% to 37.5 ± 2.0%). Fatty acid contents in *Pavlova* sp. only showed significant differences in total MUFA contents on both harvesting days between light regimes; microalgae cultured under continuous light showed the highest MUFA contents (8% and 8.3% on day 10 and day 20, respectively) (Table A3, Fig. A3f). The relative proportions of the various classes of fatty acids can be described as PUFA > SFA > MUFA (Table 5.6).

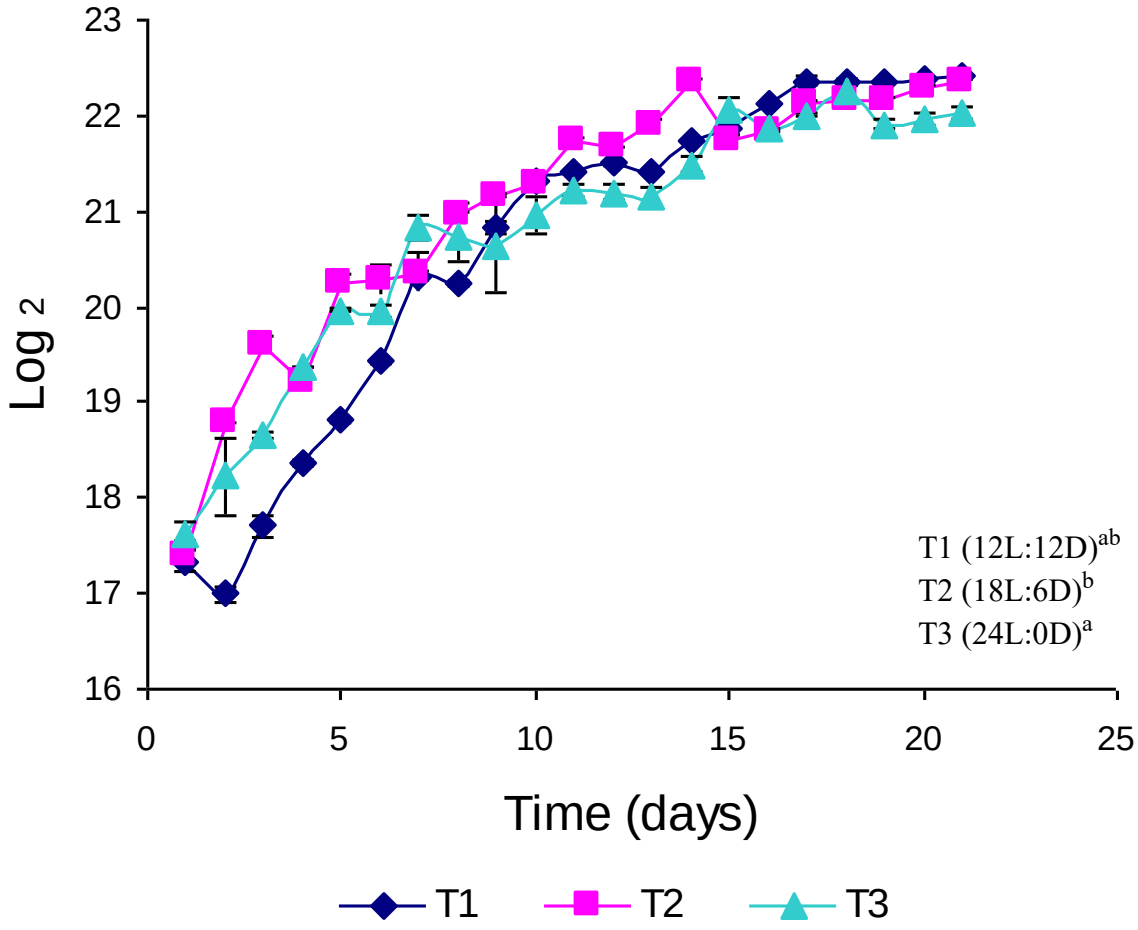


Fig. 5.3 Mean (n= 30) growth patterns (log_2 of cell density mL^{-1}) of *Pavlova* sp. cultured using three light regimes: T1 = 12L:12D; T2 = 18L:6D; and T3 = 24L:0D. The same superscript indicate no significantly difference between treatments (P >0.05).

Table 5.5 Response of *Pavlova* sp. to different light regimes in relation to the following parameters: growth rates (k, divisions day^{-1}), generation time (T_2), productivity in 16-L culture (P, grams, mean ± s.e.), chlorophyll 'a' contents (µg/mg, mean ± s.e.) and proximate composition (mean ± s.e. % of dry weight).

***Pavlova* sp.**					
Light regime	**Day 5 (cells mL^{-1})**	**Day 10 (cells mL^{-1})**	**Day 20 (cells mL^{-1})**	**k**	
12L:12D	0.70 $x10^6$	2.8 $x10^6$	5.5 $x10^6$	0.81±0.2	(Day 2-7)
18L:6D	1.3 $x10^6$	3.5 $x10^6$	5.3 $x10^6$	0.49±0.3	(Day 2-7)
24L:0D	1.0 $x10^6$	2.4 $x10^6$	4.3 $x10^6$	0.44±0.3	(Day 2-7)

	P		**Chlorophyll 'a'**	
		T_2	**Day 10**	**Day 20**
12L:12D	0.1925 ± 0.03	1.2±0.4	21.2 ± 3.9	20.0 ± 3.2
18L:6D	0.1614 ± 0.03	2.0±0.8	20.0 ± 2.8	18.1 ± 0.8
24L:0D	0.2219 ± 0.00	2.3±1.1	16.3 ± 1.6	14.2 ± 0.5

	Day 10			**Day 20**		
	Protein	**Lipid**	**Carbohydrate**	**Protein**	**Lipid**	**Carbohydrate**
12L:12D	21.0 (1.4)	26.4 (2.2)	6.0 (0.4)	47.8 (2.0)	35.9 (1.0)	6.3 (0.2)
18L:6D	31.7 (3.8)	32.4 (1.0)	4.5 (0.3)	29.5 (4.0)	33.0 (1.1)	4.9 (0.3)
24L:0D	32.2 (0.8)	48.0 (0.9)	5.8 (1.0)	26.0 (1.4)	37.5 (2.0)	5.4 (1.0)

Table 5.6. Fatty acid composition (mean ± s.e. % of total fatty acids) of *Pavlova* sp. grown under different light regimes and at different harvesting days. The table shows myristic acid (14:0) and palmitic acid (16:0) contents, the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total *n-3* and *n-6* fatty acids and *n-3/n-6* ratios. It also shows the degree of unsaturation (PUFA+MUFA/SFA) and the index (PUFA/SFA+MUFA).

	Day 10				**Day 20**		
	12L:12D	**18L:6D**	**24L:0D**		**12L:12D**	**18L:6D**	**24L:0D**
14:0	12.2 ±1.2	21.1 ±0.8	18.9 ±2.2		17.4 ±4.7	17.1 ±1.8	22.5 ±0.1
16:0	2.2 ±2.2	8.4 ±1.2	11.5 ±0.7		7.3 ±1.6	7.7 ±0.2	12.3 ±0.6
Total SFA	19.0 ±2.5	29.8 ±2.2	30.9 ±2.9		24.7 ±6.3	25.0 ±2.0	35.1 ±0.5
Total MUFA	3.5 ±3.0	4.2 ±0.9	8.0 ±0.6		2.5 ±0.	2.2 ±1.1	8.2 ±0.4
Total PUFA	79.1 ±2.1	66.0 ±3.1	61.1 ±3.4		72.8 ±5.	77.7 ±3.2	56.7 ±1.0
n-3	77.4	64.7	50.2		72.8	71.4	49.7
n-6	1.7	1.9	9.2		20.	2.9	7.0
n-3/n-6	45.2	34.9	5.5		36.2	24.3	7.1
PUFA+MUFA/SFA	4.3	2.4	2.2		3.0	3.0	1.8
PUFA/SFA+MUFA	3.4	1.9	1.6		2.7	2.7	1.3

d. ***Micromonas pusilla***

There were significant difference in *M. pusilla* growth between different light regimes and different sampling time (Fig. 5.4, Fig. A4a, Table A4). *M. pusilla* grew significantly slower for both sampling times under 12:12 light regime, but the growth pattern between 18:6 and 24:0 light regimes did not differ (Fig. 5.4, Fig. A4a, Table A4).The proximate biochemical components of *M. pusilla* were not significantly different between harvesting days and between light regimes (Table 5.7, Fig. A4,b,c,d, Table A4). Fatty acid contents (SFA and PUFA) in *M. pusilla* also showed no significant differences when cultured under the three light regimes or day of harvesting (Fig. A4,e,f, Table A4). The relative proportions of the major classes of fatty acids can be described as PUFA > SFA > MUFA (Table 5.8).

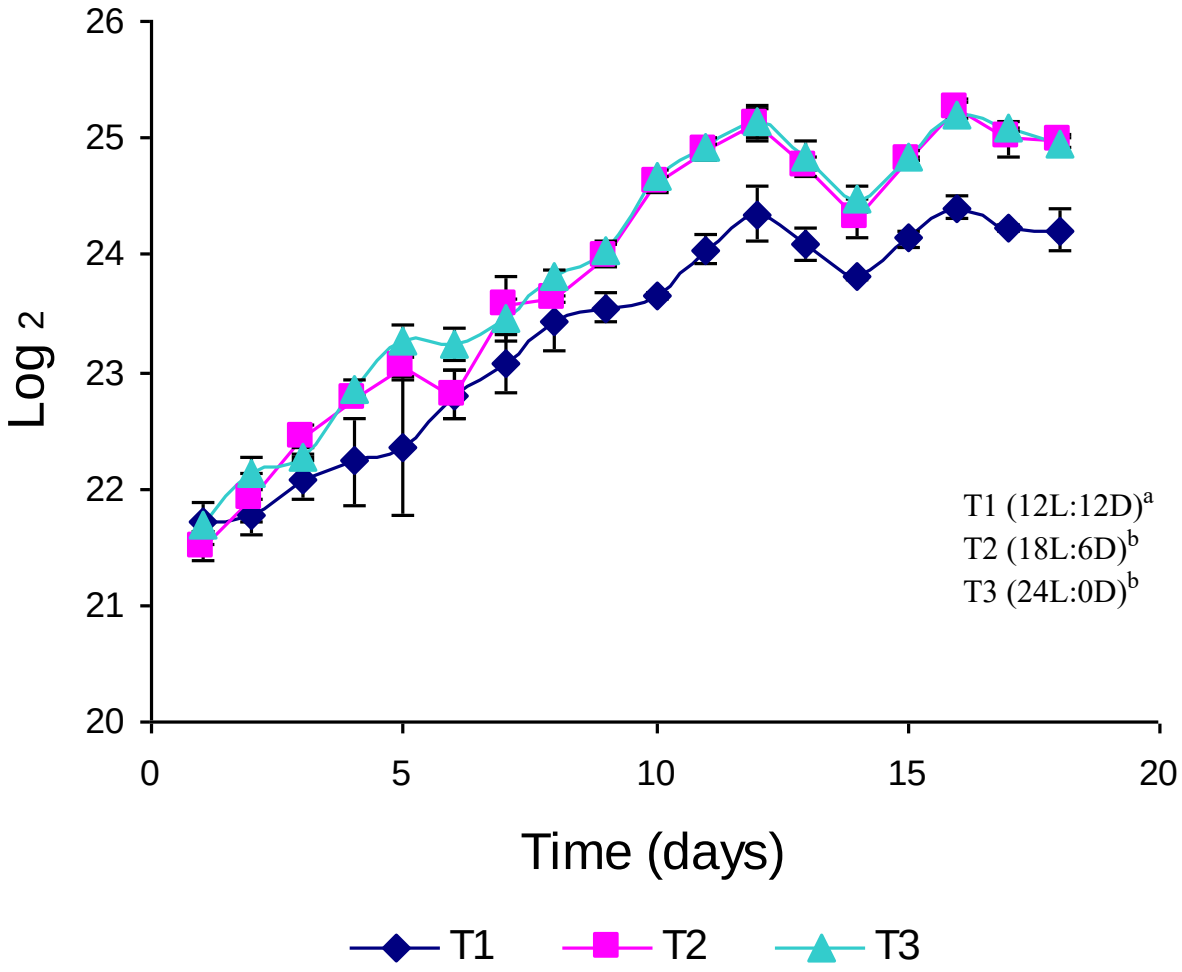


Fig. 5.4 Mean (n= 30) growth patterns (log_2 of cell density mL^{-1}) of *Micromonas pusilla* cultured using three light regimes: T1 = 12L:12D; T2 = 18L:6D; and T3 = 24L:0D. The same superscript indicate no significantly difference between treatments (P >0.05).

Table 5.7 Response of *Micromonas pusilla* to different light regimes in relation to the following parameters: growth rates (k, divisions day^{-1}), generation time (T_2), productivity in 16-L culture (P, grams, mean ± s.e.), chlorophyll 'a' contents (μg/mg, mean ± s.e.) and proximate composition (mean ± s.e. % of dry weight).

Micromonas pusilla					
Light regime	**Day 5 (cells mL^{-1})**	**Day 10 (cells mL^{-1})**	**Day 15 (cells mL^{-1})**	**k**	
12L:12D	11.3 x10^6	17.3 x10^6	22.3 x10^6	0.28±0.2	(Day 3-7)
18L:6D	12.9 x10^6	25.6 x10^6	39.7 x10^6	0.28±0.1	(Day 3-7)
24L:0D	14.7 x10^6	26.5 x10^6	38.7 x10^6	0.28±0.1	(Day 3-7)

	P		**Chlorophyll 'a'**	
		T_2	**Day 10**	**Day 15**
12L:12D	0.0628 ± 0.00	3.6±0.3	12.0 ± 4.2	24.4 ± 2.8
18L:6D	0.1174 ± 0.00	3.5±0.2	15.6 ± 3.3	29.1 ± 0.8
24L:0D	0.1118 ± 0.00	3.5±0.2	14.8 ± 0.3	15.9 ± 2.8

	Day 10			**Day 15**		
	Protein	**Lipid**	**Carbohydrate**	**Protein**	**Lipid**	**Carbohydrate**
12L:12D	33.7 (2.6)	17.3 (0.6)	4.5 (0.5)	49.2 (4.2)	50.0 (7.1)	2.8 (0.3)
18L:6D	48.7 (2.9)	29.2 (1.0)	6.3 (0.2)	57.5 (1.1)	37.0 (4.1)	3.4 (0.2)
24L:0D	42.4 (3.1)	31.4 (1.6)	5.0 (0.4)	44.1 (1.9)	37.5 (2.10^{j})	4.5 (0.5)

Table 5.8. Fatty acid composition (mean ± s.e. % of total fatty acids) of *Micromonas pusilla* grown under different light regimes and at different harvesting days. The table shows myristic acid (14:0) and palmitic acid (16:0) contents, the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total *n-3* and *n-6* fatty acids and *n-3/n-6* ratios. It also shows the degree of unsaturation (PUFA+MUFA/SFA) and the index (PUFA/SFA+MUFA).

	Day 10				**Day 15**		
	12L:12D	**18L:6D**	**24L:0D**		**12L:12D**	**18L:6D**	**24L:0D**
14:0	2.9 ±0.1	3.0 ±0.4	4.1 ±0.4		8.3 ±6.7	6.2 ±2.2	4.2 ±0.1
16:0	17.3 ±2.5	19.7 ±0.7	23.6 ±1.7		12.6 ±3.9	16.2 ±0.5	17.1 ±0.4
Total SFA	20.4 ±2.2	22.7 ±1.1	29.7 ±1.9		21.6 ±3.1	22.8 ±2.1	21.7 ±0.5
Total MUFA	3.0 ±0.9	0.7 ± 0.7	3.5 ±0.6		2.6 ±2.6	0.7 ±0.5	0.5 ±0.5
Total PUFA	76.1 ±1.5	76.6 ±0.4	68.6 ±2.4		76.2 ±0.4	76.4 ±2.6	77.9 ±1.0
n-3	68.8	65.6	56.0		105.5	66.3	66.3
n-6	7.3	11.1	12.6		2.1	9.0	11.5
n-3/n-6	9.4	5.9	4.4		51.2	7.3	5.8
PUFA+MUFA/SFA	3.9	3.4	2.6		3.7	3.4	3.6
PUFA/SFA+MUFA	3.3	3.3	2.2		3.2	3.2	3.5

e. ***Chaetoceros muelleri***

There were no significant differences detected in any of the variable measured for *C. muelleri,* though several variables (growth rates, SFA, PUFA) were perfomring slightly better under 18:6 light regime, than the other two light regimes (Fig. 5.5, 5.9, Fig. A5, Table A5)Carbohydrate contents of *C. muelleri* varied according to light regime with relatively high levels of carbohydrate found in algae cultured using continuous light on day 10 (9.5 ± 0.4%) and also in algae culture under the 12L:12D light regime (9.0 ± 1.1%) on day 15 (Table 5.9, Fig. A5d). Highest lipid content was found in microalgae cultured under the 12L:12D light regime on day 15 (37.5 ± 3.1%) (Fig. A5c). The highest protein content was recorded in algae cultured under the 12L:12D light regime (Fig. A5b). This microalgae contained different proportions of SFA, MUFA and PUFA in response to the different light regimes used (Table 5.10, Fig. A5 e,f,g), though these differences were not statistically significant (Table A5). For example, the relative proportions of the major classes of fatty acids were PUFA>SFA>MUFA under the 12L:12D light regime; however, this order became MUFA>SFA>PUFA during the continuous light regime and SFA≥MUFA≥SFA and PUFA>MUFA≥SFA under the 18L:6D light regime on day 10 and day 15, respectively (Table 5.10).

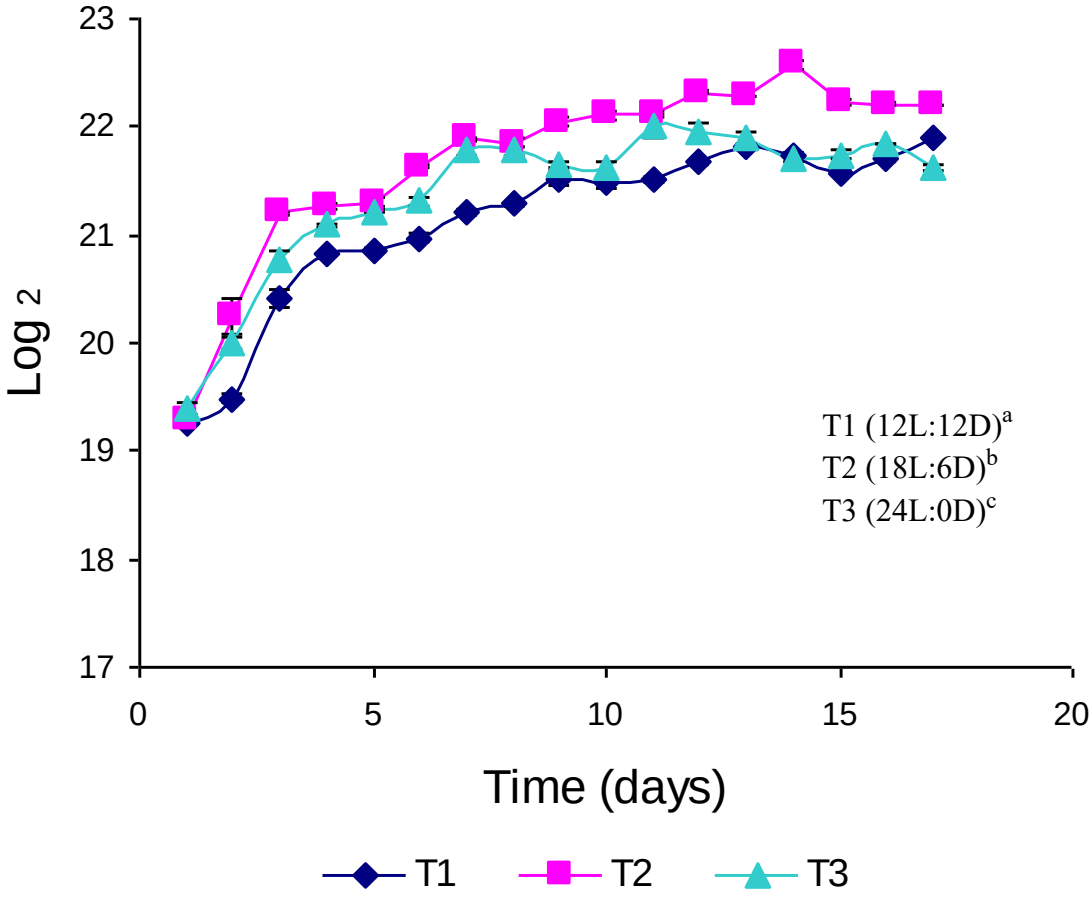


Fig. 5.5 Mean (n= 30) growth patterns (log_2 of cell density mL^{-1}) of *Chaetoceros muelleri* cultured using three light regimes: T1 = 12L:12D; T2 = 18L:6D; and T3 = 24L:0D. The same superscript indicate no significantly difference between treatments (P >0.05).

Table 5.9 Response of *Chaetoceros muelleri* to different light regimes in relation to the following parameters: growth rates (k, divisions day^{-1}), generation time (T_2), productivity in 16-L culture (P, grams, mean ± s.e.), chlorophyll 'a' contents (μg/mg, mean ± s.e.) and proximate composition (mean ± s.e. % of dry weight).

Chaetoceros muelleri					
Light regime	**Day 5 (cells mL^{-1})**	**Day 10 (cells mL^{-1})**	**Day 15 (cells mL^{-1})**	**k**	
12L:12D	2.1 x10^6	2.9 x10^6	3.4 x10^6	0.37±0.1	(Day 1-5)
18L:6D	3.3 x10^6	4.5 x10^6	4.8 x10^6	0.35±0.1	(Day 1-5)
24L:0D	2.6 x10^6	4.2 x10^6	3.8 x10^6	0.33±0.2	(Day 1-5)

	P		**Chlorophyll 'a'**	
		T_2	**Day 10**	**Day 15**
12L:12D	0.0650 ± 0.01	2.7±0.3	22.1 ± 1.2	19.6 ± 1.2
18L:6D	0.1041 ± 0.02	2.9±0.7	19.2 ± 0.7	15.3 ± 0.8
24L:0D	0.0663 ± 0.01	3.0±0.5	14.9 ± 0.2	13.4 ± 0.3

	Day 10			**Day 15**		
	Protein	**Lipid**	**Carbohydrate**	**Protein**	**Lipid**	**Carbohydrate**
12L:12D	31.5 (6.7)	25.6 (6.3)	5.3 (0.7)	29.4 (3.1)	37.5 (3.1)	9.0 (1.1)
18L:6D	18.7 (3.8)	32.7 (2.6)	5.5 (0.4)	6.3 (0.4)	29.5 (1.9)	6.8 (0.6)
24L:0D	21.5 (1.0)	21.0 (0.3)	9.5 (0.4)	26.5 (6.9)	27.1 (1.0)	5.4 (0.9)

Table 5.10. Fatty acid composition (mean ± s.e. % of total fatty acids) of *Chaetoceros muelleri* grown under different light regimes and at different harvesting day. The table shows myristic acid (14:0) and palmitic acid (16:0) contents, the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total *n-3* and *n-6* fatty acids and *n-3/n-6* ratios. It also shows the degree of unsaturation (PUFA+MUFA/SFA) and the index (PUFA/SFA+MUFA).

	Day 10				**Day 15**		
	12L:12D	**18L:6D**	**24L:0D**		**12L:12D**	**18L:6D**	**24L:0D**
14:0	23.7 ±4.9	24.2 ±8.6	17.3 ±0.7		22.7 ±3.0	22.1 ±1.7	17.3 ±1.6
16:0	6.4 ±1.3	8.3 ±2.0	5.2 ±0.9		5.2 ±0.9	5.2 ±0.9	11.7 ±0.1
Total SFA	30.1 ±6.3	32.9 ±10.9	23.0 ±1.6		28.2 ±3.9	27.5 ±2.9	30.4 ±1.9
Total MUFA	28.8 ±6.0	33.8 ±8.9	56.6 ±1.8		23.0 ±3.8	32.8 ±2.9	55.0 ±2.3
Total PUFA	41.1 ±8.6	33.4 ±13.7	20.4 ±7.7		48.9 ±12.3	39.6 ±10.2	14.6 ±4.5
n-3	77.2	37.7	20.7		29.2	38.1	4.9
n-6	4.9	4.1	4.0		41.6	5.5	3.4
n-3/n-6	15.7	9.2	5.2		0.7	7.0	1.4
PUFA+MUFA/SFA	2.3	2.0	3.3		2.6	2.6	2.4
PUFA/SFA+MUFA	0.7	0.5	0.3		1.0	0.7	0.2

5.4 Discussion

The success of hatchery production of bivalves is highly correlated to the quantity and quality of the microalgae supplied as larval food (Helm and Bourne, 2004). Because microalgae culture makes up a significant component of the production costs of bivalve spat (Müller et al., 2003), it is important that microalgae not only provide bivalve larvae with the required nutrients, but they should also be grown under conditions that maximize their productivity (growth rate and cell density) and optimise their nutritional composition for the target species. The increased availability of tropical microalgae species as a food source for tropical aquaculture species is a relatively new development and limited information is available on the nutritional value of these species. Studies on tropical microalgae species have focused primarily on describing their nutritional profile (Brown and Jeffrey, 1992; Dunstan et al., 1992; Renaud et al., 1994a,b; Brown et al., 1997; Volkman and Brown, 2005). However, some tropical microalgae have been used as food for pearl oyster larvae (Taylor et al., 1997; Southgate et al., 1998b) with very good results. The results of experiments conducted in Chapters 3 and 4 of this thesis confirmed the high nutritional value of some species of tropical microalgae for *P. margaritifera* larvae. They also showed that larval growth was positively correlated with a number of biochemical components of dietary microalgae such as total protein, lipid and carbohydrate, SFA and PUFA contents (Chapter 3). Having identified the important nutritional components of microalgae when fed to *P. margaritifera* larvae, it then becomes possible to assess the changes in nutrient levels within microalgae in response to varying culture conditions as a means of optimising the nutritional content of cultured

microalgae. This study determined the extent to which variations in light regime influence the biochemical contents of selected species of tropical microalgae when harvested at two points of the stationary phase of growth..

5.4.1 Growth patterns and chlorophyll 'a' contents

Lag or adaptation phase was practically negligible in all the microalgae species used in this study, independent of light regime, with the exception of *Pavlova* sp., for whichcontinuous light regime produced more cells (in terms of growth) compared to microalgae grown under the other light regimes during the same culture period. Similarly, *M.pusilla* produced more cells under 18L:6D and continouse light regimes than under 12L:12D. This agrees with previous studies which have shown that the growth of TISO using a 12L:12D light regime is similar to that obtained under continuous light (Toro, 1989, Tzovenis et al., 1997).

The increases in chlorophyll 'a' levels between the two days of harvesting, noted for most of the species, are presumably a response to reduced light resulting from increasing cell density (Parrish and Wangersky, 1990). It has previously been reported that PUFA accumulation (EPA in particular) could be associated with chlorophyll 'a' (Chl 'a') content of the photosynthetic membrane (Cohen et al., 1998); however, no significant relationship has been found between total PUFA and Chl 'a' in TISO (Brown et al., 1993). A relationship between the degree of unsaturation [(PUFA+MUFA)/SFA]

and Chl ‘a’ contents has, however, been reported for the diatom, *S. costatum* (Blanchemain and Grizcau, 1996). Attempts to determine whether there was a relationship between Chl ‘a’ contents and total PUFA contents in the different tropical microalgae species tested during this study, showed that this relationship was not significant, independent of day of harvest and light regime. On the other hand, a weak positive correlation was found between Chl ‘a’ content and EPA values during first harvesting day (day 7 or 10) ($r = 0.641$) but no significant correlation was found for these two parameters during the second harvesting day (day 10 or day 15). When trying to relate the degree of fatty acid unsaturation to the Chl ‘a’content for each microalga species cultured using the three different light regimes, no significant correlation was found, independent of microalgae species.

5.4.2 Proximate biochemical composition

Results showed that light regime had minor influence on the proximate composition of the most microalgae tested during this study. Accumulation of lipid on day 15 or day 20 was recorded for all species, independent of light regime, with the exception of TISO (18L:6D, continuous light), *Pavlova* sp. (12L:12D) and *C. muelleri* (12L:12D, continuous light), however there were only a few significant differences in nutrient accumulation between different light regimes Previous studies have accounted for this accumulation of lipid on the basis of nutrient limitation, specifically reduced nitrogen availability (McGinnis et al., 1997; Valenzuela-Espinoza et al., 2002; De la Peña and Villegas, 2005).

It has been suggested that when sufficient nutrients are available protein is synthesized and when nutrients are limited, cell division is suppressed and a greater amount of carbon is available for lipid storage (Johansen et al., 1990; Sukenik and Wahnon, 1991). In contrast to the other species, *M. pusilla* showed high protein levels when cultured in all light regimes on day 20. It may be possible that *M. pusilla* had sufficient nutrients to be able to synthesize protein during the late stationary phase. In our laboratory, *M. pusilla* can grow and maintain good condition for more than 20 days without apparent depletion in cells numbers.

5.4.3 Fatty acid composition

Fatty acid profiles of microalgae are susceptible to manipulation according to culture conditions such as changes in light regime (Price et al., 1998). In this study, the proportions of the major classes of fatty acids (i.e. SFA, MUFA and PUFA) were similar in golden-brown flagellates (TISO, *Pavlova* sp. and *Pav. salina*) where the relative proportions can be described as PUFA>SFA>MUFA independent of day of harvest or light regime. This agrees with the findings of previous studies which have shown relatively high levels of PUFA, relatively low levels of MUFA and intermediate levels of SFA in TISO when cultured using 12L:12D and continuous light regimes (Volkman et al.,1989; Tzovenis et al., 2003b). As reported in previous experiments, *M. pusilla* showed similar patterns to the golden-brown flagellates during this study (Chapter 3) where a the relative proportions of the major groups of fatty acids could also be described as PUFA>SFA>MUFA.

The fatty acid content of *C. muelleri* varied depending on day of harvest and light regime, however those difference were not statistically significant. The 12L:12D light regime resulted in algae where the relative proportions of the major groups of fatty acids were PUFA>SFA>MUFA independent of day of harvest. However, when *C. muelleri* was grown using a 18L:6D light regime, the relative proportions of the major groups of fatty acids was SFA≥MUFA≥PUFA on day 10 and PUFA>MUFA≥SFA on day 15. Continuous light resulted in a change in the relative proportions of the major groups of fatty acids to MUFA>SFA>PUFA independent of day of harvest. These results indicate high variability in the fatty acid content of *C. muelleri* according to culture conditions. This is consistent with previous observations that *C. muelleri* is less stable when compared to TISO in terms of responses to different culture conditions and is very susceptible to physiological manipulation (Pernet at al., 2003). Golden-brown flagellates and *M. pusilla* contained the highest PUFA contents which did not differ significantly between light regimes. In contrast, *C. muelleri* showed a trend towards a greater PUFA content during discontinuous light (18L:6D regimes) than in continuous light, independent of day of harvest, though those differences were not statistically significant. Similar findings have been reported in previous studies (McGinnis et al., 1997; Pernet et al., 2003).

The saturated fatty acids 14:0 and 16:0 have been characterized as important energy sources for metabolic processes in bivalves and as precursors for chain elongation and desaturation during synthesis of longer chain fatty acids including PUFA (Renaud et al.,

2000). All microalgae species contained these two SFA in relatively high amounts independent of light regime. Thomson et al. (1993) reported a correlation between the growth of *Crassostrea gigas* larvae and the SFA content of microalgae. Species of microalgae with high levels of SFA are thought to be more nutritious for bivalve larvae because energy is released more efficiently from saturated fats than from unsaturated fats (Thompson et al., 1993).

When the *n-3/n-6* quotient is within the 2-5 range, the resulting microalgae is considered to have acceptable nutritional quality (Delaunay et al., 1993; Tzovenis et al., 2003a,b; De la Peña and Villegas, 2005). The results showed that such indices were optimal in all golden-brown flagellates and in *M. pusilla* independent of growth phase and light regime. In contrast, the *n-3/n-6* index was less than 2 in *C. muelleri* on day 15 when this microalgae was cultured using the 12L:12D and continuous light regimes.

5.4.4. Influence of culture conditions on nutritional value

The results of Chapter 3 showed that the growth rates of *P. margaritifera* larvae were positively and significantly correlated with levels of specific nutrients within microalgae fed to them. For D-stage larvae these key nutrients were total carbohydrate, total lipid, total protein, SFA, PUFA, DHA, EPA+DHA and 14:0+16:0, and for umbone larvae growth was correlated with total carbohydrate, PUFA and EPA+DHA. The results of this Chapter have shown that the levels of many of these nutrients in microalgae are

influenced by culture conditions. It is reasonable to assume that an increase in the level of a key nutrient, brought about by a change in culture conditions, would signify a potential increase in nutritional value to *P. margaritifera* larvae. The relative levels of these key nutrients in each of the microalgae resulting from each of the culture conditions assessed in this study are shown in Tables 5.11 to 5.15.

The results of Chapter 3 showed that the two species of microalgae supporting the best larval growth of D-stage and umbo-stage *P. margaritifera* larvae as single species were *Pav. salina* and *Pavlova* sp. Furthermore, when the best performing mono-specific diets were used in binary and ternary combinations to feed larvae in Chapter 4, the results showed that *Pavlova* sp. supported a high growth rate of D-stage *P. margaritifera* larvae which was equivalent to that of larvae fed plurispecific diets. For umbo-stage *P. margaritifera* larvae, the best growth rate was achieved when the binary combination of *Pavlova* sp. and *C. muelleri* was used. Based on these results (Chapters 3 and 4), *Pavlova* sp. is the recommended diet for D-stage *P. margaritifera* larvae and a combination of *Pavlova* sp. and *C. muelleri* is the recommended diet for umbo-stage *P. margaritifera* larvae.

When *Pavlova* sp. and *C. muelleri* were cultured under different light regimes their proximate and fatty acid contents varied in response, however most of those differences were not statistically significant (Tables 5.13 and 5.15). Moreover, when looking at the resulting changes in levels of key nutrients (i.e. those that correlate to larval growth; Chapter 3), *Pavlova* sp. (day 20) and *C. muelleri* (day 15) showed relatively high levels

of these nutrients when cultured using a 12L:12D light regime and harvested during the stationary phase of growth (Tables 5.13 and 5.15, respectively). It is notable, however, that *Pavlova* sp. cultured using continuous light also showed relatively high levels of some of these components. Overall, algae cultured under 12L:12D and/or 18L:6D light regimes performed better for all species examined in this study in relation to growth and most of biochemical and fatty acid compositions. Therefore, in practical terms for pearl oyster hatcheries, culture of microalgae using a 12L:12D or 18L:6D light regime would be preferred from an economic perspective when compared to microalgae culture using continuous light.

Table 5.11. Relative proportions (low, * and high, ***) of key nutrients[#] within TISO grown under various culture regimes. [#]Those positively correlated with growth rates *P. margaritifera* larvae (Chapter 3).

TISO						
Component	12L:12D		18L:6D		24L:0D	
	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15
D-stage larvae						
Carbohydrate	*	***	*	*	***	***
Lipid	***	*	***	***	*	*
Protein	***	***	***	***	*	*
SFA	***	***	*	*	*	*
PUFA	*	*	***	***	*	***
DHA	*	*	*	*	***	*
EPA+DHA	*	*	*	*	***	***
14:0+16:0	***	***	*	*	***	***
Umbone larvae						
Carbohydrate	*	***	***	*	***	***
PUFA	*	*	***	***	*	***
EPA+DHA	*	*	*	*	*	*

Table 5.12. Relative proportions (low, * and high, ***) of key nutrients[#] within *Pavlova salina* grown under various culture regimes. [#]Those positively correlated with growth rates *P. margaritifera* larvae (Chapter 3).

Pavlova salina						
Component	12L:12D		18L:6D		24L:0D	
	Day 10	Day 15	Day 10	Day 15	Day 10	Day 15
D-stage larvae						
Carbohydrate	*	***	*	***	*	***
Lipid	***	*	***	*	***	*
Protein	***	***	*	*	***	***
SFA	***	*	*	***	*	***
PUFA	***	***	***	*	*	*
DHA	*	***	***	*	*	*
EPA+DHA	***	***	*	*	*	*
14:0+16:0	***	*	***	*	*	***
Umbone larvae						
Carbohydrate	*	***	*	***	*	***
PUFA	***	***	***	*	*	*
EPA+DHA	***	***	*	*	*	*

Table 5.13. Relative proportions (low, * and high, ***) of key nutrients[#] within *Pavlova* sp. grown under various culture regimes. [#]Those positively correlated with growth rates *P. margaritifera* larvae (Chapter 3).

Pavlova sp.						
Component	12L:12D		18L:6D		24L:0D	
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20
D-stage larvae						
Carbohydrate	***	***	*	*	***	***
Lipid	*	***	*	*	***	***
Protein	*	***	***	*	***	*
SFA	*	*	*	***	***	***
PUFA	***	***	*	*	*	*
DHA	***	***	*	*	*	*
EPA+DHA	***	***	*	***	*	*
14:0+16:0	*	*	***	*	*	***
Umbone larvae						
Carbohydrate	***	***	*	*	***	***
PUFA	***	***	*	*	*	*
EPA+DHA	***	***	*	***	*	*

Table 5.14. Relative proportions (low, * and high, ***) of key nutrients[#] within *Micromonas pusilla* grown under various culture regimes. [#]Those positively correlated with growth rates *P. margaritifera* larvae (Chapter 3).

Micromonas pusilla						
Component	12L:12D		18L:6D		24L:0D	
	Day 10	Day 15	Day 10	Day 15	Day 10	Day 10
D-stage larvae						
Carbohydrate	*	***	***	*	*	***
Lipid	*	***	***	*	***	*
Protein	*	*	***	***	*	*
SFA	*	*	*	***	***	*
PUFA	***	***	***	*	*	*
DHA	*	***	*	*	***	*
EPA+DHA	*	***	*	*	***	*
14:0+16:0	*	*	*	*	*	*
Umbone larvae						
Carbohydrate	*	***	***	*	*	***
PUFA	***	***	***	*	*	*
EPA+DHA	*	***	*	*	***	*

Table 5.15. Relative proportions (low, * and high, ***) of key nutrients[#] within *Chaetoceros muelleri* grown under various culture regimes. [#]Those positively correlated with growth rates *P. margaritifera* larvae (Chapter 3).

Chaetoceros muelleri						
Component	12L:12D		18L:6D		24L:0D	
	Day 10	Day 15	Day 10	Day 15	Day 10	Day 15
D-stage larvae						
Carbohydrate	*	***	*	*	***	*
Lipid	*	***	***	***	*	*
Protein	***	***	*	*	*	*
SFA	***	*	*	*	*	***
PUFA	***	***	*	*	*	*
DHA	*	*	***	*	***	*
EPA+DHA	***	*	*	***	*	*
14:0+16:0	***	***	*	*	*	***
Umbone larvae						
Carbohydrate	*	***	*	*	***	*
PUFA	***	***	*	*	*	*
EPA+DHA	***	*	*	***	*	*

Chapter 6

General conclusions and recommendations

6.1 Introduction

Reliable methods for hatchery culture of bivalves were developed in the 1960's and 1970's (Loosanoff and Davis, 1963; Walne 1974) for temperate species. For pearl oyster larvae the first studies in this field were those reported in a series of papers published in Japanese by Tanaka and collaborators in the 1970's (e.g. Tanaka et al., 1970 a, b, c, d). Studies with *P. margaritifera* during the 1980's were primarily limited to those dealing with natural stocks assessments and genetic diversity (i.e. Intes et al., 1985; Durand et al., 1988); only Alagarswami et al. (1989) reported, in a pioneering study, on the rearing of *P. margaritifera* larvae. It was not until the 1990's to 2000 that there were more detailed published reports of hatchery culture of *P. margaritifera* relating to culture systems (Southgate and Beer, 1997, Southgate and Ito, 1998), biochemical parameters (Doroudi et al., 1999b), larval density (Doroudi and Southgate, 2000) and feeding rations (Doroudi et al., 1999a; 2003). This study follows-on from those and has attempted to fine-tuned hatchery rearing protocols for *P. margaritifera*, particularly in relation to feeding. This study is the first to determine the nutritional value of tropical microalgae species for pearl oyster larvae on the basis of their nutrient compositions.

6.2 Nutritional value of tropical microalgae species

6.2.1 Nutritional components of tropical microalgae and their relationship with pearl oyster larval growth

The results of this study have shown that there are tropical microalgae species which have nutritional compositions that make them highly nutritious for *P. margaritifera* larvae (Chapter 3). *Pavlova salina* (CS-49) and *Pavlova* sp. (CS-50) were the two species of microalgae that supported the best growth rates of D-stage larvae (Day 2 to 11) and umbone larvae (Day 11 to 19). Microalgae species from the Prasinophycaea have been reported as preferentially ingested by young pearl oyster larvae (Doroudi et al., 2003; Martínez-Fernández et al., 2004). D-stage larvae fed *Isochrysis* sp., *C. muelleri* and *M. pusilla* showed significantly greater growth than unfed larvae, and larvae fed *Chaetoceros* sp. and CS-126 did not grow at a rate greater than unfed larvae. For umbo-stage larvae, the same microalgae supported similar growth to those used for D-stage larvae with those fed *Pav. salina* and *Pavlova* sp. again showing the greatest growth while those fed *Chaetoceros* sp. and CS-126 again grew at a rate similar to unfed larvae.

This study was the first to attempt to correlate the nutritional components of tropical microalgae with pearl oyster larval growth at two different growth stages. The results show that the growth of D-stage *P. margaritifera* larvae was positively and significantly correlated with dietary levels of protein, lipid and carbohydrate but the growth of older

umbone larvae was significantly correlated with carbohydrate content only. Furthermore, polyunsaturated fatty acid (PUFA) content, specifically the sum of EPA and DHA, was positively correlated to growth rate in both larval stages. DHA contents of microalgae were found to be positively correlated to the growth of D-stage larvae but not to that of umbone larvae. Similarly, the level of dietary SFA and 14:0+16:0 were significantly correlated with growth of D-stage larvae but not with that of umbo stage larvae. The experiments reported in Chapter 3 provided the basis for selection of microalgae species to be used in binary and ternary combinations for *P. margaritifera* larvae. Such combinations are likely to be valuable in optimising hatchery culture protocols.

6.2.2 Tropical microalgae combinations and pearl oyster larvae response

This thesis reports for the first time on the relative nutritional value of binary and ternary combinations of tropical micro-algae for *P. margaritifera* larvae (Chapter 4). There were no significant benefits in using binary or ternary algal combinations as food for D-stage larvae since binary and ternary diet did not support increased larval growth rates when compared to the best single species diet (*Pavlova* sp.) and there were no significant differences in terms of survival. *Pavlova* sp. (CS 50) is therefore the recommended diet for D-stage *P. margaritifera* larvae. For umbone larvae the results show that using diatoms as part of the algae combinations noticeably increase growth rates and survival when compared to dietary microalgae combinations without diatoms. A combination of *Pavlova* sp. and *C. muelleri* (CS-176) would be the recommended diet based on results

obtained in Chapter 4 for umbone larvae. Further research is required to determine how diatoms species contribute to improvements in larval growth and survival.

6.2.3 Biochemical response of selected tropical microalgae species to varying culture conditions

On the basis of their nutritional value for *P. margaritifera* larvae, the best five tropical microalgae selected in this study were: *Isochrysis* sp. (TISO), *Pav. salina, Pavlova* sp., *Micromonas pusilla* and *C. muelleri*. All microalgae species showed different patterns of growth and biochemical composition when culture under the three different light regimes. Photoperiods of 18L:6D and continuous light produced greater productivity of cells in all microalgae species. In terms of the nutritional components shown to be correlated to larval growth in Chapter 3 (total lipid, carbohydrate, proteins, SFA, 14:0+16:0, PUFA, DHA+EPA and DHA), the light regime of 12L: 12D produced relatively higher values of these nutrients when microalgae were harvested during the late stationary growth phase (Chapter 5). The results of Chapter 5 allow recommendations to be made relating to optimized culture conditions and harvesting stage for microalgae that result in improved nutritional composition.

6.3 Major findings of this study

The major findings of this study are shown in Fig. 6.1 together with aspects of our previous knowledge on the larval culture and feeding of *P. margaritifera* larvae. Based on the results of this study, recommendations can be made regarding the species composition of microalgae that should be fed to D-stage and umbone *P. margaritifera*

larvae in order to maximize growth and survival. As well as the compositions of such diets, the results of this thesis provide a guide to the microalgae culture conditions that will optimize the nutritional composition for *P. margaritifera* larvae. The diet recommended for D-stage larvae is a single-species diet composed of *Pavlova* sp. grown under a 12L:12D light regime and harvested during the late stationary growth phase. While, it is generally accepted that a mixture of microalgae fed to bivalve larvae provides a better balance of nutrients (Brown et al., 1997, 1998; Southgate, 2003), the results of this study showed that there was no advantage in terms of larval growth and survival resulting from the addition of other species to a diet of *Pavlova* sp. when fed to D-stage larvae. Microalgae culture has been estimated to make up 30% of hatchery production costs for bivalves; with production costs being estimated at $50 to 400 per kg of microalgae (Coutteau and Sorgeloos, 1993). On this basis, there is considerable economic benefit if good growth rates of larvae can be obtained by feeding one species of microalgae only. From a logistical perspective, this is also advantageous for *P. margaritifera* hatcheries in developing nations of the Pacific which may lack physical and technical resources and require simplified hatchery culture techniques (Southgate and Beer, 1997). The results of this study showed very clearly that the incorporation of a diatom into the diet of umbone *P. margaritifera* larvae supported increased growth rates. A binary diet composed of *Pavlova* sp. and *Chaetoceros muelleri* is recommended for umbone *P. margaritifera* larvae (Fig. 6.1). Again, algae should be cultured using a 12L:12D light regime and harvested during the stationary growth phase to maximize nutritional benefit to the larvae.

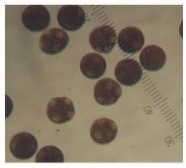
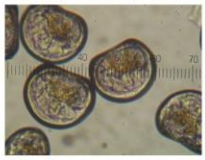
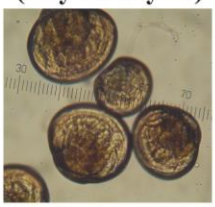
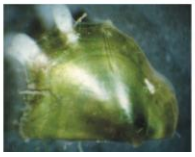
Previous knowledge		Results of this study
Thermal stimulation with addition of sperm (Southgate and Beer, 1997)	**Spawning**	
30-50 eggs mL^{-1} (Southgate et al., 1998a) 26-29ºC at 27-32‰ (Doroudi et al., 1999b)	**Egg incubation** 	
1:1 mixture of TISO & *Pavlova salina* as suitable diet. Larval density and algal ration: 1st wk 2.2 larvae mL^{-1}, 4.5-11.5 cells μL^{-1} 2nd wk 2 larvae mL^{-1}, 15 cells μL^{-1} Survival 27% (Doroudi and Southgate, 1999a; 2000) (Southgate et al., 1998a) 26-29ºC at 27-32‰ (Doroudi et al., 1999b)	**Larval culture D-Stage larvae (Day 2-day 12)** 	***Pavlova* sp**. As suitable diet Algal ration: Day 2-5 (0.235 µg mL^{-1}), DW basis Day 6-12 (0.470 µg mL^{-1}), DW basis Survival 98% Microalgae culture: 26 ± 1 ºC, 12L:12D, harvested stationary phase to obtain high levels of carbohydrate, lipid, protein, SFA and PUFA.
1:1 mix of TISO & *Pavlova salina* as suitable diet. Larval density and algal ration: 3rd wk 2 larvae mL^{-1}, 15-32 cells μL^{-1} Survival 50% (Doroudi and Southgate, 2000) 1:1:1 Tiso: *P. salina: C. muelleri* (Southgate & Beer, 1997) 1:1 *Isochrysis galbana*: *P. lutheri* (Alagarswami et al., 1999)	**Larval culture Umbone arvae (Day 13-day 26)** 	***Pavlova* sp**. - ***Chaetoceros muelleri*** as suitable diet Algal ration: Day 13-26 (0.470 - 0.705 µg mL^{-1}), DW basis Survival 81.7% Microalgae culture: 26±1ºC, 12L:12D, harvested stationary phase to obtain high levels of carbohydrate, and PUFA (EPA + DHA).
Spat transfer to ocean 3-5 weeks after settlement. (Pit & Southgate, 2000)	**Spat settlement (from day 27)** 	

Figure 6.1 Summary of the importance and application of the results of this thesis

The results of this study help identify nutrients imparting high nutritional value to tropical microalgae and allow some interpretation of the nutritional requirements of *P. margaritifera* larvae. The results provide a basis for development of more effective larval culture techniques by identifying microalgae supporting good growth of *P. margaritifera* larvae of different ages.

It is important to notice the high rates of larval survival obtained during this study compared to previous reports of *P. margaritifera* larval culture (Alagarswami et al., 1989; Southgate and Beer, 1997, Doroudi and Southgate, 2000) (Table 6.1). The use of tropical microalgae species may have enhanced larval performance in terms of survival and further research is required to determine whether such enhanced survival during larval culture results in greater yields of spat and more efficient hatchery production.

Table 6.1. Survival of D-stage and umbo-stage *P. margaritifera* larvae in this and previous studies

Study	**D-stage**	**Umbo-stage**
Alagraswami et al. (1989) (Microalgae used: *Isocrysis galbana*, *Pav. lutheri*)		6.3%
Southgate and Beer (1997) (Microalgae used: *Pav.salina,* TISO, *Chaetoceros muelleri*)		4.3%
Doroudi and Souhtgate, (2000) (Microalgae used: TISO, *Pav. salina, C. muelleri*)	23.2 %	7.7 %
Bellais et al. (2005) (Microalgae used: TISO, *Pav. lutheri, C. minus*)		30%
This study (Microalgae used: *Pavlova* sp, *Micromonas pusilla*, *C. muelleri*.)	98%	81.7%

References

Acosta-Salmón, H., Southgate, P.C. 2004. Use of a biopsy technique to obtain gonad tissue from the blacklip pearl oyster *Pinctada margaritifera* (L.). Aquacult. Research 35 (1) 93-96.

Alagarswami, K., Dharmaraj, S., Chellam, A., Velayudhan, T.S., 1989. Larval and juvenile rearing of black-lip pearl oyster, *Pinctada margaritifera* (Linnaeus). Aquaculture 76, 43-56.

Albentosa, M., Perez-Camacho, A., Labarta, U., Fernandez-Reiriz, M.J., 1996. Evaluation of live microalgae diets for the seed culture of *Ruditapes decussates* using physiological and biochemical parameters. Aquaculture 148, 11-23.

Anon. 2006. Goyla's Global View. Pearl World: The International Pearling Journal. 15 (1), 7-12.

Arnaud-Haond, S. Vonau, V., Bonhomme, F., Boudry, P., Prou, J., Seaman, T., Veyret, M., Goyard, E., 2003. Spat collection of the pearl oyster (*Pinctada margaritifera cumingii*) in French Polynesia: an evaluation of the potential impact on genetic variability on wild and farmed populations after 20 years of commercial explotation. Aquaculture 219, 181-192.

Balwin, B.S., Newell, R.I.E. 1991. Omnivorous feeding by planktotrophic larvae of eastern oyster *Crassostrea virginica*. Mar. Ecol. Prog. Ser. 78, 285-301.

Balwin, B.S., Newell, R.I.E. 1995. relative importance of different size food particles in the natural diet of oyster larvae (*Crassostrea virginica*). Mar. Ecol. Prog. Ser. 120, 135-145.

Barnes, H., Blackstock, J., 1973. Estimation of lipids in marine animals tissues: detailed investigation of sulphophosphovanillin method for 'total' lipids. J. Exp. Mar. Biol. Ecol. 12 (1), 103-118.

Bellais, M., Lo C. M.; Langy, S., Talfer, S.A. 2005 Hatchery as a research's tool. World Aquaculture 2005.Book of Abstracts. 9-13th of May Nusa Dua Bali, Indonesia. 80p.

Blanchemain, A., Grizcau, D. 1996. Eicosapentaenoic acid content of *Skeletonema costatum* as a function of growth and irradiance; relation with chlorophyll *a* content and photosynthetic capacity. J. of Exp. Mar. Biol. Ecol., 196, 177-188.

Blight, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (8), 911-917.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brown, M.R. 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol. 145, 79-99.

Brown, M.R., Jeffrey, S.W., 1992. The nutritional properties of microalgae used in mariculture - a review. NSW Fisheries, Salamander Bay, NSW, Australia, pp. 174-179.

Brown, M.R., Garland, C.D., Jeffrey, S.W., Jameson, I.D., Leroi, J.M., 1993. The gross and amino acid compositions of batch and semi-continuous cultures of *Isochrysis* sp. (clone T.ISO), *Pavlova lutheri* and *Nannochloropsis oculata*. J. Appl. Phycol. 5, 285-296.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151, 315-331.

Brown, M.R., McCausland, M.A.,Kowalski, K., 1998. The nutritional value of four Australian microalgal strains fed to Pacific oyster *Crassostrea gigas* spat. Aquaculture 165, 281-293.

Brown, M.R., Mccausland, M.A., Kowalski, K. 2001. The nutritional value of four Asutralian microalgal strains fed to pacific oyster *Crassostrea gigas* spat. Aquaculture, 165, 281-293.

Carvahlo, A.P.; Malcata, X.F. 2003. Kinetic modelling of the autotrophic growth of *Pavlova lutheri*: study of the combined influence of light and temperature. Biotechnol. Prog. 19, 1128-1135.

Chu, F.-L., Webb, K.L., 1984. Polyunsaturated fatty acids and neutral lipids in developing larvae of the oyster, *Crassostrea virginica*. Lipids 19, 815-820.

Cohen, Z., Vonshak, A., Richmond, A. 1998. Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum:* correlation to growth rate. J. Pgycol., 24, 328-332.

Cook, J.R. 1963. Adpatations in growth and division in *Euglena* effected by energy supply. J. Protozool. 10, 436-440.

Curatolo, A. Ryan, M.J., Mercer, J.P. 1993. An evaluation of the performance of Manila clam spat (*Tapes philippinarum*) fed on different ratios of spray-dried algae (*Tetraselmis suecica*). Aquaculture, 112, 179-186.

Culliney, J.L., Boyle, P.J. and Turner, R.D. 1974. New approaches and techniques for studying bivalve larvae. In: W.L. Smith, Chanley, M.H. (Ed.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 257-271.

Coutteau, P., Sorgeloos, P. 1993. Substitute diets for live algae in the intensive rearing of bivalve molluks – a state of art report. World Aquacult. 24 (2), 45-52.

Dawes, C.J. 1991. Marine Botany. 2nd Ed. John Wiley Press, New York. 454pp.

Delaunay, F., Marty, Y., Moal, J., Samain, J.F., 1993. The effect of monospecific algal diets on growth and fatty acid composition of *Pecten maximus* (L.) larvae. J. Exp. Mar. Biol. Ecol. 173, 163-179.

De la Peña, M.R., Villegas, C.T. 2005. Cell growth, effect of filtrate and nutritive value of the tropical Prasinophyte Tetraselmis tetrathele (Butcher) at different phases of culture. Aquacult. Res. 36, 1500-1508.

Doroudi, M.S., Southgate, P.C., Mayer, R.J., 1999a. Growth and survival of black-lip pearl oyster fed different densities of microalgae. Aquacult. Int. 7, 179-187.

Doroudi, M.S., Southgate, P.C., Mayer, R.J. 1999b. The combined effects of temperature and salinity on embryos and larvae of the black-lip pearl oyster, *Pinctada margaritiferai* (L.). Aquacult. Res. 30, 271-277.

Doroudi, M., Southgate, P.C., 2000. The influence of algal ration and larval density on growth and survival of blacklip pearl oyster *Pinctada margaritifera* (L.) larvae. Aquacult. Res. 31, 621-626.

Doroudi, M., Southgate, P. C., Mayer, R.J., 2002. Evaluation of partial substitution of live algae with dried *Tetraselmis* for larval rearing of black-lip pearl oyster, *Pinctada margaritifera* (L.). Aquacult. Inter. 10, 265-277.

Doroudi, M., Southgate, P., Lucas, J., 2003. Variation in clearance and ingestion rates by larvae of the black-lip pearl oyster (*Pinctada margaritifera* , L.) feeding on various microalgae. Aquacult. Nutr. 9, 11-16.

Dubois, M. Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. 18, 350-356.

Dunstan, G.A., Volkman, J.K., Jeffrey, S.W., Barrett, S.M., 1992. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 2. Lipid classes and fatty acids. J. Exp. Mar. Biol. Ecol. 161, 115-134.

Durand, P., Blanc, F., 1988. Genetic diversity in tropical marine bivalve: *Pinctada margaritifera* (Linne, 1758). Bull.Soc.Zool. France 113, 293-304.

Emata, A.C.,Ogata, H.Y., Garibay, E.S., Furuita, H., 2003. Advanced broodstock diets for the mangrove red snapper and potential importance of arachidonic acid in egss and fry. Fish Physiol. Biochem. 28, 489-491.

Enright, C.T., Newkirk, G.F., Craigie, J.S. Castell, J.D., 1986. Growth of juvenile *Ostrea edulis* L. fed *Chaetoceros gracilis* Schutt of varied chemical composition. J. Exp. Mar. Biol. Ecol. 96, 15-26.

Epifanio, C.E., 1979. Comparision of yeast and algal diets for bivalve molluscs. Aquaculture 16, 187-192.

Ellis, S., Haws, M. 1999. Producing pearls using the black-lip pearl oyster (*Pinctada margaritifera*). Aquafarmer Information Sheet. Center for tropical and subtropical aquaculture. Publication number 141. 8pp.

Ewart, J.W., Pruder, G.D., 1981. Comparative growth of *Isochrysis galbana* Parke and *Isochrysis aff. Galbana*, clone T-iso, at four temperatures and three light intensities. J. World. Maricult. Soc., 12, 333-339.

Falkowski, P.G. 1977. The adenylate energy charge in marine phytoplankton: the effect of temperature on the physiological state of *Skeletonema costatum* (Cleve). J. Exp. Mar. Biol. Ecol. 27, 37-45.

Fassler, C.R., 1997. Opportunities for investing in pearl farming. World Aquaculture Society, World Aquaculture'97 Book of abstracts. Seattle, WA, USA, Feb. 19-23.

Finlayson, A.J. 1964. Amino acid recoveries an the analysis of some feed samples. Can. J. Plant Sci. 45, 184-188.

Fritz, L.W. ,Lutz, R.A., Foote, M.A., Van Dover, C.L., Ewart, J.W. 1984. Selective feeding and grazing rates of oyster (*Crassostrea vitginica*) larvae on natural phytoplankton assemblages. Estuaries, 7, 513-518.

Folch, A.J., Less, M., Sloan-Stanley, G.H., 1957. A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Gallager, S.M., Mann, R., Sasaki, G.C., 1986. Lipid as an index of growth and viability in three species of bivalve larvae. Aquaculture 56, 81-103.

Gervis, M.H., Sims, N.A., 1992. The biology and culture of pearl oysters (Bivalvia: Pteriidae). ICLARM Stud.Rev.,Manila, Philippines 49 pp.

Guillard, R.R.L., 1973. Division rates. In: Stein, J.R. (Ed.). Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, pp. 289-311.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: W.L. Smith, Chanley, M.H. (Ed.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 26-60.

Helm, M.M., Bourne, N. 2004. Hatchery culture of bivalves . A practical manual. FAO. CITY, Italy. 177pp.

Helm, M.M., Laing, I., 1987. Preliminary observations on the nutritional value of "Tahiti *Isochrysis*" to bivalve larvae. Aquaculture 62, 281-288.

Helrich, K. 1990. Official methods of analysis of the association of official analytical chemists. Vol. 2, Fifteenth Edition. 1101-1102 pp.

Hofmann, E.E., Powell, E.N., Bochenek, E.A., Klinck, J.M., 2004. A modelling study of the influence of environment and food supply on survival of *Crassostrea gigas* larvae. J. Mar. Sci. 61, 596-616.

Holland, D.L., Gabbott, P.A., 1971. A micro-analytical scheme for determination of protein, carbohydrate, lipid and RNA levels in marine invertebrate larvae. J. Mar. Biol. Ass. U.K. 51, 659-668.

Intes, A., Coeroli, M., 1985. Evolution and condition of natural stocks of pearl oysters (*Pinctada margaritifera* Linne) in French Polynesia. Atenne Museum-EPHE, Moorea (French Polynesia), 545-550.

Isik, O., Sarihan, E., Gul, O., Erbatur, O., 1999. Comparasion of the fatty acid composition of the freshwater fish larvae *Tilapia zillii*, the rotifer *Brachionus calyciforus*, and the microalgae *Scenedesmus abundans*, *Monoraphidium minitum* and *Chlorella vulgaris* in the algae-rotifer-fish larvae food chains. Aquaculture, 174, 299-311.

James, C.M., Al-Hinty, S., Salmon A.E., 1989. Growth and ω3 fatty acid and amino acid composition of microalgae under different temperature regimes. Aquaculture 77, 337-351.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem.Physiol. Pflanzen, Bd. 167. S. 191-194.

Jeffrey, S.W., LeRoi, J.M., Brown, M.R. 1992. Characteristics of microalgal species for Australian mariculture. In: Allan, G.L. and Dall, W. (Eds.). Proceedings of the National Aquaculture Workshops, Pt. Stephens, NSW Australia, April 1991. 164-173.

Johansen, J.R. JR, Barclay WR, Nagle, N., 1990. Physiological variability within strains of *Chaetoceros muelleri* (Bacillariophyceae). J. Phycol. 26, 271-278.

Jonsson, P.R., Berntsson, K.M., André, C., Wängberg, S.A., 1999. Larval growth and settlement of the European oyster (*Ostrea edulis*) as a function of food quality measured as fatty acid composition. Mar. Biol., 134, 559-570.

Knauer, J., Southgate, P.C., 1999. A review of the nutritional requirements of bivalves and the development of alternative and artificial diets for bivalve aquaculture. Rev. Fish. Sci. 7, 241-280.

Knuckey, R.M., Brown, M.R., Barrett, S.M., Hallegraeff, G.M., 2002. Isolation of new nanoplnktonic diatom strains and their evaluation as diets for juvenile Pacific oyster (*Crassostrea gigas*). Aquaculture 211, 253-274.

Laing, I., Millican, P.F., 1986. Relative growth and growth efficiency of *Ostrea edulis* L. spat fed various algal diets. Aquaculture 54, 245-262.

Laing, I., Millican, P.F., 1991. Dried-algae diets and indoor nursery cultivation of Manila clam juveniles. Aquaculture 95, 75-97.

Langdon, C.J., Waldock, M.J., 1981. The effect of algal and artificial diets on the growth and fatty acid composition of *Crassostrea gigas* spat. J. Mar. Biol. Assoc. U.K. 61, 431-448.

Langdon, C.J. 1983. Growth studies with bacteria-free oyster (*Crassostrea gigas*) larvae fed on semidefined artificial diets. Biol.Bull. 164, 227-235.

Lavens, P., Sorgeloos, P. 1996. Manual on the production and use of live food in aquaculture. *Fisheries Technical Paper.* No. 361. Rome, FAO. 1996. 295p.

Lee, Y-K., Shen, H. 2004. Basic culturing techniques. In: A. Richmond (Ed.), Handbook of microalgal culture: biotechnology and applied phycology. Blackwell Science Publishing, Oxford, UK. pp 40-56.

Leonardos, N., Lucas, I.A.N., 2000. The nutritional value of algae grown under different culture conditions for *Mytilus edulis* L. larvae. Aquaculture 182, 301-315.

Le Pennec, M., Rangel-Dávalos, C. 1985. Observation en microscopie à épifluorescence de l'ingestion et de la digestion d'algues unicellulaires chez des jeunes larves de *Pecten maximus* (Pectinidae, Bivalvia). Aquaculture, 47. 39-51.

Loosanoff, V.L., Davis, H.C. 1963. Rearing Bivalve Mollusks. In: Rusell, F.S., (Ed.) Advances in Marine Biology. Vo. 1. Academic Press. 1-136.

Lora-Vilchis, M.C., Ruiz-Velazco- Cruz, E., Reynoso-Granados, T; Voltolina, D. 2004a. Evaluation of five microalgae diets for juvenile pen shells *Atrina maura*. J. of the World Aquacult. Soc., 35 (2): 232-236.

Lora-Vilchis, M.C., Robles-Mungaray, M., Doktor, N., Voltolina, D. 2004b. Food value of four microalgae for juveniles of the Lion's Paw scallop *Lyropecten subnodosus* (Sowerby, 1833). J. of the World Aquacult. Soc., 35 (2): 297-303.

Lourenco, S.O., Barbarino, E., Mancini-Filho, J., Schinke, K.P., Aidar, E., 2002. Effects of different nitrogen sources on the growth and biochemical profile of 10 marine microalgae in batch culture: an evaluation for aquaculture. Phycologia 41, 158-168.

Lucas, A. Rangel-Dávalos, C. 1981, Vitesses d'ingestion et de digestion du phytoplankton observées au microscope à épifluorescence chez les larves de *Mytilus edulis* (L.) (Bivalvia, Mollusca). Haliotis, 11, 171-180.

Mansour, M.P., Frampton, D.M.F., Nichols, P.D., Volkman, J.K. 2005. Lipid and fatty acid yield of nine stationary-phase microalgae: Applications and usual C_{24}-C_{28} polyunsaturated fatty acids. J. of Appl. Phycol., 17, 287-300.

Martínez-Fernández, E., Acosta-Salmón, H., Rangel-Dávalos, C., 2004. Ingestion and digestion of 10 species of microalgae by winged pearl oyster *Pteria sterna* (Gould, 1851) larvae. Aquaculture 230, 417-423.

Marty, Y., Dalaunay, F., Moal, J, Samain, J.-F. 1992. Changes in the fatty acid composition of the scallop *Pecten maximus* (L.) during larval development. J. Exp. Mar. Biol. Ecol. 163, 221-234.

McGinnis, K.M., Dempters, T.A., Sommerfeld, M.R. 1997. Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri*. J. Appl. Phycol. 9, 19-24.

Minaur, J., 1969. Experiments on the artificial rearing of the larvae of *Pinctada maxima* (Jameson) (Lamellibranchia) Aust. J. Mar. Freshwater Res. 20, 175-187.

Moore, S. 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. 238, 235-237.

Müller-Feuga, A., Robert, R., Cahu, C., Robin, J., Divanach, P., 2003. Uses of microalgae in aquaculture. In: Stottrup, J.G., McEvoy, L.A. (Ed.), Live Feeds in Marine Aquaculture. Blackwell Science Ltd., Oxford, UK. pp. 252-299.

Nell, J.A., O'Connor W.A. 1991. The evaluation of fresh algae and stored algal concentrates as a food source for Sydney rock oyster, *Saccostrea commercialis*, larvae. Aquaculture 99, 277-284.

O'Connor, W.A., Heasman, M.P., 1997. Diet and feeding regimens for larval doughboy scallops, *Mimachlamys asperrima*. Aquaculture 158, 289-303.

O'Sullivan, D. Savage, J. 2003. Status of Australian aquaculture in 2001/2002. Austasia Aquaculture trade directory. 5-22.

Opute, F.I. 1974. Lipid and fatty acid composition of diatoms. J. Expt. Bot., 25, 823-835.

Papandroulakis, N., Divanach, P., Anastasiadis, P, Kentouri, M. 2002. The pseudo green water technique for intensive rearing of sea bream *Sparus aurata* larvae. Aquacult. Int., 9, 205-216.

Parris, C.C., Wangersky, P.J. 1990. Growth and lipid class composition of the marine diatom, *Chaetoceros gracilis*, in laboratory and mass culture turbidostats. J. of Plank.Res. 12 (5), 1011-1021.

Pernet,F., Tremblay, R., Demers, E., Roussy, M., 2003. Variation of lipid class and fatty acid composition of *Chaetoceros muelleri* and *Isochrysis* sp. grown in a semicontinuos system. Aquaculture 221, 393-406.

Pernet F., Tremblay, R., 2004. Effect of varying levels of dietary essential fatty acid during early ontogeny of sea scallop *Placopecten magallanicus*. J. Exp. Mar. Biol. Ecol. 310, 73-86.

Pit, J.H., Southgate, P.C. 2000. When should pearl oyster, Pinctada margaritifera (L.), spat be transferred from the hatchery to the ocean?. Aquaculture Research 31 (10), 773-778.

Price, L.L., Yin, K., Harrison, P.J. 1998. Influence of continuos light and L:D cycles on the growth and chemical composition of Prymnesiophyceae including coccolithophores. J. Exp.Mar. Biol. Ecol., 223, 223-234.

Ponis, E, Probert, I. Véron, B., Le Coz, J.R., Mathieu, M. and Robert, R. 2006. Nutritional value of six Pavlovophyceae for *Crassostrea gigas* and *Pecten maximus* larvae. Aquaculture 254, 544-553.

Pouvreau, S., Bacher, C., Heral, M. 2000. Ecophysiological model of growth and reproduction of the black pearl oyster, *Pinctada margaritifera*: potential applications for pearl farming in French Polynesia. Aquaculture 186, 117-144.

Powell, E.N., Bochenek, E.A., Klinck, J.M., Hofmann, E.E., 2002. Influence in food quality on the growth and development of *Crassostrea gigas* larvae: A modelling approach. Aquaculture 210, 89-117.

Powell, E.N., Bochenek, E.A., Klinck, J.M., Hofmann, E.E., 2004. Influence of short-term variations in food on survival of *Crassostra gigas* larvae: A modelling study. J. Mar. Res. 62, 117-152.

Price, L.L., Yin, K., Harrison, P.J. 1998. Influence of continuos light and L:D cycles on the growth and chemical composition of Prymnesiophyceae including coccolithophores. J. of Exp.Mar. Biol. Ecol., 223, 223-234.

Raven, J.A., Geider, R.J. 1988. Temperature and algal growth. New Phycol. 110, 441-461.

Renaud, S.M., Parry, D.L., 1994a. Microalgae for use in tropical aquaculture 2: Effect of salinity on growth, gross chemical composition and fatty acid composition of three species of marine microalgae. J. Appl. Phycol. 6, 347-356.

Renaud, S.M., Parry, D.L., Thinh, L.-V., 1994b. Microalgae for use in tropical aquaculture 1: Gross chemical and fatty acid composition of twelve species of microalgae from the Northern Territory, Australia. J. Appl. Phycol. 6, 337-345.

Renaud, S.M., Thinh, L.V., Parry, D.L., 2000. The gross biochemical composition and fatty acid composition of 18 species of tropical microalgae for possible use in mariculture. Aquaculture, 170, 147-159.

Renaud, S.M., Thinh, L.V., Lambrinidis, G., Parry, D.L., 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical microalgae grown in batch cultures. Aquaculture 211, 195-214.

Robert, R.;Trintignac, P., 1997. Microalgues et nutritrion larvaire en ecloserie de mollusques. Haliotis 26, 1-13.

Rodhouse, P.G., Roden, C., Somerville-Jacklin, M.E., 1983. Nutritional value of microalgae mass culture to the oyster *Ostrea edulis* L. Aquaculture 32, 11-18.

Rose, R.A., Baker, S.B., 1994. Larval and spat culture of the Western Australian silver- or goldlip pearl oyster, *Pinctada maxima* Jameson (Mollusca: Pteriidae). Aquaculture 126, 35-50.

Ryan, M., O'Sullivan, D. 2001. Culture techniques of the Australian pearl industry. Austasia Aquaculture, 32- 37.

Sanchez, M.R. 1986. Rearing of mysid stages of *Penaeus vannamei* fed cultured algae of three species. Aquaculture, 58, 139-144.

Sandnes, J.M., Källqvist, T., Wenner, D., Gislerod, H.R., 2005. Combined influence of light and temperature on growth rates of *Nannochloropsis oceanica*: linking cellular responses to large biomass production. J. of Appl. Phycol. 17, 515-525.

Shifrin, N.S., Chisholm, S.W. 1980 Phytoplankton lipids: environmental influences on production and possible commercial applications. In: Shelef, G. Soeder, C.J. eds. Algae Biomass. 627-645.

Sorokin, C. 1973. Dry weight, packed cell volume and optical density. In: Stein, J.R. (Ed.). Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, pp.

Southgate, P.C., Beer, A.C., 1996. Hatchery production of the black-lip pearl oyster. Austasia Aquaculture 10(4), 58-60.

Southgate, P.C., Beer, A.C., 1997. Hatchery and early nursery culture of the blacklip pearl oyster (*Pinctada margaritifera* L.). J. Shellfish Res. 16, 561-567.

Southgate, P.C., Ito M., 1998. Evaluation of a partial flow-through culture technique for pearl oyster (*Pinctada margaritifera* L.) larvae. Aquacult. Eng. 18, 1-7.

Southgate, P.C., Taylor, J.J., Ito M. 1998a. The effects of egg density on hatch rate of pearl oyster (*Pinctada maxima* and *P. margaritifera*) larvae. Asian Fisheries Science 10: 265-268.

Southgate, P.C., Beer, A.C., Duncan, P.F., Tamburri, R., 1998b. Assessment of the nutritional value of three species of tropical microalgae dried *Tetraselmis* and a yeast-based diet for larvae of the blacklip pearl oyster, *Pinctada margaritifera* (L.). Aquaculture 162, 249-259.

Southgate, P.C. 2003. Feeds and feed production. In : Lucas, J.S., Southgate, P.C. (Eds). Aquaculture: Farming Aquatic Animals and Plants. Blackwell Publishing. Oxford, U.K. pp 172-198.

Spackman, D.H., Stein, W.H., Moore, S. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30, 1190.

Strugnell, J.M., Southgate, P.C., 2003. Changes in tissue composition during larval development of the blacklip pearl oyster, *Pinctada margaritifera* (L.). Molluscan Res. 23, 179-183.

Sukenik, A., Wahnon, R. 1991. Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*. Aquaculture, 97, 61-72.

Sweeny, R.A., 1989. Generic combustion method for determination of crude protein in feeds. J. Assoc. Off. Anal. Chem. 72, 770-774.

Tanaka, Y., Inoha, S., Kakazu, K., 1970a. Studies on seed production of black-lip pearl oyster, *Pinctada margaritifera*, in Okinawa –I Spawning induction by thermal stimulation. Bull. Tokai Regional Fisheries Res. Lab. 63, 75-78.

Tanaka, Y., Inoha, S., Kakazu, K., 1970b. Studies on seed production of black-lip pearl oyster, *Pinctada margaritifera*, in Okinawa –II Food micro-organisms available for the larvae. Bull. Tokai Regional Fisheries Res. Lab. 63, 79-85.

Tanaka, Y., Inoha, S., Kakazu, K., 1970c. Studies on seed production of black-lip pearl oyster, *Pinctada margaritifera*, in Okinawa – III Culture experiment of *Monochrysis lutheri* at high water temperature levels. Bull. Tokai Regional Fisheries Res. Lab. 63, 87-90.

Tanaka, Y., Inoha, S., Kakazu, K., 1970d. Studies on seed production of black-lip pearl oyster, *Pinctada margaritifera*, in Okinawa – V Rearing of the larvae. Bull. Tokai Regional Fisheries Res. Lab. 63, 97-106.

Taylor, J.J., Southgate, P.C., Wing, M.S., Rose, R.A. 1997. The nutritional value of five species of microalgae for spat of the silver-lip pearl oyster, *Pinctada maxima* (Jameson)(Mollusca: Pteriidae). Asian Fish. Sci. 10, 1-8

Thinh, L.V., Renaud, S.M., Parry, D.L. 1999. Evaluation of recent isolated Australian tropical microalgae for the enrichment of the dietary value of brine shrimp, *Artemia nauplii*. Aquaculture 170, 161-173.

Thomas, W.H., Seibert, D.L.R., Alden, M., Neori, A., Eldridge, P. 1984. Yields, photosynthetic efficiencies and proximate composition of dense marine microalgae cultures. III. *Isochrysis* sp. and *Monollantus salina* experiments ans comparative conclusions. Biomass 5, 299-316.

Thompson, P.A., Guo, M., Harrison, P.J., 1993. The influence of irradiance on the biochemical composition of three phytoplankton species and their nutritional value for larvae of the Pacific oyster (*Crassostrea gigas*). Mar.Biol. 117, 259-268.

Thompson, P.A., Guo. M.-X., Harrison, P.J. 1996. Nutritional value of diets that vary in fatty acid composition for larval Pacific oysters (*Crassostrea gigas*). Aquaculture 143, 379-391.

Toro J.E., 1989.The growth rate of two species of microalgae used in shellfish hatcheries cultured under two light regimes. Aquaculture and Fisheries Management 20, 249-254.

Tranter, D.J. 1958. Reprodcution in Australian pearl oysters (Lamellibranchia). IV. *Pinctada margaritifera* (Linnaeus). Aust. J. Mar. Freshw. Res. 9 (4), 509-525.

Tzovenis, I., De Pauw, N., Sorgeloos, P., 1997. Effect of different light regimes on the docosahexaenoic acid (DHA) content of Isochrysis aff. galbana (clone T-ISO). Aqua. Int. 5 (6), 489-507.

Tzovenis, I., De Pauw, N., Sorgeloos, P. 2003a. Optimisation of T-ISO biomass production rich in essential fatty acids: I. Effect of different light regimes on growth and biomass production. Aquaculture 216, 203-222.

Tzovenis, I., De Pauw, N., Sorgeloos, P. 2003b. Optimization of T-ISO biomass production rich in essential fatty acids. II. Effect of different light regimes on the production of fatty acids. Aquaculture 216, 223-242.

Utting, S.D., 1986. A preliminary study on growth of *Crassostrea gigas* larvae and spat in relation to dietary protein. Aquaculture 56, 123-138.

Valenzuela-Espinoza, E., Millan-Nunez, R., Nunez-Cebrero, F., 2002. Protein,

carbohydrate, lipid and chlorophyll a content in *Isochrysis* aff. *galbana* (clone T-Iso) cultured with a low cost alternative to the f/2 medium. Aquacult. Eng. 25, 207-216.

Van den Hoek, C., Mann D.G., Jahns, H.M. 1995. Algae: an introduction to phycology. Cambridge University Press, 623pp.

Van Wijngaarden, D., 1967. Modified rapid preparation of fatty acids esters from lipids for gas chromatographic analysis. Anal. Chem. 39, 848-849.

Volkman, J.K., Jeffrey, S.W., Nichols, P.D., Rogers, G.I., Garland, C.D., 1989. Fatty acid and lipid composition of ten species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol. 128, 219-240.

Volkman, J.K., Dunstan, S.W., Jeffrey, S.W., Kearney, P.S. 1991. Fatty acids from microalgae of genus *Pavlova*. Phytochemistry 30, 1855-1859.

Volkman, J.K., Brown, M.R., Dunstan, G.A., Jefrrey, S.W. 1993. The biochemical composition of marine microalgae from the class Eustigmatophyceae. J.Phycol. 29, 69-78.

Volkman, J.K., Brown, M.R. 2005. Nutritional value of microalgae and applications. In: Subba Rao, D.V. (Editor) Algal Cultures, Analogues of Blooms and Applications. Science Publishers. Plymouth, U.K. pp 407-457.

Waldock, M.J., Holland, D.L., 1984. Fatty acid metabolism in young oysters, *Crassostrea gigas*: polyunsaturated fatty acids. Lipids 19, 332–336.

Walne, P.R., 1963. Observations on the food value of seven species of algae to the larvae of *Ostrea edulis*: I. Feeding experiments. J. Mar.Biol. Assoc. UK 43, 115-134.

Walne, P.R. 1974. Culture of bivalve molluscs. 50 years experience at Conwy. Fishing News Books. Whitefriars Press Ltd. London, U.K. 190 pp.

Webb, K.L., Chu, F.L., 1982. Phytoplankton as a food source for bivalve larvae. In: Pruder, G.D., Langdon, C.J., Conklin, D.E. (Eds.). Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition. Louisiana State University, Baton Rouge, pp. 272-291.

Whyte, J.N.C., 1987. Biochemical composition and energy content if six species of phytoplankton used in mariculture of bivalves. Aquaculture 60, 231-241.

Whyte, J.N.C., Bourne N., Hodgson, C.A., 1990. Nutritional condition of Rock scallop, *Crassadoma gigantea* (Gray), larvae fed mixed algal diets. Aquaculture 86, 25-40.

Wikfors, G.H. 1986. Altering growth and gross biochemical composition of two microalgal molluscan food species by varying nitrate and phosphate. Aquaculture, 59, 1-14.

Zar, J.H., 1999. Biostatistical Analysis. Fourth edition. Prentice Hall International, Inc. New Jersey, 123pp.

Zhu, C.J., Lee, Y.K., Chao, T.M. 1997. Effects of temperature and growth phase on lipid and biochemical composition of Isochrysis galbana TK1. J. of Appl. Phycol. 9, 451-457.

APPENDIX 1

Biochemical methods used in this study

A. Microalgae paste were freeze dried. Each pre-dried and pre-weighed sample was placed in a labelled micro-centrifuge tube and homogenised with and IKA UltraTurrax T25 homogeniser with 1-mL of distilled water

LIPID ASSAY

1. Add 300-µL homogenate to a 5 mL polypropylene centrifuge tube
2. Add 100-µL H_2O + 1,500-µL 1:2 $CHCl_3$:CH_3OH – 1st Extraction (Blight and Dyer, 1959)
3. Shake or sonicate
4. Stand for 10 minutes
5. Centrifuge at 1000 g for 10 minutes
6. Remove supernatant to a second centrifuge tube
7. To precipitate from 6. add 1,500-µL 2:1 $CHCl_3$:CH_3OH – 2nd Extraction (Folch et al., 1957)
8. Shake or sonicate
9. Stand for 10 minutes
10. Centrifuge at 1000 g for 10 minutes
11. Remove supernatant and pool with supernatant from 6. above
12. Add 950-µL of 0.7% NaCl to the pooled supernatant –Purification of lipid extract (Folch et al., 1957)

13. Mix thoroughly, stand at 4 ºC for > 30 min
14. Centrifuge at 500 g (1,500 rpm) for 10 min
15. Take 1,000-µL of bottom layer ($CHCl_3$) (which contain lipid) to a micro-tube and dry at 60 °C
16. Add 500 µL H_2SO_4
17. Shake
18. Close the micro-tube and heat in a water bath at 100°C for 10 minutes
19. Cool down at room temperature
20. Transfer 20-µL to a new micro-tube (3-5 replicates)
21. Add 500-µL of the H_3PO_4 – Vanillin reagent (0.5 g of Vanillin (Univar 574 – 100 G) in 250 mL Orthophosphoric Acid [4:1 Phosphoric Acid: Water]) (Barnes and Blackstock, 1973)
22. Shake
23. Stand for 20 minutes
24. Transfer 200 µL to microplate
25. Read @ **520 nm**
    a. Calibration versus cholesterol or tripalmitin dissolved in 2:1 v/v $CHCl_3$: CH_3OH at 1. above
    b. Reagent blank: solvent only at 1. Above

Lipid standard (to be prepared at the beginning of the process, so from each concentration take 300-µL in centrifuge tubes as 1. above in 3 replicates)

- Cholesterol 8 mg mL^{-1} dissolved in 2:1 v/v $CHCl_3$:CH_3OH

Reagent/concentration	0	2.5	5.0	7.5	10
Cholesterol Standard sol.	0	0.25-mL	0.5-mL	0.75-mL	1-mL
2:1 v/v $CHCl_3$:CH_3OH	1-mL	0.75-mL	0.5-mL	0.25-mL	0

PROTEIN AND CARBOHYDRATE ASSAYS (Modified from Bradford, 1976 and Dubois et al., 1956)

1. Add 500-µL homogenate to micro-tube
2. Add 250-µL of 15% Trichloroacetic Acid (TCA)
3. Shake
4. Stand > 10 minutes (preferably overnight) at 4°C
5. Centrifuge @ 1000 g for 10 minutes
6. Add 100-µL supernatant to a new micro-tube (3-5 replications)
7. Add 100-µL H_2O + 100 µL 5% Phenol
8. Mix
9. Add 500-µL H_2SO_4
10. Mix
11. Stand > 25 minutes
12. Transfer 200-µL to microplate
13. Read @ **490 nm**
14. Calibration versus glucose dissolved in 5% w/v TCA at 6. above
    a. Reagent blank: solvents only at 6. above

Carbohydrate standard (to be prepared at the beginning of the process, so from each concentration take 100-µL in micro- tubes as 1. above in 3 replicates)

- Glucose 1 mg mL^{-1} dissolved in 5% TCA

Reagent/concentration	0	0.25	0.50	0.75	1.0
Glucose standard sol.	0	0.25-mL	0.50-mL	0.75-mL	1-mL
5% TCA	1-mL	0.75-mL	0.50-mL	0.25-mL	0

15. To precipitate *(remove supernatant*) of 5. above add 1-mL of 0.1 N NaOH
16. Mix
17. Dissolved precipitate by putting samples in the oven at 90 ºC for one hour
18. Add 250-µL to micro-tube (3-5 replicates)
19. Add 990-µL H_2O
20. Mix
21. Add 250-µL BioRad Dye reagent
22. Mix
23. Incubate for 5 minutes
24. Transfer 200-µL to microplate
25. Read @ **595 nm**
    a. Calibration versus bovine serum albumin dissolved in distilled water at 14. above
    b. Reagent blank: distilled water at 14. above

Protein standard (to be prepared at the beginning of the process, so from each concentration take 100-µL in micro- tubes as 1. above in 3 replicates)

- BSA 6 mg mL^{-1} dissolved in H_2O

Reagent/concentration	0	1.5	3	4.5	6
BSA standard sol.	0	0.25-mL	0.50-mL	0.75-mL	1-mL
H_2O	1-mL	0.75-mL	0.50-mL	0.25-mL	0

APPENDIX 2

ANOVA Results for Chapter 5

Two-way ANOVA Results for Chapter 5

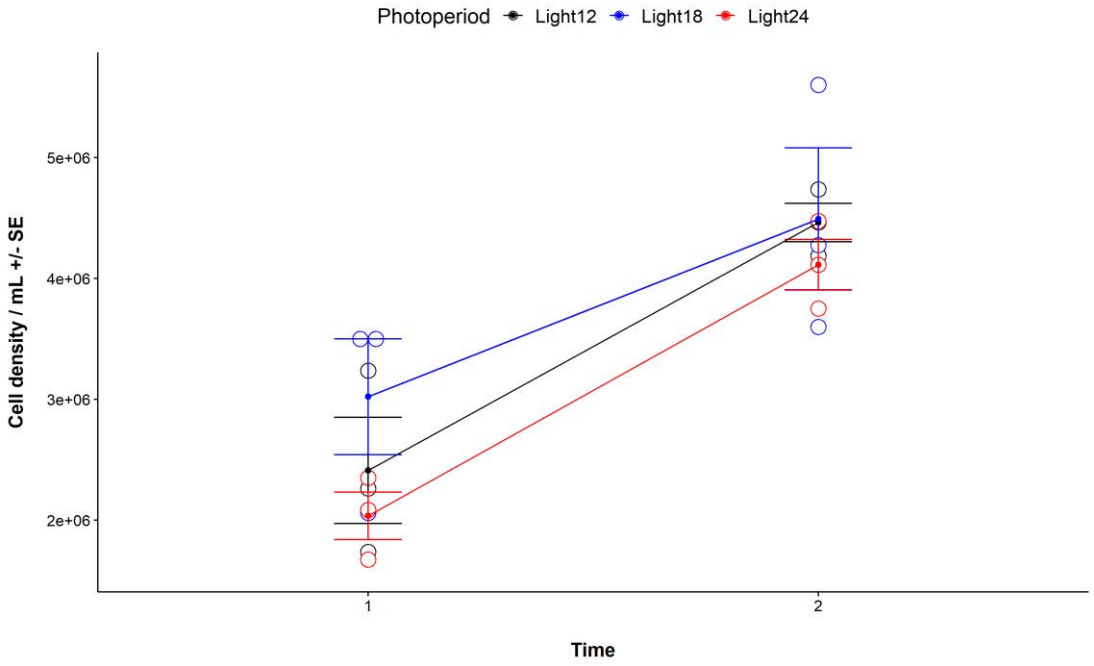
a. *Isochrysis* sp. (TISO)

Table A1 Repeated measures ANOVA & one-way ANOVA results for TISO growth rates (cell density/ mL), biochemical composition (protein, lipid and carbohydrate contents) and fatty acid content (SFA, MUFA, PUFA). Significance accepted at adjusted $p < 0.007$ and is highlighted in bold and red. **Note:** Protein data did not have a full set of samples for time 2, therefore only data for time 1 has been analyzed.

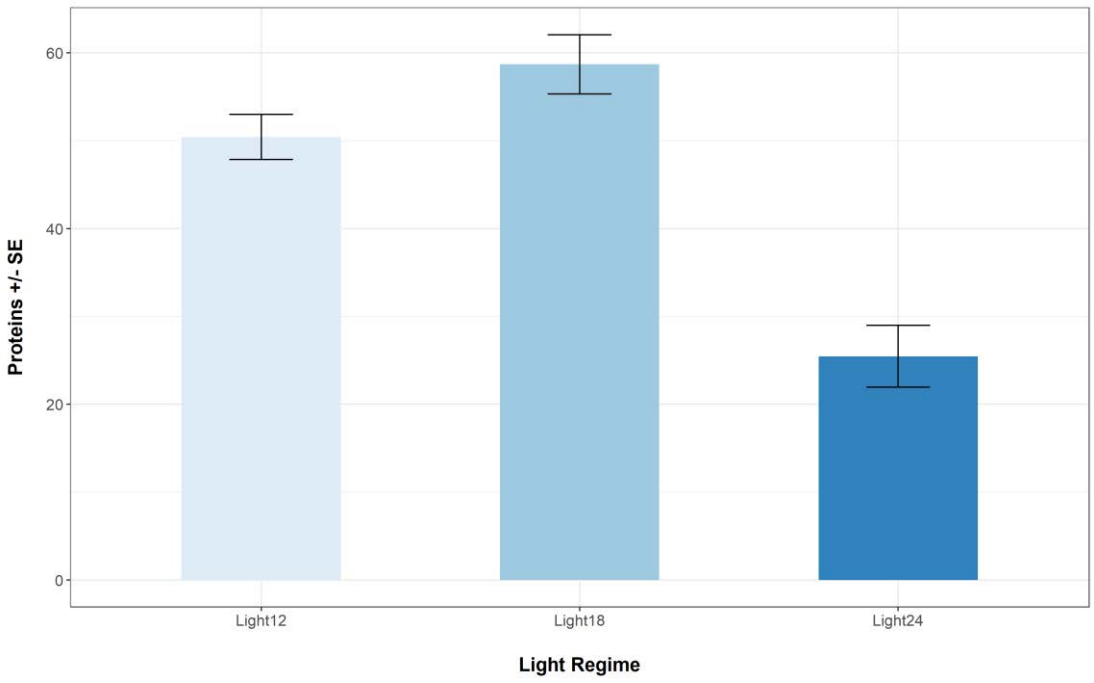
Variable	Factor	df	SS	MS	F	p
Growth rates	Light Regime (LR)	2	1.394e+12	6.971e+11	1.59	0.244
	Sampling time (ST)	1	1.566e+13	1.566e+13	35.75	**0.000**
	LR:ST	2	3.505e+11	1.753e+11	0.4	0.679
	Residuals	12	5.255e+12	4.379e+11		
Protein (One-way ANOVA for t1)	LR	2	1198.0	599.0	29.58	0.0106
	Residuals	3	60.7	20.2		
Lipid	LR	2	1431.0	715.5	17.516	**0.006**
	ST	1	2.0	2.0	0.049	0.834

	LR:ST	2	637.5	318.8	7.803	0.03
	Residuals	5	204.2	40.8		
Carbohydrate	LR	2	156.94	78.47	2.992	0.14
	ST	1	0.42	0.42	0.016	0.9
	LR:ST	2	47.80	23.90	0.91	0.46
	Residuals	5	131.12	26.22		
SFA	LR	2	36.24	18.122	1.004	0.43
	ST	1	0.13	0.125	0.007	0.937
	LR:ST	2	15.49	7.747	0.429	0.673
	Residuals	5	90.22	18.045		
MUFA	LR	2	120.06	60.03	4.71	0.0708
	ST	1	36.97	36.97	2.901	0.1493
	LR:ST	2	1.54	0.77	0.06	0.942
	Residuals	5	63.72	12.74		
PUFA	LR	2	192.85	96.42	4.406	0.0788
	ST	1	32.79	32.79	1.499	0.2754
	LR:ST	2	7.33	3.67	0.168	0.8503
	Residuals	5	109.42	21.88		

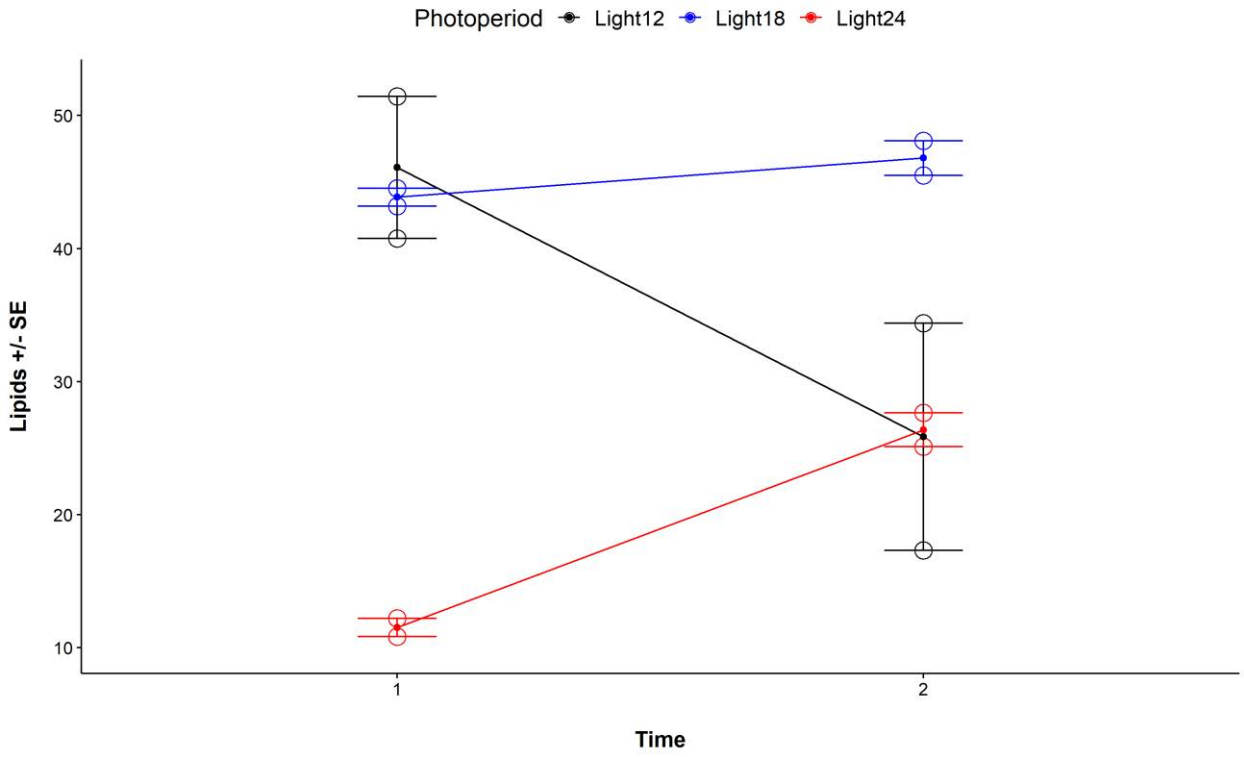
a.


Photoperiod
Light12
Light18
Light24
Cell density / mL +/- SE
5e+06
4e+06
3e+06
2e+06
1
2
Time

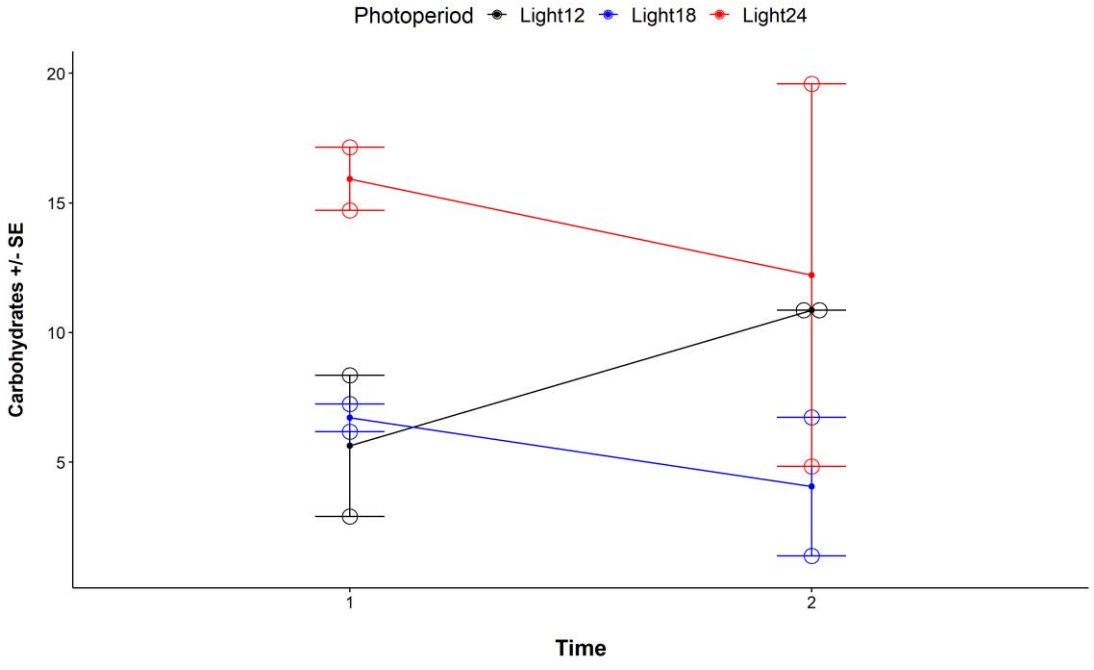
b.


Proteins +/- SE
60
40
20
0
Light12
Light18
Light24
Light Regime

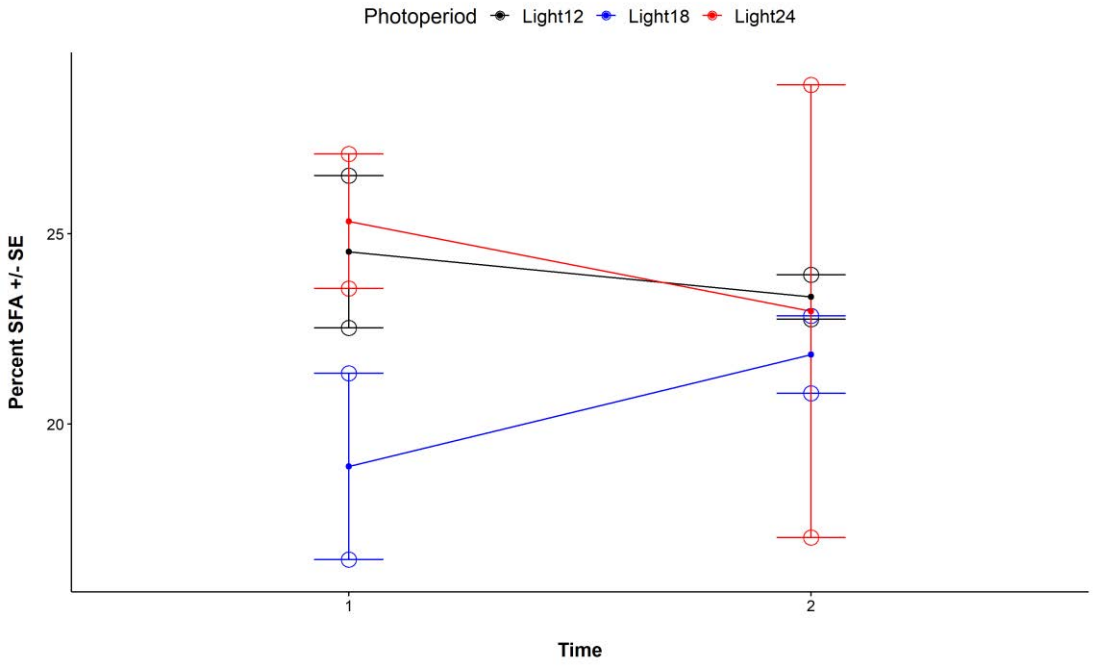
c.


Photoperiod
Light12
Light18
Light24
Lipids +/- SE
50
40
30
20
10
1
2
Time

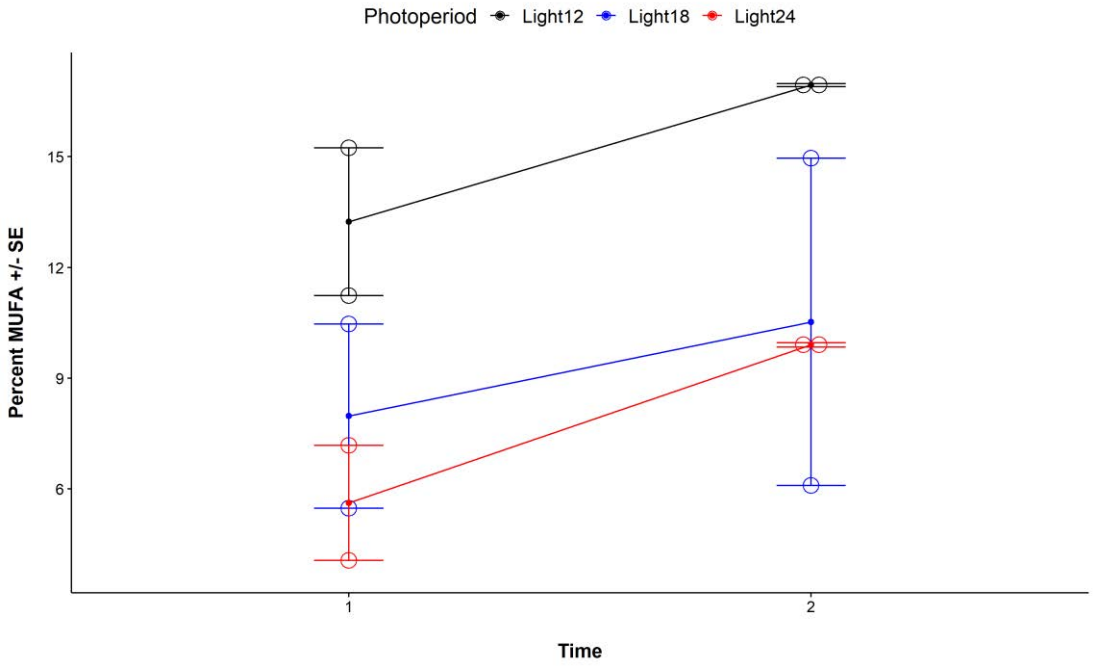
d.


Photoperiod
Light12
Light18
Light24
Carbohydrates +/- SE
20
15
10
5
1
2
Time

e.


Photoperiod
Light12
Light18
Light24
Percent SFA +/- SE
25
20
1
2
Time

f.


Photoperiod
Light12
Light18
Light24
Percent MUFA +/- SE
15
12
9
6
1
2
Time

g.

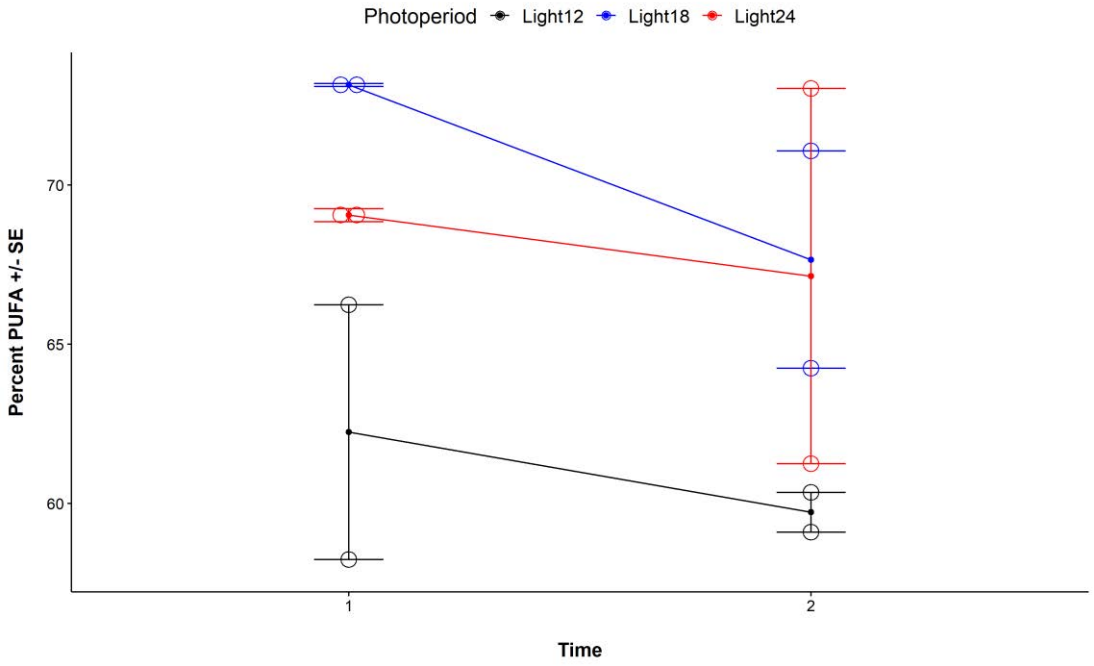


Figure A1 Interaction plots for TISO **a.** growth rates (cell density/ mL), biochemical composition (**b.** protein, **c.** lipid and **d.** carbohydrate contents; displayed as % of dry weight) and fatty acid content (**e.** SFA, **f.** MUFA, **g.** PUFA) at two different sampling times (Day 7 and Day 15) between 3 different light regimes. **Note:** Protein data did not have a full set of samples for time 2, therefore only data for time 1 for all three light regimes is shown.

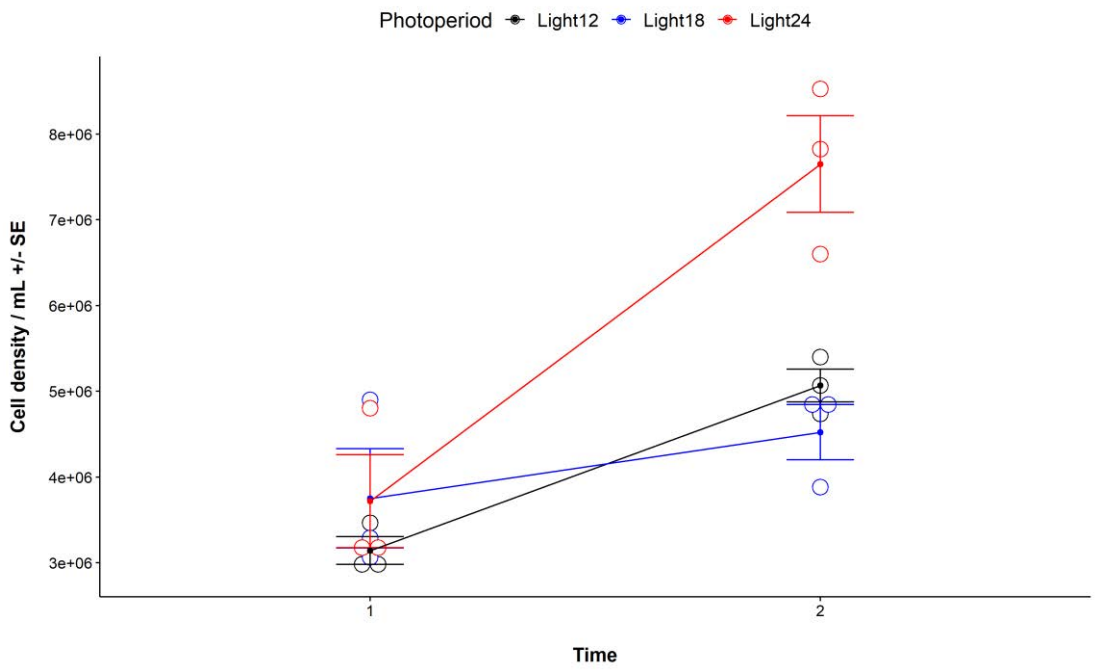
b. *Pavlova salina*

Table A2 Repeated measures ANOVA & one-way ANOVA results for *P.salina* growth rates (cell density/ mL), biochemical composition (protein, lipid and carbohydrate contents) and fatty acid content (SFA, MUFA, PUFA). Significance accepted at adjusted $p < 0.007$ and is highlighted in bold and red. . **Note:** Protein data did not have a full set of samples for time 1, therefore only data for time 2 has been analyzed.

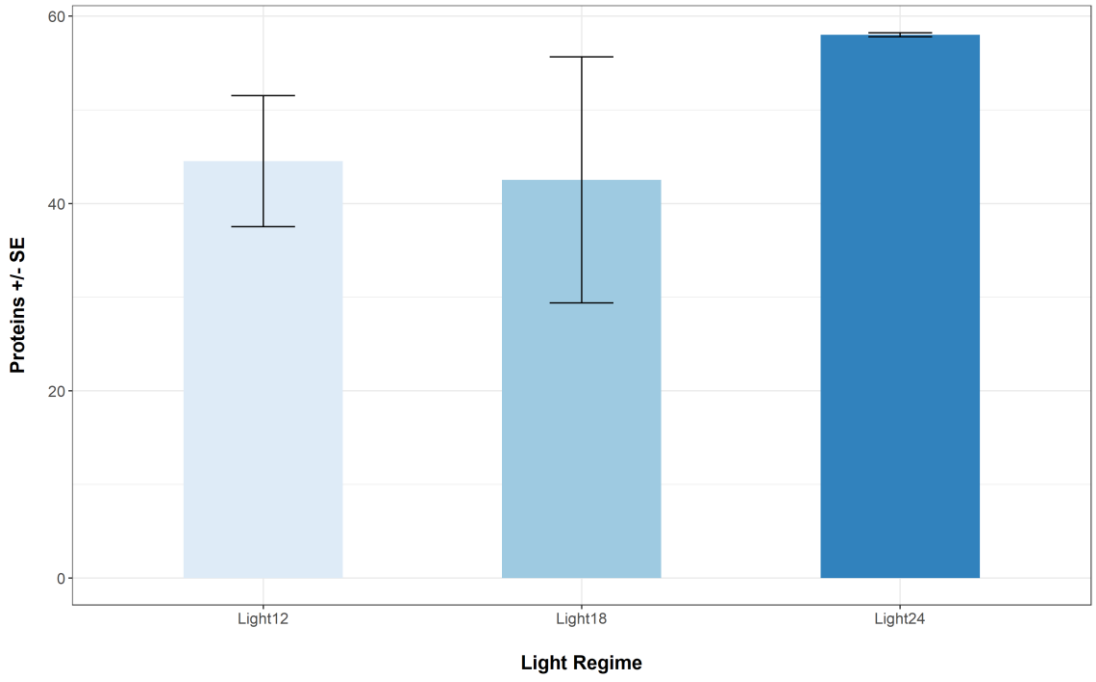
Variable	Factor	df	SS	MS	F	p
Growth rates	Light Regime (LR)	2	9.769e+12	4.884e+12	8.79	**0.005**
	Sampling time (ST)	1	2.200e+13	2.200e+13	39.59	**0.0000**
	LR:ST	2	7.673e+12	3.836e+12	6.90	0.01
	Residuals	12	6.668e+12	5.557e+11		
Protein (one-way ANOVA for t2)	LR	2	284.2	142.1	0.96	0.476
	Residuals	3	443.9	148.0		
Lipid	LR	2	601.3	300.66	28.409	**0.002**
	ST	1	311.3	311.3	29.418	**0.003**
	LR:ST	2	87.6	43.78	4.137	0.087
	Residuals	5	52.9	10.58		
Carbohydrate	LR	2	1.562	0.781	0.254	0.785
	ST	1	0.367	0.367	0.119	0.744

	LR:ST	2	6.949	3.474	1.128	0.394
	Residuals	5	15.404	3.081		
SFA	LR	2	0.408	0.204	0.098	0.908
	ST	1	5.478	5.478	2.639	0.1652
	LR:ST	2	23.651	11.825	5.698	0.0514
	Residuals	5	10.377	2.075		
MUFA	LR	2	193.63	96.82	5.317	0.0578
	ST	1	40.84	40.84	2.243	0.1945
	LR:ST	2	172.38	86.19	4.734	0.0702
	Residuals	5	91.04	18.21		
PUFA	LR	2	205.54	102.77	6.763	0.0378
	ST	1	16.40	16.40	1.08	0.3464
	LR:ST	2	159.78	79.89	5.258	0.059
	Residuals	5	75.97	15.19		

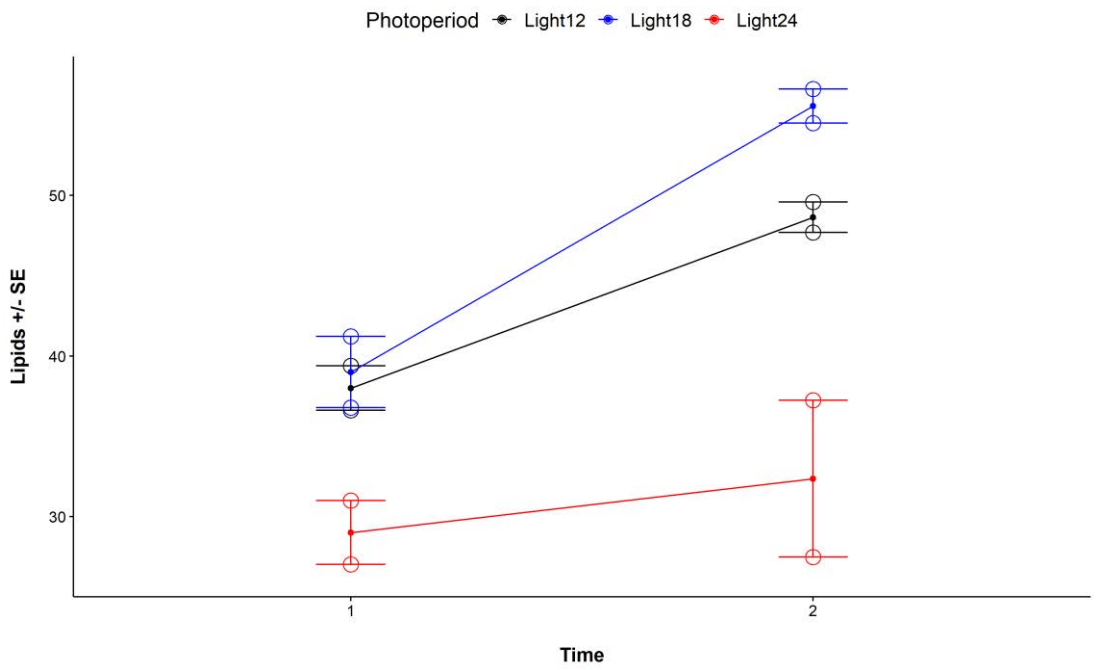
a.


Photoperiod
Light12
Light18
Light24
Cell density / mL +/- SE
8e+06
7e+06
6e+06
5e+06
4e+06
3e+06
1
2
Time

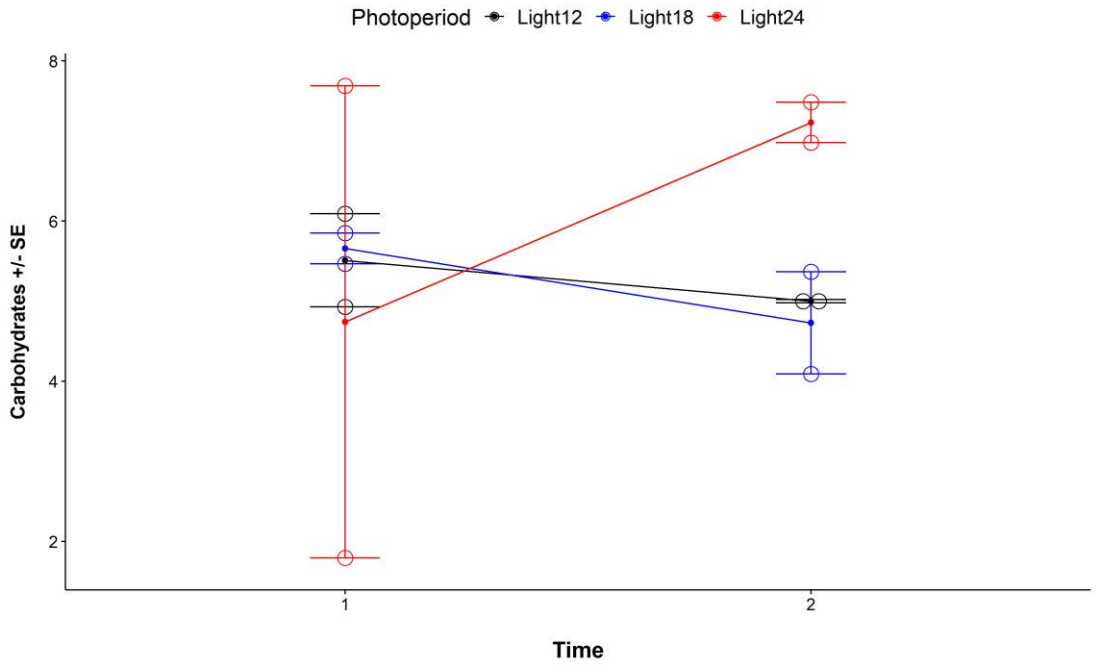
b.


Proteins +/- SE
60
40
20
0
Light12
Light18
Light24
Light Regime

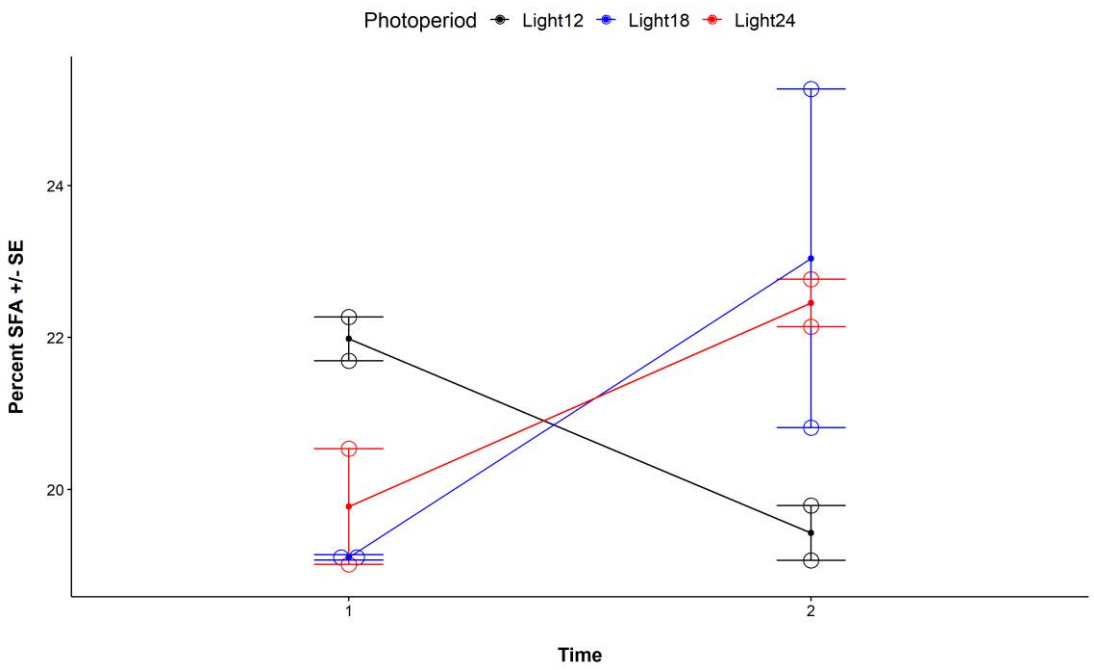
c.


Photoperiod
Light12
Light18
Light24
Lipids +/- SE
50
40
30
1
2
Time

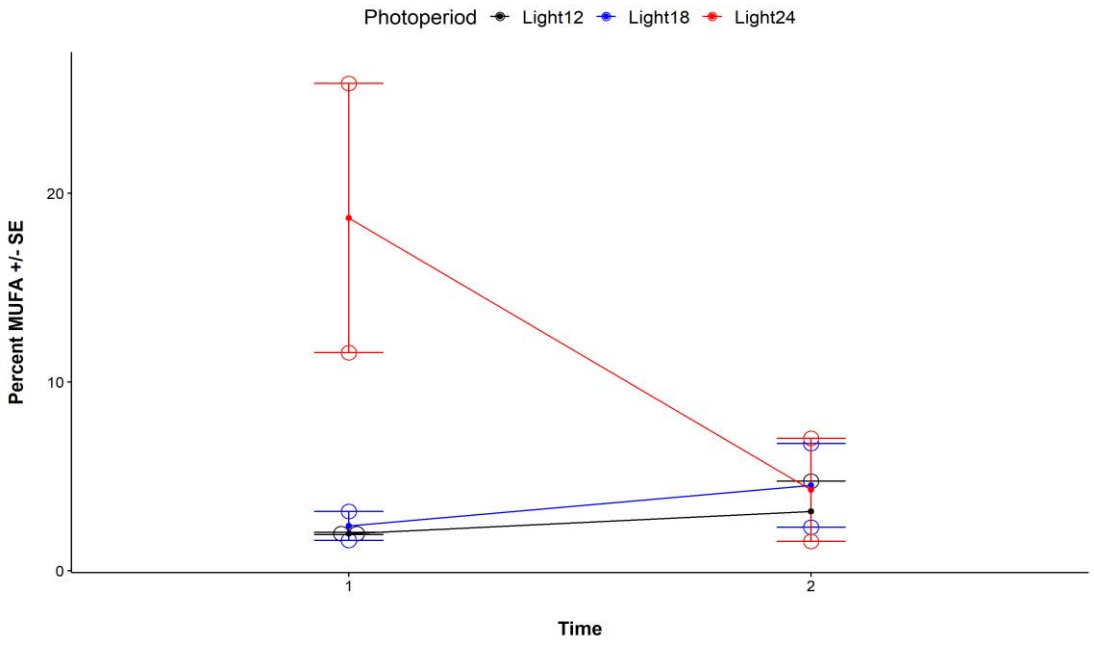
d.


Photoperiod
Light12
Light18
Light24
Carbohydrates +/- SE
8
6
4
2
1
2
Time

e.


Photoperiod
Light12
Light18
Light24
Percent SFA +/- SE
24
22
20
1
2
Time

f.


Photoperiod
Light12
Light18
Light24
Percent MUFA +/- SE
20
10
0
1
2
Time

g.

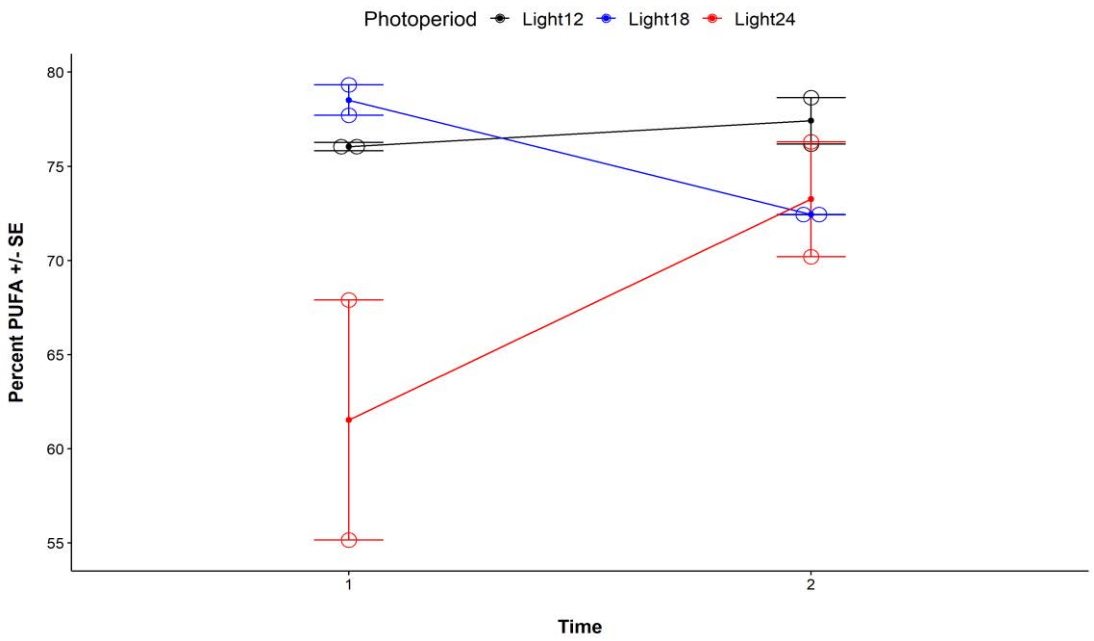


Figure A2 Interaction plots for *P. salina* **a.** growth rates (cell density/ mL), biochemical composition (**b.** protein, **c.** lipid and **d.** carbohydrate contents; displayed as % of dry weight) and fatty acid content (**e.** SFA, **f.** MUFA, **g.** PUFA) at two different sampling times (Day 10 and Day 15, exp for protein) between 3 different light regimes. **Note:** Protein data did not have a full set of samples for time 1, therefore only data for time 2 for all three light regimes is shown.

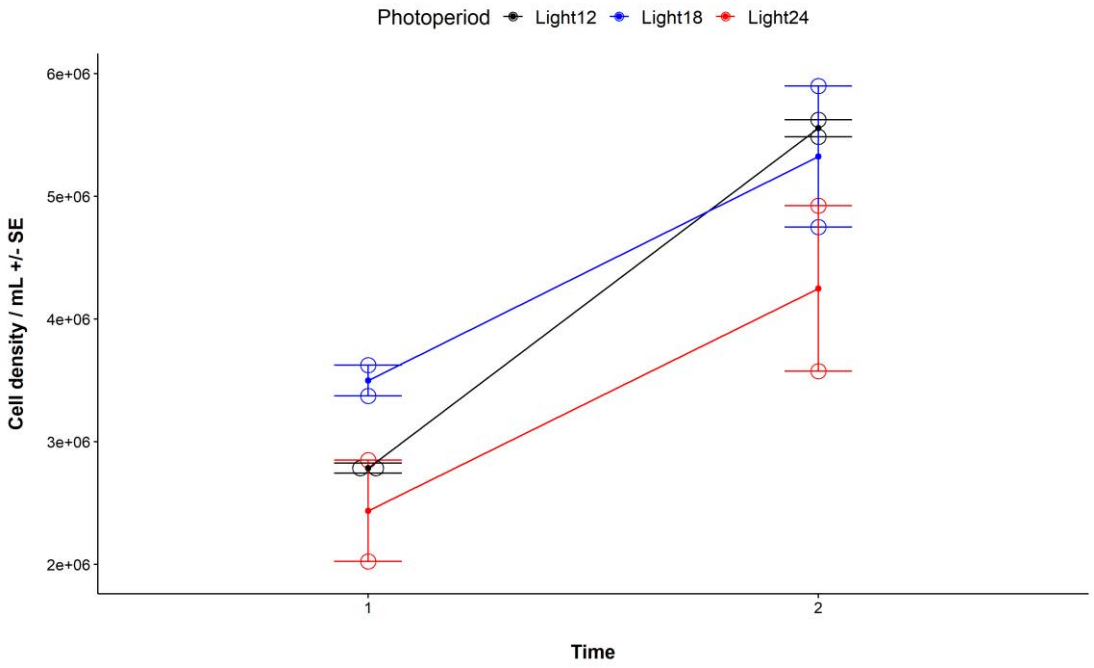
c. *Pavlova* sp.

Table A3 Repeated measures ANOVA results for *Pavlova* sp. growth rates (cell density/ mL), biochemical composition (protein, lipid and carbohydrate contents) and fatty acid content (SFA, MUFA, PUFA). Significance accepted at adjusted $p < 0.007$ and is highlighted in bold and red.

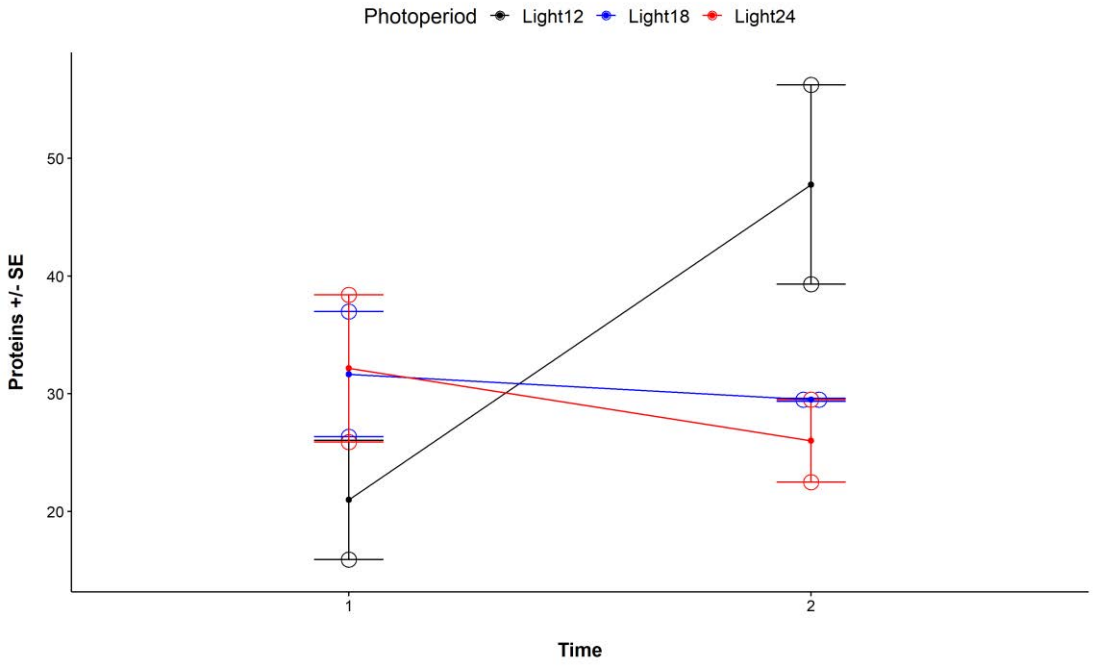
Variable	Factor	df	SS	MS	F	p
Growth rates	Light Regime (LR)	2	2.512e+12	1.256e+12	3.85	0.0839
	Sampling time (ST)	1	1.369e+13	1.369e+13	41.99	**0.0006**
	LR:ST	2	6.057e+11	3.029e+11	0.93	0.4453
	Residuals	6	1.957e+12	3.261e+11		
Protein	LR	2	59.6	29.8	0.608	0.5803
	ST	1	113.5	113.5	2.317	0.1884
	LR:ST	2	646.1	323.1	6.595	0.0396
	Residuals	5	244.9	49.0		
Lipid	LR	2	317.5	158.76	22.454	**0.003**
	ST	1	0.1	0.05	0.007	0.935
	LR:ST	2	200.8	100.42	14.203	0.009
	Residuals	5	35.4	7.07		
Carbohydrate	LR	2	4.177	2.0887	2.505	0.176
	ST	1	0.016	0.0164	0.020	0.894
	LR:ST	2	0.323	0.1616	0.194	0.83
	Residuals	5	4.17	0.8339		
SFA	LR	2	248.38	124.19	6.488	0.0408

	ST	1	8.84	8.84	0.462	0.5269
	LR:ST	2	64.78	32.39	1.692	0.2746
	Residuals	5	95.70	19.14		
MUFA	LR	2	66.89	33.44	26.16	**0.0023**
	ST	1	2.56	2.56	2.002	0.216
	LR:ST	2	2.31	1.15	0.902	0.4629
	Residuals	5	6.39	1.28		
PUFA	LR	2	542.6	271.28	12.162	0.012
	ST	1	1.9	1.89	0.085	0.783
	LR:ST	2	84.4	42.19	1.892	0.245
	Residuals	5	111.5	22.31		

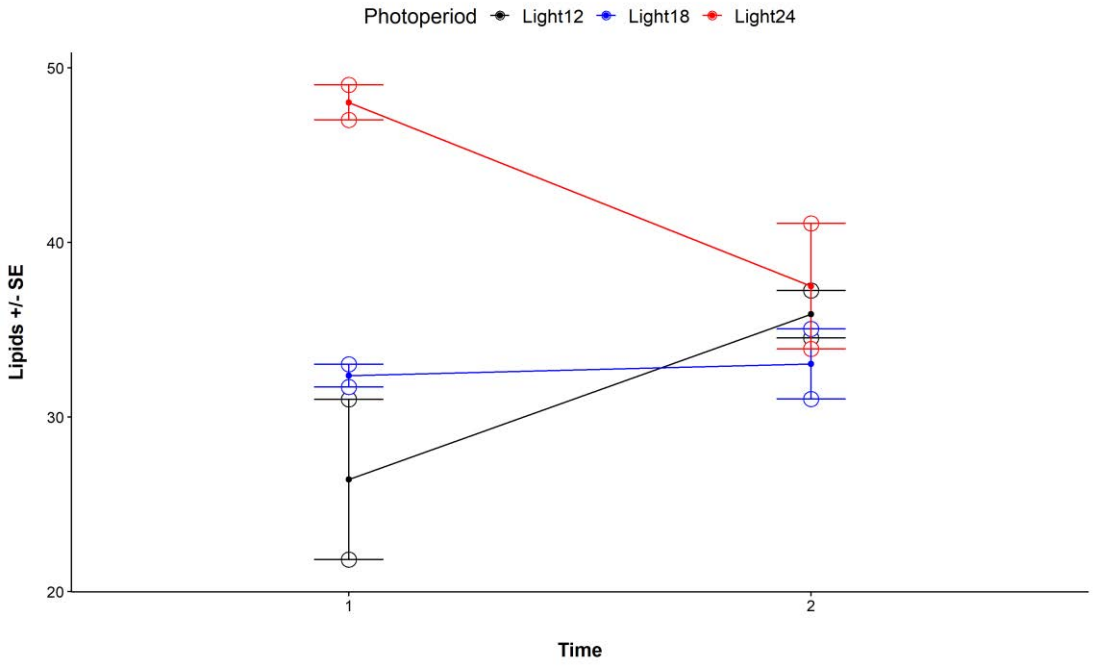
a.


Photoperiod
Light12
Light18
Light24
6e+06
5e+06
4e+06
3e+06
2e+06
Cell density / mL +/- SE
1
2
Time

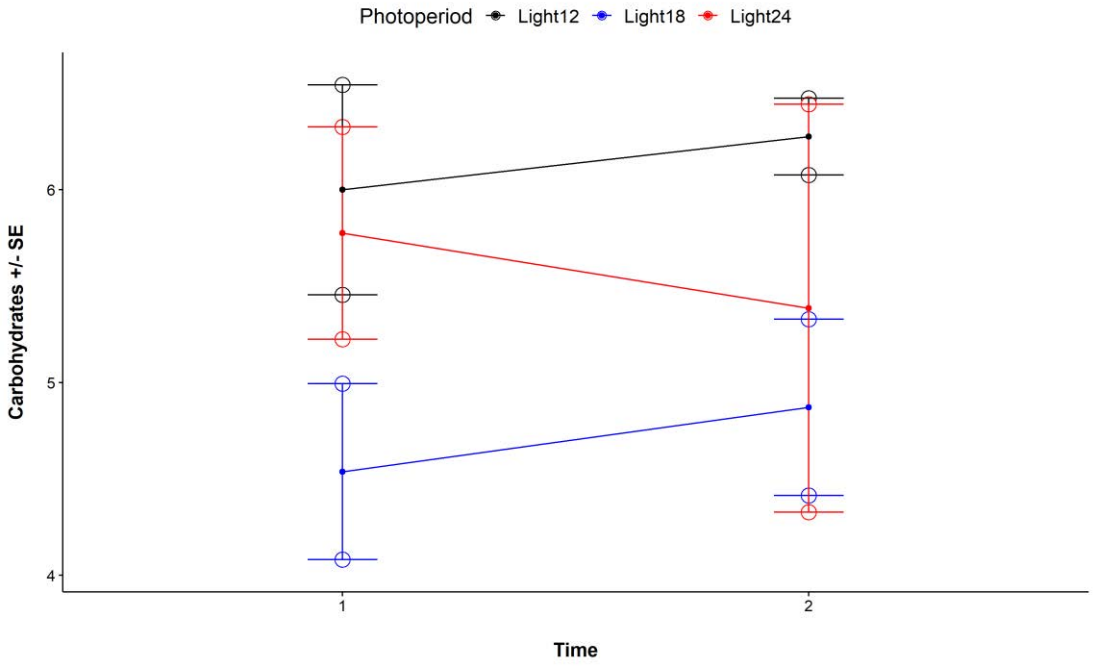
b.


Photoperiod
Light12
Light18
Light24
50
40
30
20
Proteins +/- SE
1
2
Time

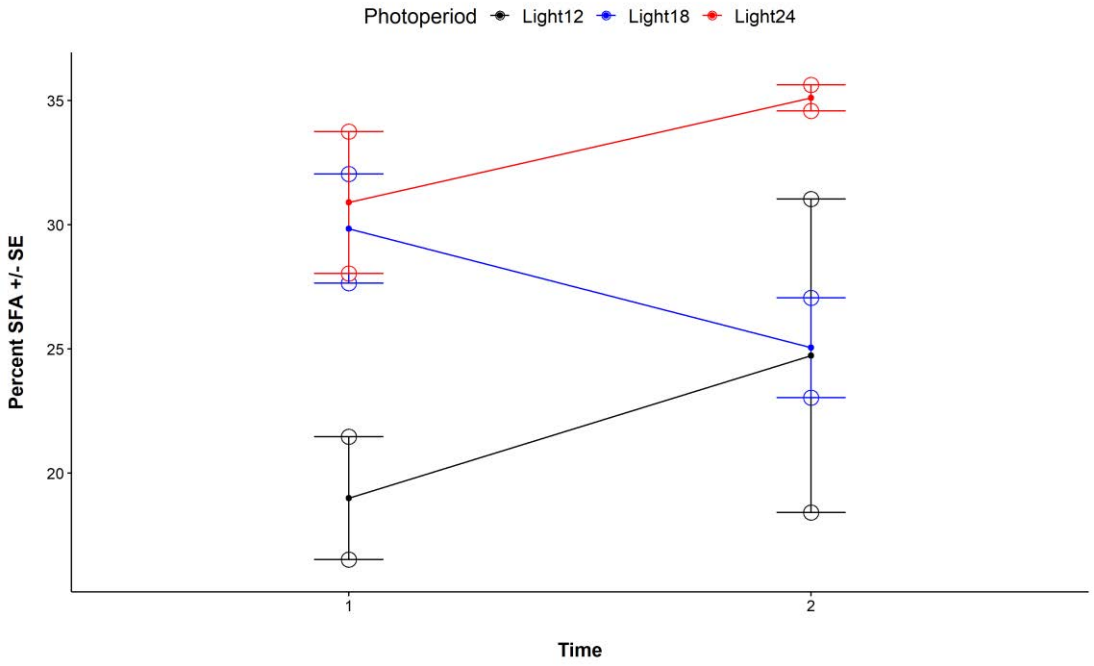
c.


Photoperiod
Light12
Light18
Light24
Lipids +/- SE
50
40
30
20
1
2
Time

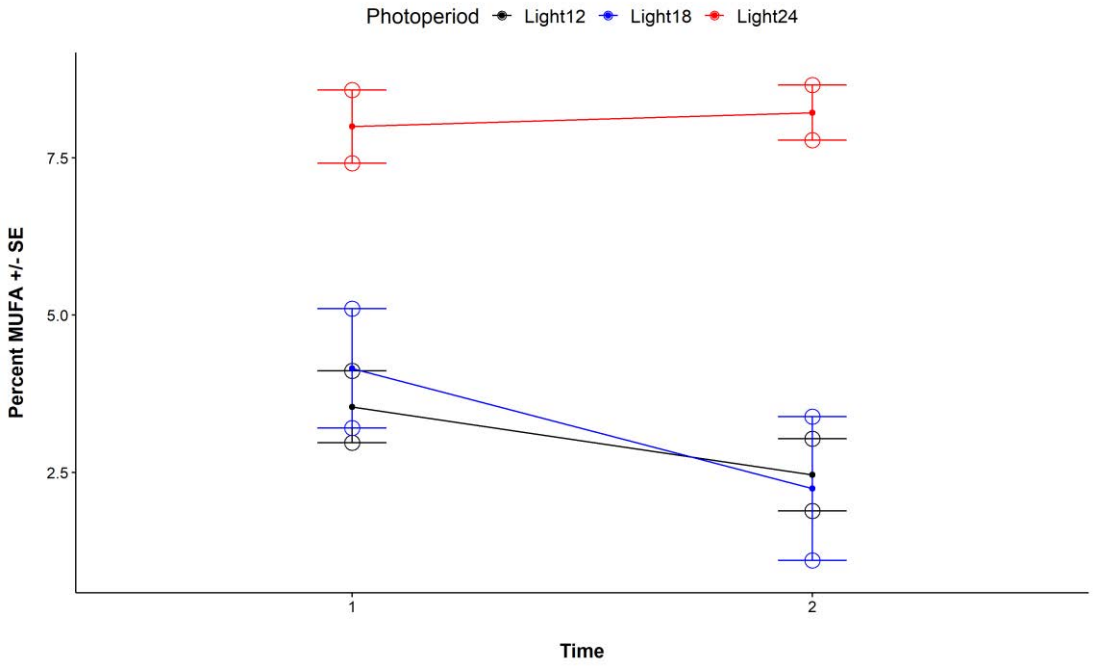
d.


Photoperiod
Light12
Light18
Light24
Carbohydrates +/- SE
6
5
4
1
2
Time

e.


Photoperiod
Light12
Light18
Light24
Percent SFA +/- SE
35
30
25
20
1
2
Time

f.


Photoperiod
Light12
Light18
Light24
Percent MUFA +/- SE
7.5
5.0
2.5
1
2
Time

g.

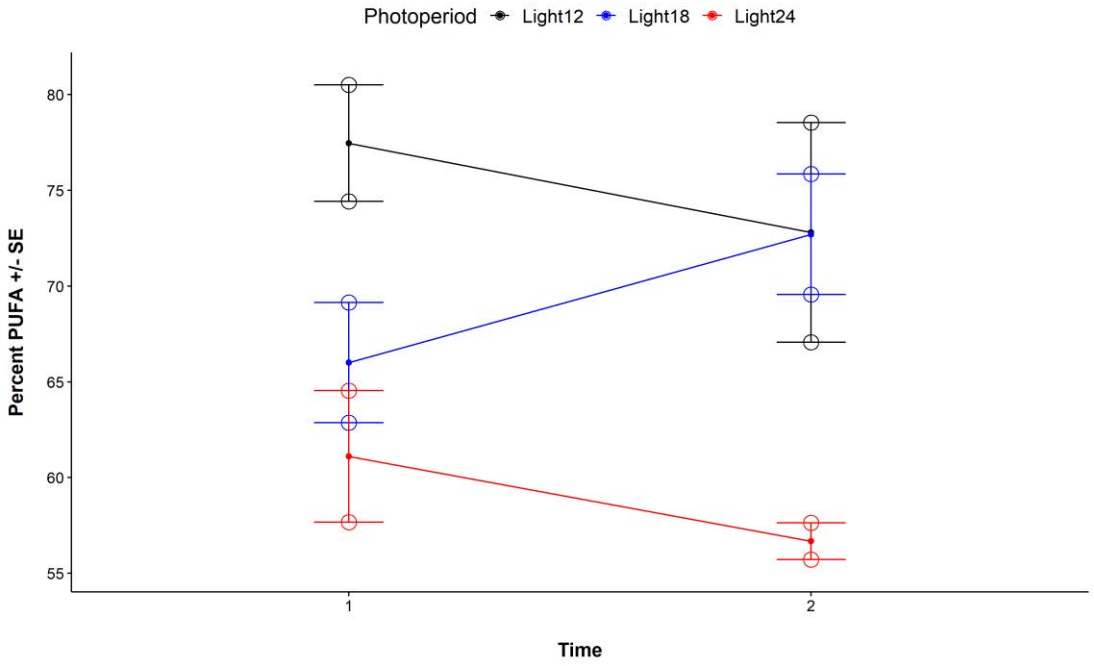


Figure A3 Interaction plots for *Pavlova* sp. **a.** growth rates (cell density/ mL), biochemical composition (**b.** protein, **c.** lipid and **d.** carbohydrate contents; displayed as % of dry weight) and fatty acid content (**e.** SFA, **f.** MUFA, **g.** PUFA) at two different sampling times (Day 10 and Day 20) between 3 different light regimes.

d. *Micromonas pusilla*

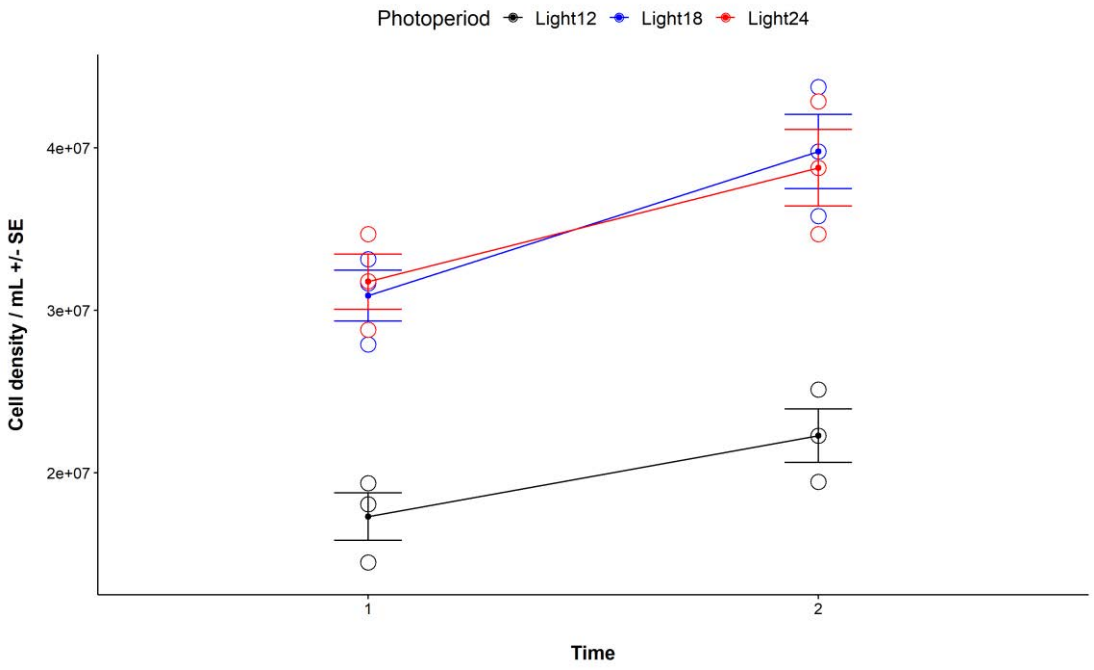
Table A4 Repeated measures ANOVA results for *M. pusilla* growth rates (cell density/ mL), biochemical composition (protein, lipid and carbohydrate contents) and fatty acid content (SFA, PUFA). Significance accepted at adjusted $p < 0.007$ and is highlighted in bold and red.

Note: Protein data did not have a full set of samples for the light regime 24:0, therefore only data for the light regimes 18:6 and 12:12 has been analyzed.

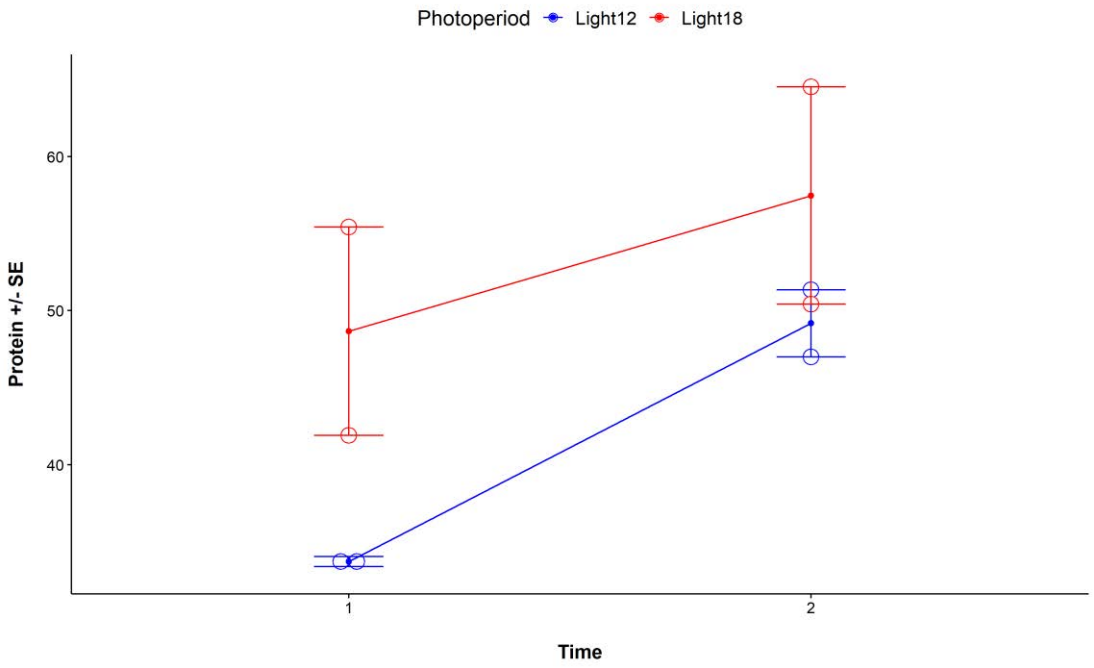
Variable	Factor	df	SS	MS	F	p
Growth rates	Light Regime (LR)	2	9.635e+14	4.817e+14	45.93	**0.0000**
	Sampling time (ST)	1	2.179e+14	2.179e+14	20.78	**0.0006**
	LR:ST	2	1.133e+13	5.665e+12	0.54	0.5962
	Residuals	12	1.259e+14	1.049e+13		
Protein **LR: 18:6 & 24:0 only**	LR	1	294.6	294.59	4.44	0.126
	ST	1	270.0	269.98	4.069	0.137
	LR:ST	1	22.01	22.1	0.333	0.604
	Residuals	3	199.0	66.35		
Lipid	LR	2	3.4	1.7	0.019	0.981
	ST	1	725.3	725.3	8.249	0.035
	LR:ST	2	443.9	222.0	2.524	0.175
	Residuals	5	439.6	87.9		
Carbohydrate	LR	2	7.802	3.901	1.104	0.401
	ST	1	4.681	4.681	1.325	0.302
	LR:ST	2	2.43	1.215	0.344	0.725
	Residuals	5	17.667	3.533		

SFA	LR	2	31.68	15.841	1.673	0.278
	ST	1	9.32	9.32	0.984	0.367
	LR:ST	2	30.68	15.341	1.62	0.287
	Residuals	5	47.35	9.469		
PUFA	LR	2	27.66	13.83	0.762	0.514
	ST	1	25.33	25.33	1.395	0.291
	LR:ST	2	60.74	30.37	1.673	0.278
	Residuals	5	90.75	18.15		

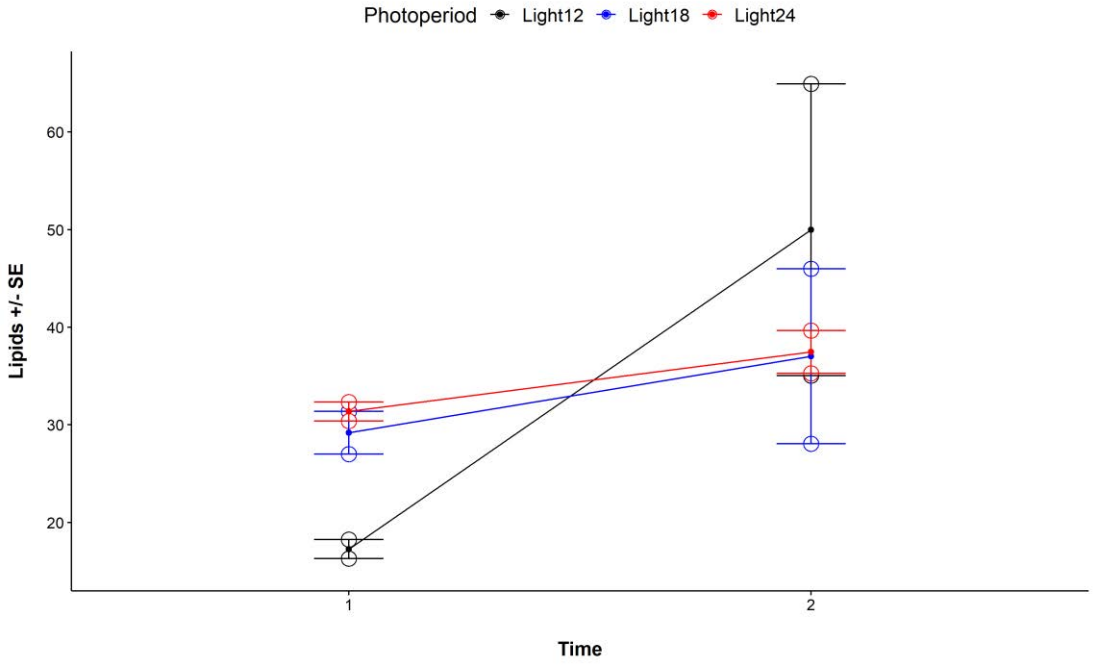
a.



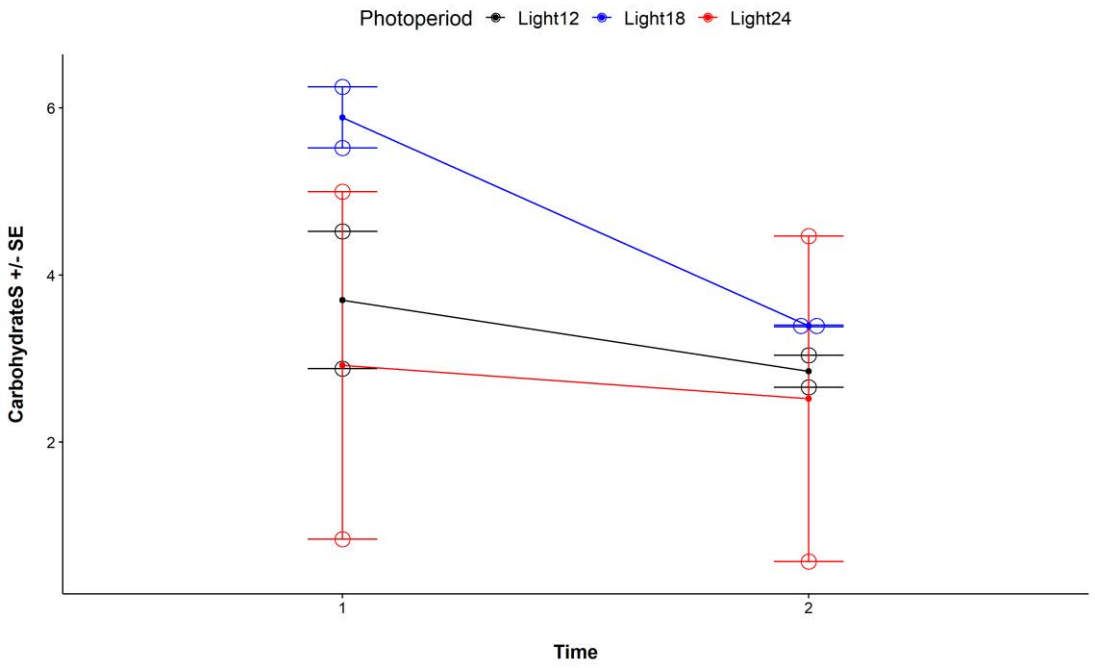
b.


Photoperiod
Light12
Light18
Protein +/- SE
60
50
40
1
2
Time

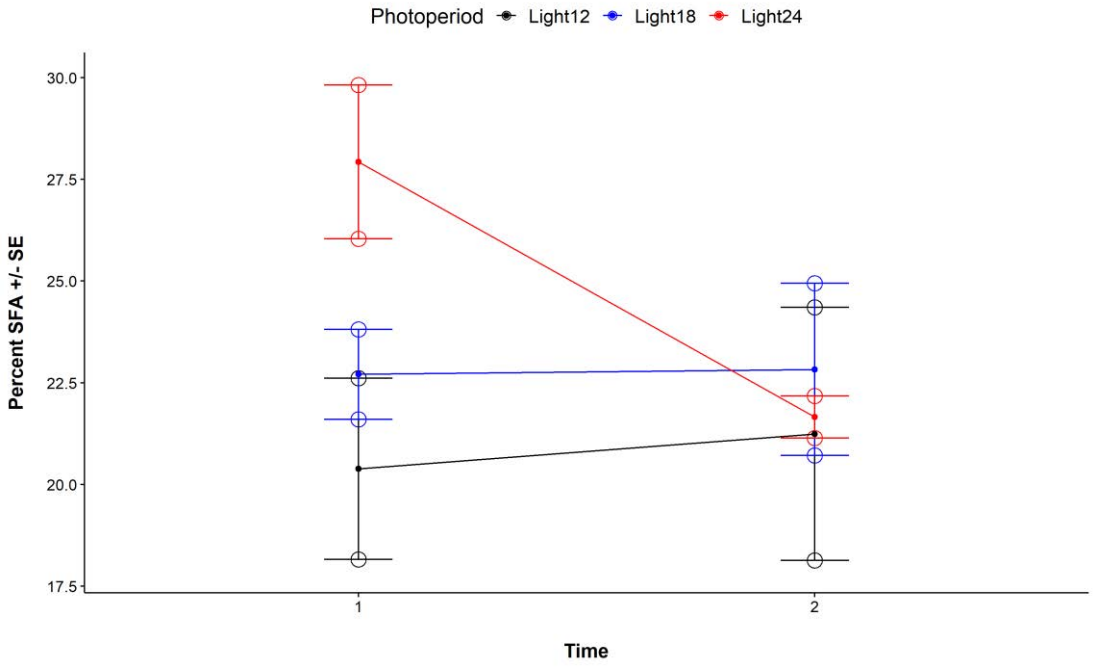
c.


Photoperiod
Light12
Light18
Light24
Lipids +/- SE
60
50
40
30
20
1
2
Time

d.


Photoperiod
Light12
Light18
Light24
CarbohydrateS +/- SE
6
4
2
1
2
Time

e.


Photoperiod
Light12
Light18
Light24
Percent SFA +/- SE
30.0
27.5
25.0
22.5
20.0
17.5
1
2
Time

f.

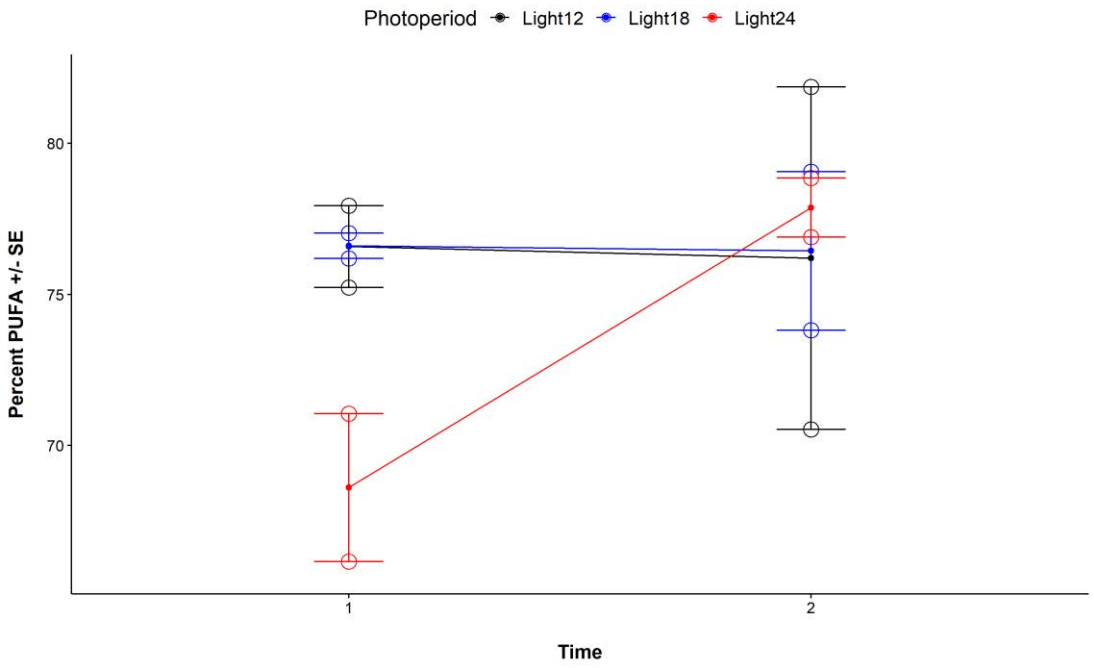


Figure A4 Interaction plots for *M. pusilla* **a.** growth rates (cell density/ mL), biochemical composition (**b.** protein, **c.** lipid and **d.** carbohydrate contents; displayed as % of dry weight) and fatty acid content (**e.** SFA, **f.** PUFA) at two different sampling times (Day 10 and Day 15) between 3 different light regimes. **Note:** Protein data did not have a full set of samples for the light regime 24:0, therefore only data for the light regimes 18:6 and 12:12 is shown.

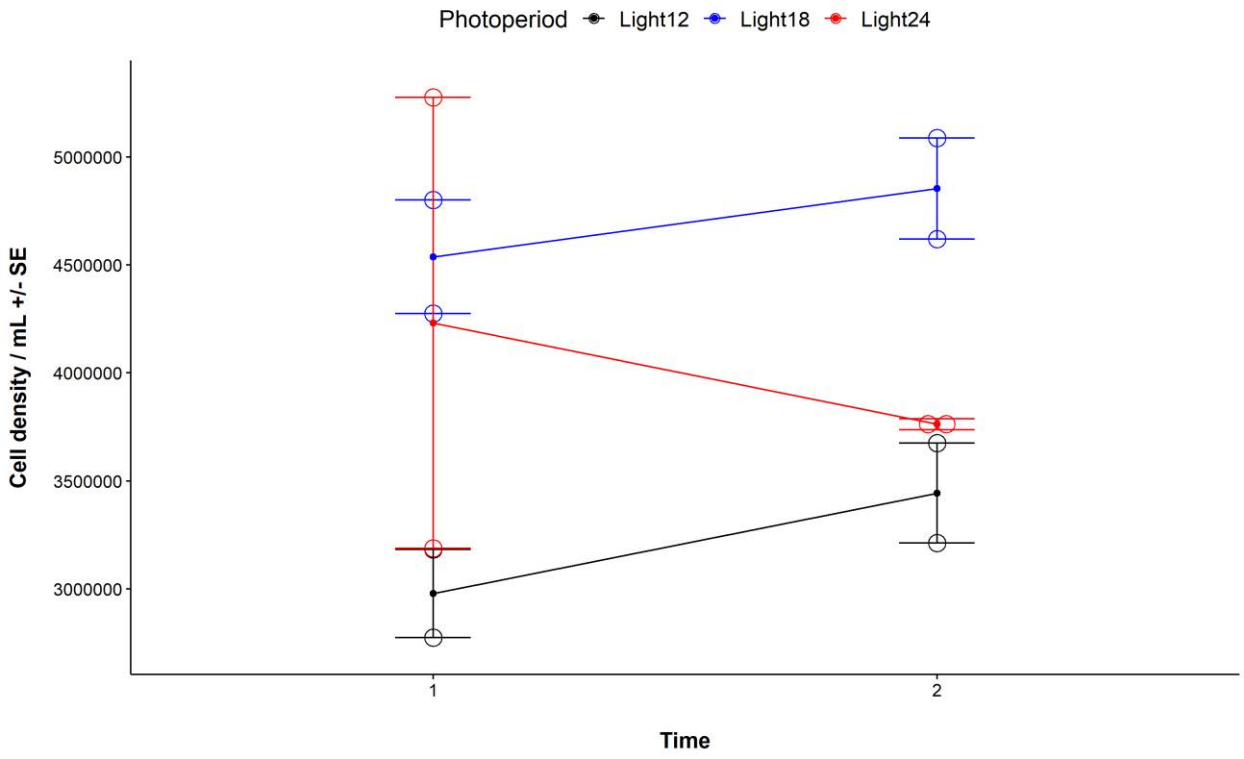
e. *Chaetoceros muelleri*

Table A5 Repeated measures ANOVA results for *C. muelleri* growth rates (cell density/ mL), biochemical composition (protein, lipid and carbohydrate contents) and fatty acid content (SFA, MUFA, PUFA). Significance accepted at adjusted $p < 0.007$ and is highlighted in bold and red.

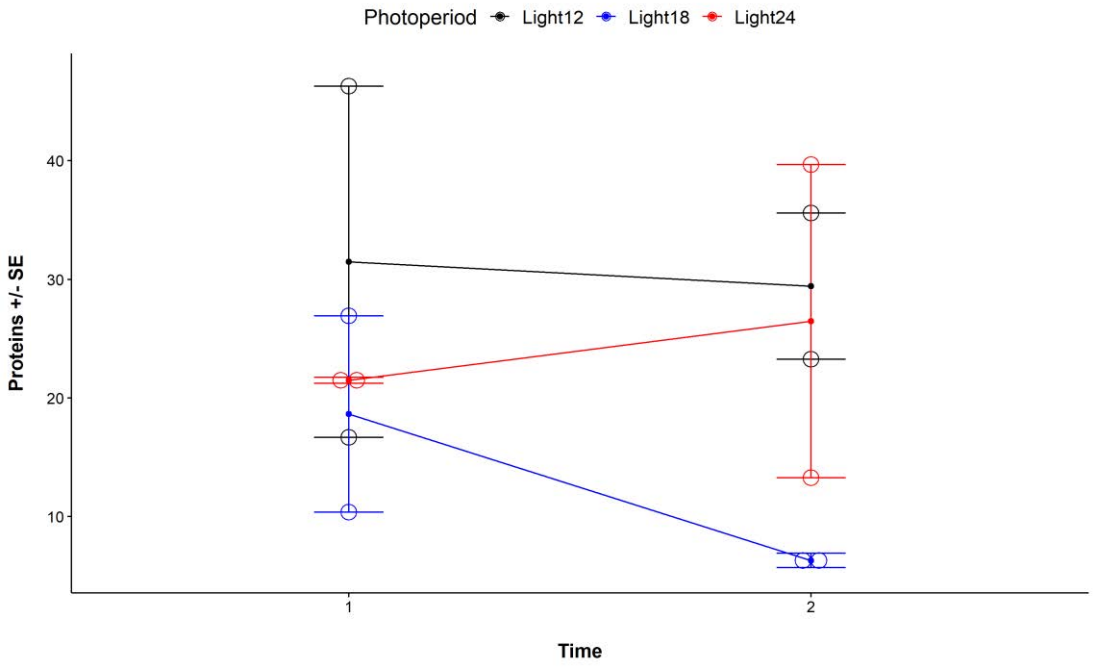
Variable	Factor	df	SS	MS	F	p
Growth rates	Light Regime (LR)	2	4.409e+12	2.204e+12	5.052	0.0517
	Sampling time (ST)	1	3.234e+10	3.234e+10	0.074	0.7946
	LR:ST	2	5.028e+11	2.514e+11	0.576	0.5903
	Residuals	6	2.618e+12	4.363e+11		
Protein	LR	2	663.6	331.8	2.093	0.219
	ST	1	29.6	29.6	0.186	0.684
	LR:ST	2	152	76	0.479	0.645
	Residuals	5	792.5	158.5		
Lipid	LR	2	142.0	70.99	0.638	0.567
	ST	1	72.6	72.62	0.652	0.456
	LR:ST	2	113.5	56.76	0.51	0.629
	Residuals	5	556.8	111.36		
Carbohydrate	LR	2	3.62	1.808	0.395	0.693
	ST	1	0.41	0.409	0.089	0.777
	LR:ST	2	32.02	16.010	3.495	0.112

	Residuals	5	22.90	4.58		
SFA	LR	1	32.94	32.94	1.29	0.339
(LR18:6&24:0)	ST	1	0.42	0.42	0.016	0.906
	LR:ST	1	66.97	66.97	2.622	0.204
	Residuals	3	76.63	25.54		
MUFA	LR	1	854.4	854.4	21.991	0.0183
(LR18:6&24:0)	ST	1	18.8	18.8	0.485	0.5363
	LR:ST	1	9.1	9.1	0.233	0.6624
	Residuals	3	116.6	38.9		
PUFA	LR	1	551.8	551.8	5.093	0.109
(LR18:6&24:0)	ST	1	13.6	13.6	0.126	0.746
	LR:ST	1	26.8	26.8	0.247	0.653
	Residuals	3	325.1	108.4		

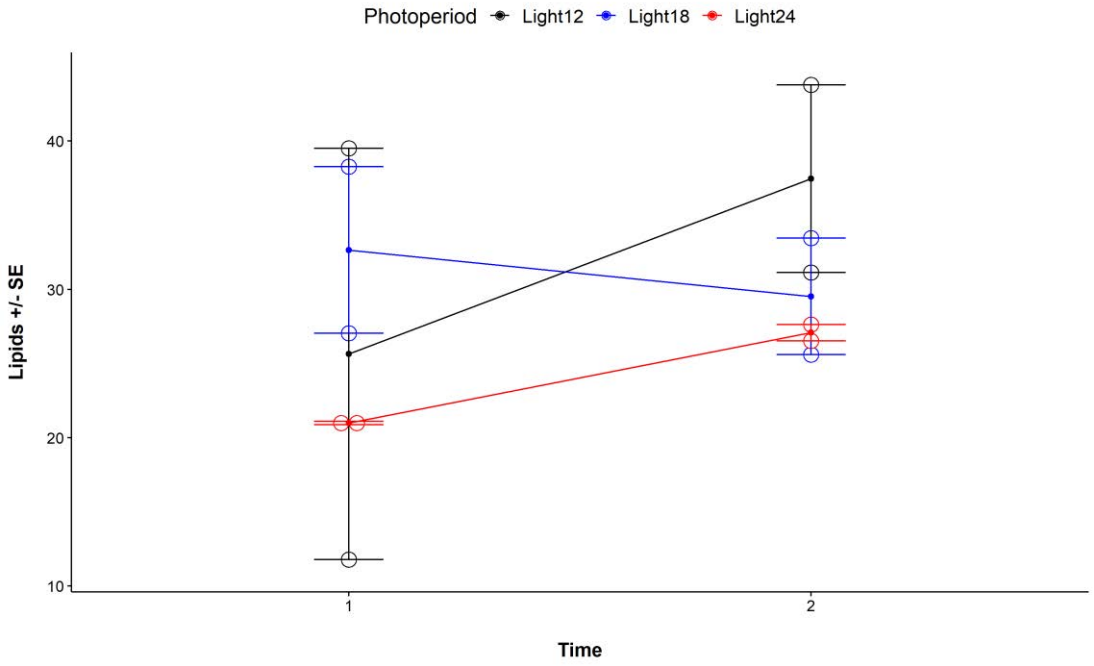
a.


Photoperiod
Light12
Light18
Light24
Cell density / mL +/- SE
5000000
4500000
4000000
3500000
3000000
1
2
Time

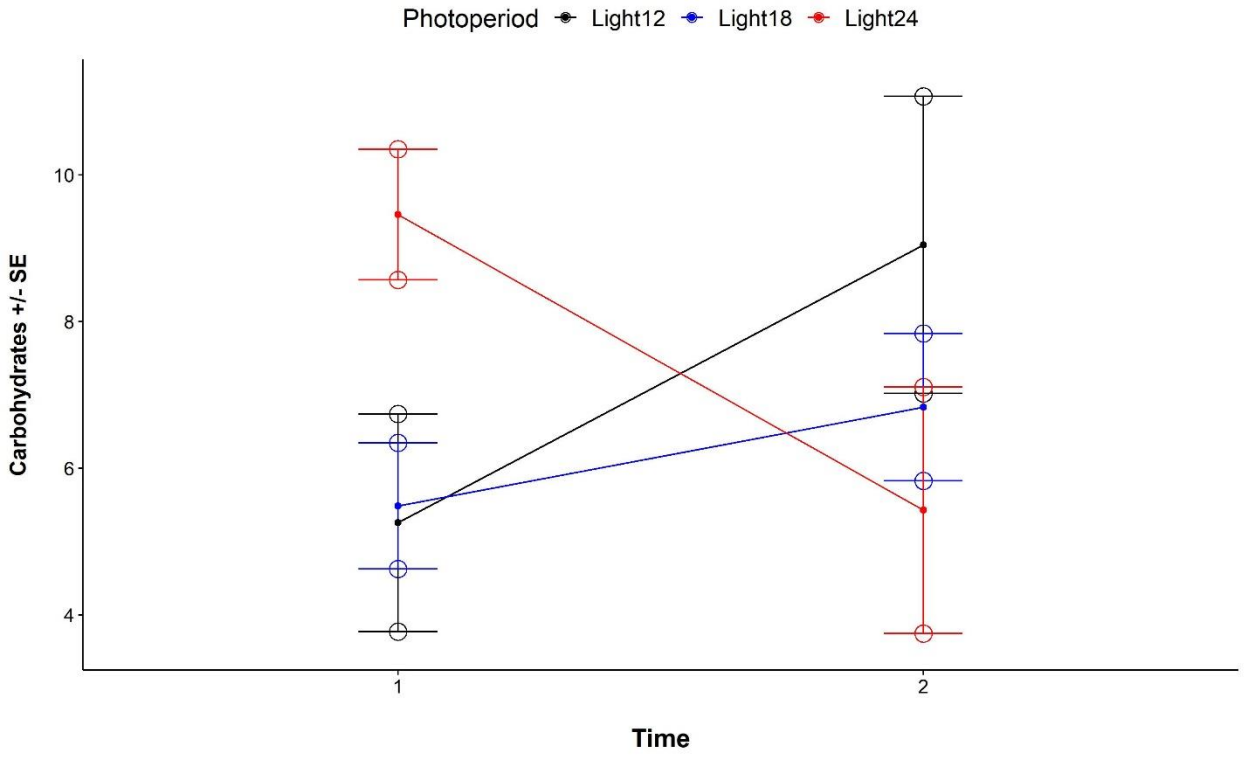
b.


Photoperiod
Light12
Light18
Light24
Proteins +/- SE
40
30
20
10
1
2
Time

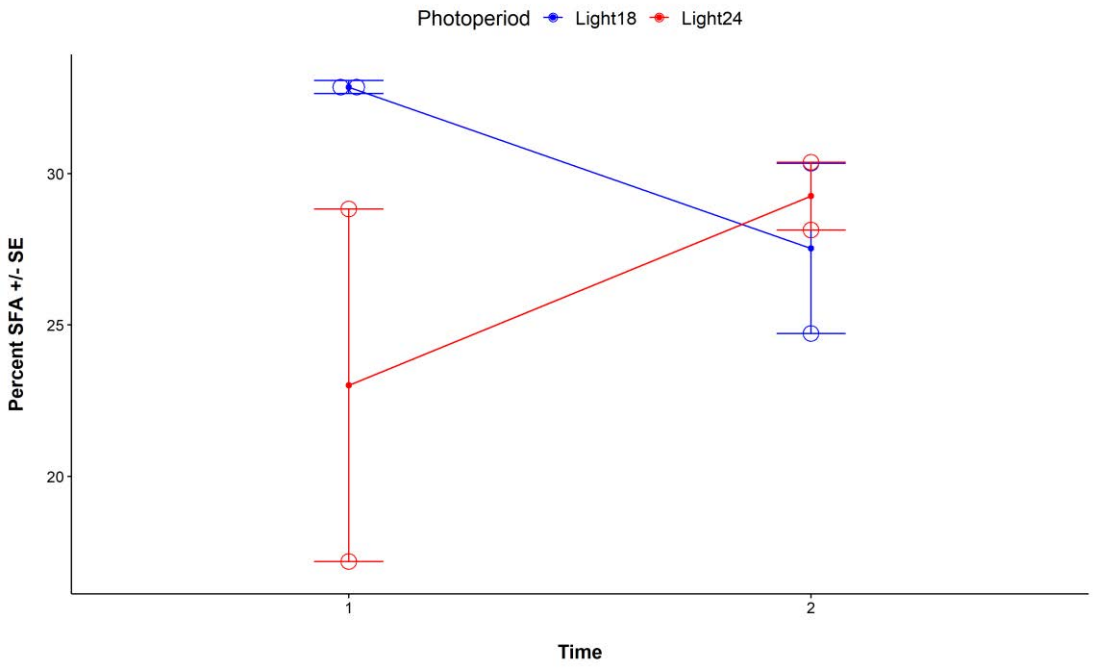
c.


Photoperiod
Light12
Light18
Light24
Lipids +/- SE
40
30
20
10
1
2
Time

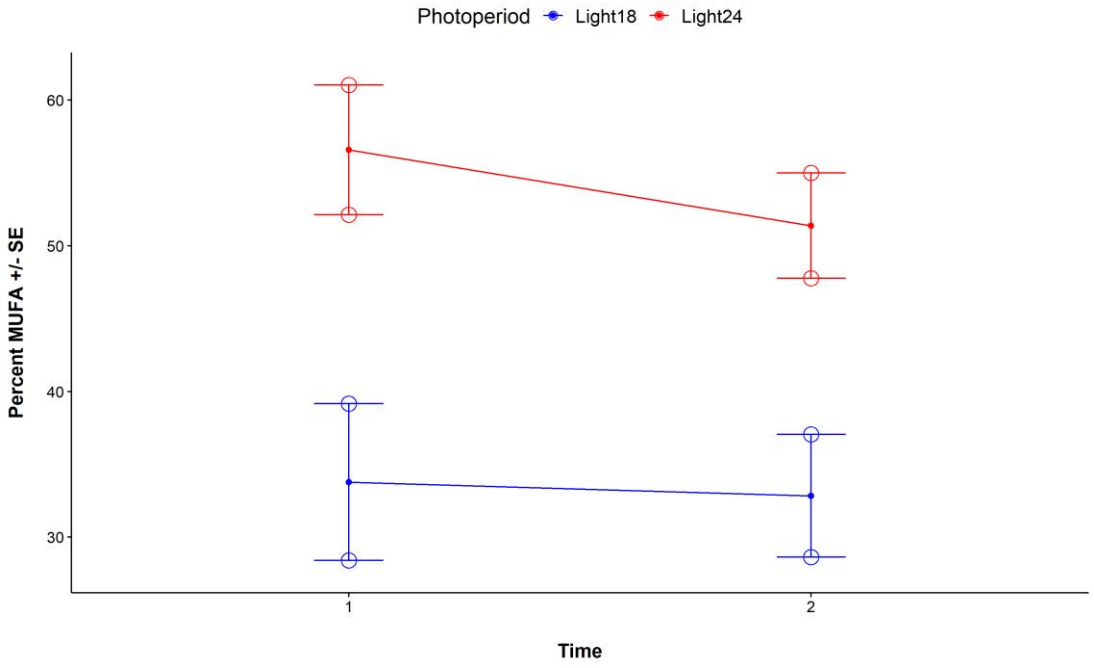
d.


Photoperiod
Light12
Light18
Light24
Carbohydrates +/- SE
10
8
6
4
1
2
Time

e.


Photoperiod
Light18
Light24
Percent SFA +/- SE
30
25
20
1
2
Time

f.


Photoperiod
Light18
Light24
Percent MUFA +/- SE
60
50
40
30
1
2
Time

g.

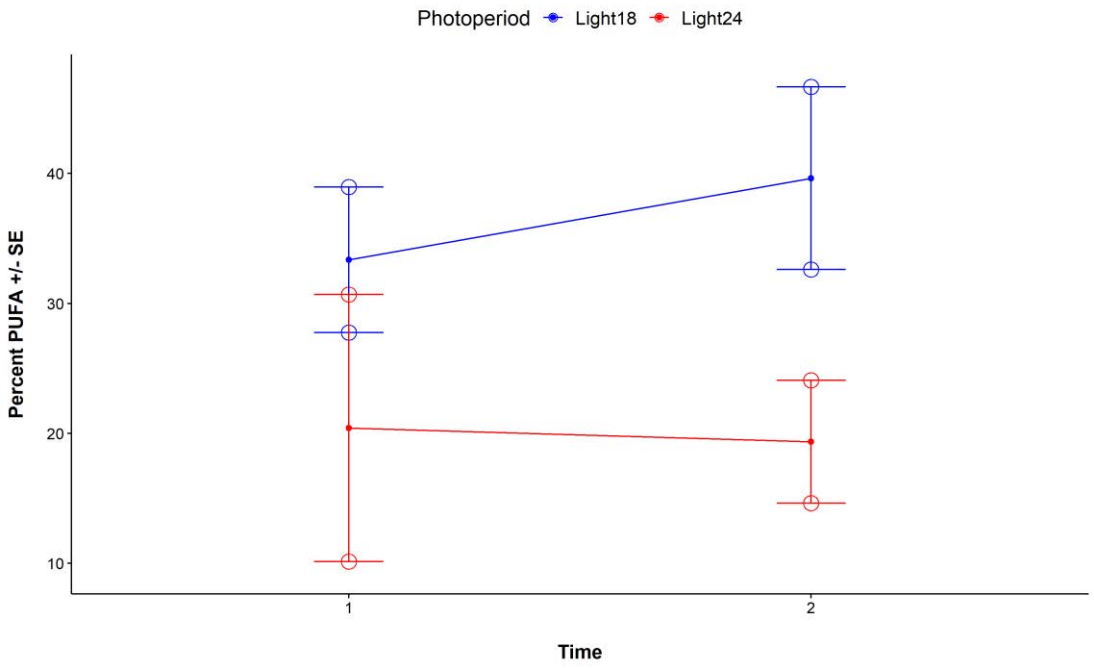


Figure A5 Interaction plots for *C. muelleri* **a.** growth rates (cell density/ mL), biochemical composition (**b.** protein, **c.** lipid and **d.** carbohydrate contents; displayed as % of dry weight) and fatty acid content (**e.** SFA, **f.** MUFA, **g.** PUFA) at two different sampling times (Day 10 and Day 15) between 3 different light regimes.

APPENDIX 3

Publications resulting from this thesis

Martínez-Fernández, E., Acosta-Salmón, H., Southgate, P.C. 2006. The nutritional value of tropical microalgae for black-lip pearl oyster (*Pinctada margaritifera*, L.) larvae. Aquaculture 257, 491- 503.

Martínez-Fernández, E., Southgate, P.C. 2007. Use of tropical microalgae as food for larvae of the black-lip pearl oyster *Pinctada margaritifera*. Aquaculture 263, 220-226.
